The song of lipids and proteins: dynamic lipid–protein interfaces in the regulation of plant cell polarity at different scales

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Abstract

Successful establishment and maintenance of cell polarity is crucial for many aspects of plant development, cellular morphogenesis, response to pathogen attack, and reproduction. Polar cell growth depends on integrating membrane and cell-wall dynamics with signal transduction pathways, changes in ion membrane transport, and regulation of vectorial vesicle trafficking and the dynamic actin cytoskeleton. In this review, we address the critical importance of protein–membrane crosstalk in the determination of plant cell polarity and summarize the role of membrane lipids, particularly minor acidic phospholipids, in regulation of the membrane traffic. We focus on the protein–membrane interface dynamics and discuss the current state of knowledge on three partially overlapping levels of descriptions. Finally, due to their multiscale and interdisciplinary nature, we stress the crucial importance of combining different strategies ranging from microscopic methods to computational modelling in protein–membrane studies.

Key words: Cell polarity, endocytosis, exocytosis, membrane trafficking, membrane domain, microscopy, phosphatic acid, phosphatidylinositol (4,5)-bisphosphate, pollen tube.

Introduction

Polar cell growth is one of the most fundamental processes in plant development. It determines cellular morphogenesis and ultimately defines plant phenotype. Membrane trafficking, especially exo- and endocytosis, is of crucial importance for the establishment and maintenance of cell polarity. Most processes of membrane trafficking involve membrane–protein interfaces. In this review, we aim to emphasize the critical importance of protein–membrane crosstalk in the determination of plant cell polarity and to summarize the role of membrane lipids in the regulation of membrane traffic. Since this is a topic of an inherently complex multiscale nature, for the sake of clarity we will describe this continuum in three partially overlapping spatial categories, which roughly relate to the readouts of particular research techniques: (i) membrane domains corresponding to confocal laser-scanning microscopy (CLSM) imaging with a resolution >1 µm or classical biochemical techniques; (ii) membrane domains describing processes taking place on a submicrometer scale, typically
observed with advanced optical microscopy techniques such as super-resolution, spinning-disk or total internal reflection fluorescence/variable angle epifluorescence microscopy (TIRF/VAEM); and (iii) membrane dynamics at the nanometer level, where molecular details of individual lipid–lipid and protein–lipid molecular interactions can be addressed primarily by computational approaches such as molecular dynamics simulations (Fig. 1). Due to space limitations, we do not cover most reports relying on highly advanced physical–chemical approaches (such as solid state nuclear magnetic resonance, X-ray scattering, surface plasmon resonance, FTIR spectroscopy) and we focus mainly on methods allowing the visualization of protein–membrane interface dynamics in polar membrane trafficking.

While protein complexes involved in exocytosis and endocytosis are relatively well known across eukaryotes, data on the involvement of lipid membrane components lag significantly behind, despite the obvious fact that membrane fusion processes must also involve phospholipids. This is especially true for exocytosis, where there are few data directly showing mechanistic role of phospholipids, even for animal/yeast models, and where only indirect evidence exists for plant cells. Here, we review our current knowledge of the dynamic protein–membrane interface in plant cells at different scales and point out the molecular mechanisms possibly shared with other eukaryotic models.

A game of membranes: cellular ‘macrodomains’ in plant cell polarity

Membrane components often differ not only among different organelles of a cell but also among different regions of the single cell plasma membrane (PM, Alassimone et al., 2010). Notoriously known examples involve the large membrane domains of polarized cells. Animal epithelial cells have a drastically different composition of the apical and basolateral membranes in terms of both lipids and proteins. The diffusion barrier formed by tight junctions prevents the membrane domains from mixing (Nicolson, 2014). In plants, at least four distinct membrane domains with different compositions can co-exist in one cell (Šáský et al., 2009; Alassimone et al., 2010) - the apical, basal, inner lateral and outer lateral domains. The endodermal/exodermal casparian strip is an example of a cell-type-specific plant cell polar domain (Roppolo et al., 2010).

Polar exocytosis, selective endocytosis, limitation of the diffusion rate, and the presence of diffusion barriers can all play a role in membrane protein polarity establishment and maintenance (Kleine-Vehn et al., 2011). Limiting diffusion of membrane proteins by their attachment to the cell wall is an important mechanism of plant membrane protein polarization (Martinière et al., 2012).

The discovery of the rapid changes in the spatio-temporal dynamics of minor signalling lipids (and their interacting proteins) brought about new challenges in finding approaches through which such changes can be visualized and quantified. Thanks to the existence of protein modules that selectively recognize distinct phospholipids, rapid changes in their levels and distribution can be monitored by CLSM (Balla and Várnai, 2002; Vermeer and Munnik, 2013). Although this technique is one of the few that provides information on lipid dynamics in live cells with subcellular resolution, it has also limitations that need to be taken into account when interpreting the data (Balla, 2007). The most importantly, it is not yet clear whether all pools of a particular lipid are visualized by the marker constructs. They may recognize the lipid in a very specific context and not image the whole population of the studied lipid in every cellular compartment. It is therefore imperative to remember that the absence of evidence is not evidence of absence, and to interpret the data with caution. Alongside imaging of protein–lipid dynamics in vivo, microscopic studies of giant unilamellar vesicles are excellent model systems for the determination of membrane properties and protein–lipid interactions on the macrodomain scale. Membrane phase separation, lipid sorting, membrane rigidity, and stretching elasticity have been studied using these giant unilamellar vesicles (Zhao and Lappalainen, 2012).

A feast for phosphoinositides: rare but powerful master players in the membrane

Phosphoinositides (PPIs) comprise a group of phospholipids consisting of the backbone molecule phosphatidylinositol (PI) and its mono-, bis-, or tris-phosphorylated derivatives. A variety of lipid kinases reversibly phosphorylate positions 3, 4, and 5 on the inositol ring so that three modifications results in seven PPI species. PPIs have been implicated in a plethora of processes and their classic ‘textbook’ role is to serve as substrates for the generation of secondary messengers (typically diacylglycerol (DAG) and inositol 1,4,5-trisphosphate
produced from phosphatidylinositol 4,5-bisphosphate). In exocytosis, PPIs function more as ‘local’ organizers of membrane domains and regulators of membrane deformation and sorting machineries (Thole and Nielsen, 2008), or in the control of cytoskeletal dynamics (Pleskot et al., 2014). This can be also achieved by the very ‘regional’ character of PPI metabolism, where various kinases and phosphatases are located on various organelles, thus creating distinct pools of different PPIs (Boutté and Moreau, 2014).

In plant cells, five of the seven PPIs were detected. Phosphatidylinositol 3,4,5-trisphosphate (PIP3) has never been detected in plant cells, and the reported detection of plant phosphatidylinositol 3,4-bisphosphate PI(3,4)P2 was most probably a misinterpretation of then unknown PI(3,5)P2 (Meijer and Munnik, 2003). Simon et al. (2014) used different genetically encoded biosensors to characterize localization of PPIs within Arabidopsis root epidermal cells, showing that PI(4,5)P2 is localized almost exclusively at the PM and that phosphatidylinositol 4-phosphate (PI4P) exhibits a gradient of increasing localization from the Golgi apparatus to post-Golgi compartments to the PM. During cell division, the PI4P marker showed strong labelling of the cell plate (Vermeer et al., 2009). The role of PI4P and PI(4,5)P2 in polar growth has been studied most extensively in plant-tip growing cells, pollen tubes, and root hairs. The PI4P marker is enriched at the tips of emerging and growing root hairs (Vermeer et al., 2009). Similarly, the PI(4,5)P2 marker localizes to the tip of both root hairs (van Leeuwen et al., 2007) and pollen tubes (Ischebeck et al., 2011; Potocký et al., 2014). Polar localization and gradients of phospholipids are sustained by restricted localization and the activity of PI-converting enzymes (Heilmann and Heilmann, 2014; Helling et al., 2006).

PI4P has traditionally been understood only as a precursor for the synthesis of PI(4,5)P2. However, recent lines of evidence showing PI4P as an important organizer of membrane trafficking put this molecule back into the spotlight. While in animals regulated secretion relies on PI(4,5)P2, constitutive secretion appears to be mostly under the control of PI4P, generated from PI by the phosphatidylinositol 4-kinases (PI4Ks). In mammals, four different PI4K isoforms have been identified so far. Two of these, PI4KIIα and PI4KIIIβ, are localized to the Golgi, which appears to be the organelle where PI4P has a prominent and direct role. Indeed, many PI4P-binding proteins have been identified at the Golgi, such as clathrin adaptors and lipid-transfer proteins (D’Angelo et al., 2008).

In yeast, two PI4K isoforms are present, Pik1p (type IIIα of PI4K present at the Golgi and nucleus) and Stt4p (type IIβ of PI4K localized to the PM) serving as primary sources of PI4P for secretion (Audhya et al., 2000).

In Arabidopsis, the only known enzymes with PI4K activity belong to the IIβ type. In contrast to yeast, disruption of both plant IIIβ PI4Ks does not lead to lethality, although the double mutant displays disturbed morphology of root hairs. PI4P production and membrane trafficking are further linked by the interaction of PI4Kβ1 with the small GTPase RabA4b, which controls post-Golgi trafficking to the PM (Preuss et al., 2006). Interestingly, while PI4Kβ1 co-localizes with RabA4b in the TGN-like compartment, the majority of PI4P resides at the PM (Vermeer et al., 2009). This apparent ambiguity was solved by identification of the root-hair-defective 4 (rhd4) mutant. In Arabidopsis, RHD4 is required for normal root hair development and encodes a Sac1p-like PI4P-selective phosphatase. RHD4/SAC7 also co-localizes with RabA4b and PI4Kβ1 and probably functions as the regulator of PI4P levels, restricting the levels of PI4P to the vesicles at the tips of growing root hairs. In rhd4 mutant plants, loss of the phosphatase activity results in PI4P accumulation, leading to mislocalization and accumulation of PI4P-labelled secretory vesicles (Thole et al., 2008).

The Arabidopsis genome contains 11 paralogues of PI4P 5-kinases (PIP5K, Heilmann and Heilmann (2014)) divided into a type A subfamily with a domain structure similar to mammalian and yeast enzymes (PIP5K10 and -11) and a type B subfamily with N-terminal Lin and MORN domains. PIP5K1, -2, -7, -8, and -9 are expressed ubiquitously in most vegetative tissues, PIP5K3 is trichoblast and root hair specific, and PIP5K2, -4, -5, -6, -10 and -11 are also expressed in pollen (Ischebeck et al., 2008, 2011; Kusano et al., 2008; Sousa et al., 2008; Stenzel et al., 2008; Zhao et al., 2010). Both mutations and overexpressions of PIP5K paralogues cause various defects in tip growing cells. This indicates the necessity of fine-tuned PI-converting machinery in tip growing cells (Ischebeck et al., 2010).

Localization of PIP5K3 in growing root hairs is apical (Kusano et al., 2008; Stenzel et al., 2008) and the root hairs of pip5k3 mutants are shorter and swollen. Arabidopsis root hairs overexpressing PIP5K1, -2, or -3 (Ischebeck et al., 2013; Stenzel et al., 2008) are also short and swollen.

Localization of all fluorescently tagged pollen-expressed PIP5K isoforms 2, 4, 5, 6, 10, and 11 in tobacco pollen share similar characteristics. In growing cells, PIP5Ks are localized in the subapical region, while in non-growing cells, the localization is also expanded into the very tip (Ischebeck et al., 2008, 2010, 2011; Stenzel et al., 2008, 2012; Zhao et al., 2010). Pollen from pip5k4 mutant Arabidopsis plants has a lower germination rate and slower growth with the occasional occurrence of branched pollen tubes. The germination rate is even more reduced in pip5k4 pip5k5 double mutant Arabidopsis pollen (Ischebeck et al., 2008). There are two types of phenotype associated with PIP5K overexpressors. PIP5K2, -10, and -11 overexpression causes swelling of the tobacco pollen tube tip (Ischebeck et al., 2011; Stenzel et al., 2012), while overexpression of PIP5K4, -5, and -6 results into pollen tube waving, tip branching or invagination of the tip membrane with detachment from the cell wall (‘protoplast trapping’, Ischebeck et al., 2008, 2010; Sousa et al., 2008; Stenzel et al., 2012; Zhao et al., 2010).

The protoplast-trapping phenotype of PIP5K4 and -5 overexpressors is associated with increased pectin deposition. Pollen tubes with low overexpression of PIP5K6 grow slower and show slightly slower targeting of receptor-like kinase to the membrane, indicating slower exocytosis. In contrast, Arabidopsis PIP5K6 RNAi pollen and tobacco PIP5K6 overexpressors have impaired FM dye internalization and recruit less clathrin heavy chain to the membrane. Simultaneous expression
of dominant-negative clathrin heavy chain alleviates the phenotype of strong PIP5K6 overexpressors, indicating that the phenotype is caused by accumulation of aborted endocytotic events (Zhao et al., 2010). In summary, disruption of the delicate PI(4,5)P₂ balance probably influences both exocytosis and endocytosis in the pollen tube tip.

The pollen tip-swelling phenotype of PIP5K2, -10, and -11 mutants/overexpressors is actin related, and the mechanism responsible for altered actin structure and swelling seems to be activation of the small GTPase NtRac5 (Ischebeck et al., 2012). A domain deletion and domain swapping study by Stenzel et al. (2012) identified regions of B-type PIP5Ks responsible for membrane localization and different overexpression phenotypes of PIP5K2 and PIP5K5/PIP5K6. These regions differ from the catalytic domain and could facilitate protein–protein interactions driving PIP5Ks in specific signalling contexts.

Besides systematic investigations of PI(4,5)P₂ and PIP5Ks in tip growth, their role in recycling of PIN auxin transporters has been addressed. Growth and gravitropic phenotypes of pip5k1 (Ischebeck et al., 2013), pip5k2 (Mei et al., 2012; Ischebeck et al., 2013), and mostly pip5k1 pip5k2 (Mei et al., 2012; Ischebeck et al., 2013; Tejos et al., 2014) double mutants indicate defective auxin transport. pip5k2 mutants showed reduced FM4-64 internalization, which could be rescued by external addition of PI(4,5)P₂. FM4-64 accumulation was more sensitive to brefeldin A (BFA, Mei et al., 2012). In the pip5k2 mutant background, PIN2–green fluorescent protein (GFP) and PIN3–GFP are more readily accumulated in BFA bodies and retained in the bodies after BFA washout (Mei et al., 2012). In the pip5k1 pip5k2 double mutant, PIN2 polarization is disrupted. Overexpression of PIP5K1 and PIP5K2 also causes auxin-related growth defects and reduced PIN2 localization to the PM with internalization/retention in bodies. Endocytotic cycling of PIN1 and PIN2 is impaired in the pip5k1 pip5k2 double mutant (Ischebeck et al., 2013). Localization of PIP5K1 and PIP5K2 in root cells is enriched at the apical and basal membrane in several root tissues (Ischebeck et al., 2013; Tejos et al., 2014). PM enrichment at the apical and basal membrane can also be observed for PI4P and PI(4,5)P₂ (Ischebeck et al., 2013; Tejos et al., 2014). Intact PI(4,5)P₂ metabolism with precise spatial distribution of PI(4,5)P₂ and PI(4,5)P₂-forming enzymes is thus important for both internalization and exocytosis of PIN proteins. It is noteworthy that the EXO70 subunit of the secretary vesicle tethering complex exocyst directly binds PI(4,5)P₂ in mammals and yeasts (He et al., 2007; Liu et al., 2007). The Arabidopsis homologue EXO70A1 has a conserved PI(4,5)P₂ binding site (Záský et al., 2009) and plays a role in PIN delivery to the PM (Janková Drdová et al., 2013).

The spatial restriction and steady-state levels of PI(4,5)P₂ can be also regulated by controlled hydrolysis via either phospholipases or phosphatases with fundamentally different physiological consequences. Cleavage by phospholipases gives rise to secondary messengers that propagate and amplify signalling, whereas dephosphorylation controls PI(4,5)P₂ steady-state levels and turns off its function. Indeed, in animal endocytosis, dephosphorylation removes PI(4,5)P₂ from internalized membranes, thus restricting the localization of PI(4,5)P₂ to the PM without coupling the endocytic reaction to the generation of signalling metabolites (Cremona et al., 1999). In the Arabidopsis genome, several genes from the 5PTase phosphatase family have been implicated in PI(4,5)P₂ breakdown. This family includes FRA3, a member of the plant type II 5PTase subfamily with highest substrate affinity towards PI(4,5)P₂, which controls actin organization and secondary cell-wall synthesis in fibre cells (Zhong et al., 2004), and 5PTase9, implicated in the regulation of endocytosis after salt stress (Golani et al., 2013). Furthermore, plants also possess the SAC (suppressor of actin) family, which contains both 4- and 5-specific phosphatase activities. Besides the previously mentioned PI4P-specific SAC7/RHD4, SAC9 is also implicated in PI(4,5)P₂ cleavage and is involved in the regulation of this lipid in stress responses (Williams et al., 2005). Other members of the SAC family were shown to cleave the 3-phosphate group and were implicated in vacuolar fusion (Nováková et al., 2014).

PI–phospholipase C (PLC) cleaves PI(4,5)P₂ into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate in a calcium-dependent manner. Although most plant PI–PLC studies have focused on its role in stress responses (Meijer and Munnik, 2003), PI–PLC was also shown to be important for the polar growth of pollen tubes. In both Petunia and tobacco, PI–PLC is localized to the PM but excluded from the very apex (Dowd et al., 2006; Helling et al., 2006). Together with the distribution of PIPK isofoms, this seem to restrict PI(4,5)P₂ distribution to the apex of tip growing cells. Intriguingly, a signalling role of DAG in plant cells is not obvious. Meijer and Munnik (2003) showed that DAG in plant cells is rapidly phosphorylated by DAG kinase to phosphatidic acid (PA), which plays active roles in plant signalling processes. However, data showing DAG as a signalling platform in plants are emerging (Helling et al., 2006; Pejchar et al., 2010; Pejchar et al., 2015).

Phosphatidic acid: simple but still powerful

PA represents the simplest glycerophospholipid, consisting of a hydrophobic DAG body and a single phosphate as the polar hydrophilic headgroup. Although PA can be produced by two distinct pathways (see Pleskot et al., 2012a), its production by proteins from the phospholipase D (PLD) family is by far the most investigated. While the functions of PA in various stress responses (Testerink and Munnik, 2011) and cytoskeletal regulation (Pleskot et al., 2012b, 2013) are well described, molecular details about its direct involvement in plant cell polarity are still scarce and are mostly confined to pharmacological studies and circumstantial evidence (Potocký et al., 2003; Monteiro et al., 2005; Pleskot et al., 2012a). In recent work, Potocký et al. (2014) visualized PA in living pollen tubes utilizing a PA biosensor derived from the PA-binding domain of yeast Spo20p. The authors found that PA localized in the subapical region of tobacco pollen tube PM and partially overlapped with the signal for PI(4,5)P₂. To the best of our knowledge, this is the only evidence of PA localization and dynamics in the living plant cells.
Interestingly, PLDζ2, a member of the PLD subfamily most closely related to animal PLDs, emerged as a regulator of auxin transport by affecting vesicle trafficking (Li and Xue, 2007). Arabidopsis pldζ2 mutants have a less pronounced gravitropic response, root growth, and hypocotyl elongation in response to external auxin than wild-type (WT) plants. Overexpression of PLDζ2 or externally added PA stimulates a root gravitropic response and FM internalization, while n-butanol, a PLD inhibitor, inhibits it. PLDζ2 overexpressors are less sensitive to the inhibitory effect of BFA on root growth, hypocotyl elongation, and gravitropism, and also form BFA bodies less frequently than WT controls. PA treatment and PLDζ2 overexpression stimulate PIN2 return to the PM from BFA bodies after BFA washout. These results show that PLDζ2-generated PA facilitates both exocytosis and endocytosis. A negative halotropic response-growth of the root away from soil regions with a high-salt concentration—was preceded by asymmetrical relocalization of PIN2 in Arabidopsis (Galvan-Ampudia et al., 2013) with PIN2 internalization by clathrin-mediated endocytosis (CME). The process involves PLDζ2-mediated clathrin light chain (CLC) recruitment (Galvan-Ampudia et al., 2013).

Minor acidic phospholipids regulate both exocytosis and endocytosis in plant cells and their local concentrations need to be fine-tuned for proper cell morphogenesis. The mechanisms involved are often unknown, but both PI(4,5)P₂ (Martin, 2014) and PA (Liu et al., 2013) directly bind various components of the exocytotic and endocytotic pathways in Opisthokont systems. Many of the interactions probably exist in plants as well. Minor acidic phospholipids also regulate cytoskeleton dynamics (Pleskot et al., 2013, 2014), which in turn affects trafficking.

Clash of sterols: from membrane rigidity to endocytic deformations

Besides selected phospholipid species, sterols have also been shown to be indispensable for proper trafficking in plant cells. Mild concentrations of filipin are suitable for sterol staining, whereas cells treated with higher concentrations sequester sterols from the membrane and inhibit sterol-dependent endocytosis (Boutté et al., 2011). Sterols are distributed along the whole PM (Boutté et al., 2011), within the cell plate (Men et al., 2008), and in various endosomal compartments (Grebe et al., 2003). Filipin staining of root hairs and pollen tubes showed enrichment in the prospective root hair outgrowth site and growing root hair and pollen tube tip (Liu et al., 2009; Ovečka et al., 2010). Proper sterol composition is required for endocytosis-driven maintenance of PIN2 polarity (Men et al., 2008). In BFA-treated cells, sterols get trapped in BFA bodies together with PIN2 (Grebe et al., 2003), and both PIN2 and FM4-64 internalization to BFA bodies is inhibited by high concentrations of filipin (Men et al., 2008). Disruption of sterol synthesis by genetic or pharmacological methods also affects post-cytokinetic sequestration of KNOLLE from the cell plate, and KNOLLE diffuses from the plate to lateral membranes in the sterol biosynthesis cpl1 mutant and in fenpropimorph-treated cells (Boutté et al., 2009; Frescatada-Rosa et al., 2014). In Picea meyeri pollen tubes, disruption of membrane sterols leads to partial shifting of NOX and reactive oxygen species production activity away from the tip (Liu et al., 2009). This is probably due to impaired endocytosis, which restricts NOX at the pollen tube tip. Use of ratiometric lipid order sensing dye (Frescatada-Rosa et al., 2014; Zhao et al., 2014) showed that the lipid order of the PM is higher than that of endosomes (Zhao et al., 2014) but lower than the lipid order of the cell plate (Frescatada-Rosa et al., 2014). A cpl1 mutant background and sterol synthesis inhibitors decrease the cell plate lipid order. Besides erroneous recruitment of endocytic machinery to the cell plate with decreasing order, the lipid order remains similar to that of the PM without functional DRP1A at the cell plate. There is thus mutual feedback between high lipid order maintenance and functional endocytosis at the cell plate (Frescatada-Rosa et al., 2014).

A storm of very-long-chain fatty acids (VLCFAs): the impact of VLCFAs

Besides head groups, the tail composition of membrane lipids plays an important role in membrane trafficking. In Arabidopsis, both exocytosis of selected membrane proteins (Markham et al., 2011) and proper cytokinesis (Molino et al., 2014) require lipids with VLCFAs. LOH1 and LOH3 ceramide synthases are responsible for VLCFA sphingolipid synthesis in Arabidopsis, and both can be inhibited simultaneously with fumonisin B1 (FB1, Markham et al., 2011; Molino et al., 2014). FB1 treatment inhibits polar auxin transport, and newly synthesized AUX1 and PIN1 accumulate in TGN-positive intracellular bodies after FB1 treatment, without reaching the PM, indicating a defect in the exocytotic pathway (Markham et al., 2011). Molino et al. (2014) showed that glycosphingolipids with VLCFAs enhance the membrane vesicle fusion rate in vitro. FB1 treatment also affects the later stages of cytokinesis and leads to accumulation of Golgi-derived vesicles at the cell plate. Fusion of RabA2 and VHAa1 normally occurring during late cytokinesis is slower in FB1-treated cells (Molino et al., 2014). Interestingly, trafficking of some membrane proteins (PIN2, aquaporin PIP2, and LTI6b) is not affected by FB1 (Markham et al., 2011).

The winds of traffic: the dynamic nature of membrane ‘microdomains’

In addition to the long-known examples of large co-existing membrane domains in a single eukaryotic cell, recent methodically advanced studies clearly demonstrate membrane heterogeneity at a smaller spatial scale and the presence of many co-existing stable membrane microdomains with distinct composition (Spira et al., 2012; Jarsch et al., 2014). For many years, the idea of such microdomains was mostly represented by the so-called ‘lipid raft theory’. As an extension to the original fluid mosaic concept of biological membranes, this postulated the existence of more rigid sterol- and sphingolipid-enriched fractions floating within
the rest of the membrane, which behaves in a fluid mosaic manner (Nicolson, 2014). Protein partitioning into these domains was originally tested biochemically by its presence in isolated non-ionic detergent-resistant fractions of the PM. The concept of lipid rafts has been criticized in terms of its possible artefact nature, and both the spatial and temporal dimensions of these microdomains have been discussed extensively. With the advent of super-resolution microscopy and other techniques allowing high spatiotemporal observations inside cells, evidence of multiple long-lived co-existing microdomains within one membrane is accumulating. As reported by Nicolson (2014), control of membrane microorganization may be more complex than previously thought, and many types of membrane-associated cytoskeletal structures probably exist, some of them being less susceptible to cytoskeleton-disrupting drugs. Microdomains could form due to partitioning into sterol- and sphingolipid-enriched membrane fractions, but interactions of proteins with other lipid species, protein–protein interactions, lipid clustering, confinement by the cytoskeleton, and other mechanisms seem to play a role in PM heterogeneity regulation at small spatial dimensions.

Increasing availability and usage of high-end microscopy approaches is bringing new insights into protein localizations in plant cells. Fine spatial information with regard to localization and partitioning into different PM microdomains can now be observed directly. TIRF/VAEM microscopy is being used increasingly to observe events near the membrane and in the cortical cytoplasm (Konopka and Bednarek, 2008). Use of super-resolution microscopy in plant science is also emerging; structured illumination was used for dynamic localization of cortical microtubules (Komis et al., 2014), and stimulated emission depletion (STED) was successfully applied to visualize remorins (Demir et al., 2013) and PIN cycling (Kleine-Vehn et al., 2011). Techniques like single-molecule tracking, fluorescence correlation spectroscopy (H. Li et al., 2011) and fluorescence energy resonance transfer/fluorescence lifetime imaging (FRET/FLIM; Jarsch et al., 2014) are also appearing in plant cell biology. We will not cover the details of high-end fluorescence microscopy techniques with regard to membrane microdomain organization, as the topic has been reviewed elsewhere (Owen and Gaus, 2013).

A number of different biochemical methods for identification of lipid–protein interactions on the micrometer scale have been described. For the first identification of novel lipid-binding proteins, lipid affinity beads assay were used to isolate candidate proteins from cell extracts followed by proteomic analysis (Lim et al., 2002). To identify less abundant lipid-binding proteins, additional purification and fractionation steps should be done (McLoughlin et al., 2013). Where a lipid-binding protein is presumed (e.g. containing a known lipid-binding domain), the lipid–protein interaction can be verified and particular lipid-binding specificity can be detected by a so-called protein–lipid overlay, a method that is fast, cheap, and requires only small amount of protein (Munnik and Wierzchowiecka, 2013). However, the main disadvantage of both methods is that the lipids presented are not in their natural form, which may lead to false results. Thus, the interaction of lipid and candidate proteins should be further confirmed by co-sedimentation with large unilamellar vesicles, a method that enables analysis of protein–lipid binding affinity and specificity (Julkowska et al., 2013).

A large-scale study comparing yeast PM proteins covering different functional categories and types of membrane anchor (Spira et al., 2012) combined TIRF microscopy and deconvolution to address protein distribution heterogeneity. The distribution of all tested proteins turned out to be heterogeneous, with different patterns ranging from patches to networks. The patterns of transmembrane proteins were stable within minutes, whereas peripheral proteins (and lipid markers) turned out to be highly dynamic. Co-localization of tested proteins was addressed and revealed the presence of many co-existing domains. Various microdomains were shown to require a specific membrane lipid composition and/or actin-based structures for their existence (Spira et al., 2012).

Recent systematic work on mapping localization of 20 Arabidopsis membrane proteins by TIRF/VAEM microscopy demonstrated multiple co-existing microdomains in plant cell PM (Jarsch et al., 2014). This demonstration of possible (and cell-type-specific) microdomain co-existence within the PM is of outstanding importance, since current practice is to use co-localization with remorin or flotillin to prove that the studied protein resides in ‘microdomains’. Therefore, the frequently used tag of ‘membrane microdomain-associated protein’ may be a rather vague category.

The mechanistic basis of localization of plant proteins into microdomains is not clear. It is possible that polar exocytosis, regulated endocytosis and restricted diffusion—the mechanisms responsible for asymmetric localization into large membrane domains—act in the sorting of proteins into microdomains as well. In the case of remorin At4g00670, FRAP experiments have indicated the stability of the microdomain and targeted delivery of the microdomain-resident protein. In Opisthokont systems, diffusion barriers at a small spatial scale can be formed by fine cortical cytoskeletal structures (Nicolson, 2014) and the decoration of cytoskeletal fibres may represent a general protein micropatterning mechanism. Indeed, remorin Atg13920 localizes into a filament-like pattern, which is sensitive to oryzalin treatment (Jarsch et al., 2014). Other mechanisms for keeping plant membrane proteins in specific microdomains may be limiting of the diffusion by binding to the cell wall or partitioning into areas of specific lipid composition and clustering with other proteins. Typically, post-translational modifications like acylations affect protein partitioning into the microdomains, as was shown for remorin SYREM1 (Konrad et al., 2014) and small GTPase ROP6 (Sorek et al., 2010).

The differential lipid composition of plant microdomains still needs to be addressed. Furt et al. (2010) reported existence of PI(4,5)P2 clusters within the PM of tobacco BY2 cells. However, such clusters may be induced solely by Triton X-100, used for membrane extraction (van Rheenen et al., 2005). The functional significance of plant membrane microdomains is often not clear. A lipid microenvironment can influence the functional properties of membrane proteins, as was shown by...
Spira et al. (2012), and is also generally known for membrane channels (Coskun and Simons, 2011). Microdomains may have distinct mechanical properties due to specific membrane composition and thus facilitate membrane deformations during membrane trafficking, and can harbour protein–protein interactions (Jarsch et al., 2014).

The concept of microdomains is of course complementary rather than exclusive to investigation of differences in membrane composition in large plant cell domains (apical, outer, lateral, etc.). The composition of large membrane domains/regions can also mirror the prevalence of a particular micro-compartment. FRAP measurements of protein kinetics in the apical membrane of Madin–Darby canine kidney (MDCK) epithelial cells by Meder et al. (2006) showed that, in comparison with the PM of fibroblasts, ‘raft’ proteins freely diffuse within the MDCK apical membrane and ‘non-raft’ proteins are dispersed into isolated microdomains within this ‘macro-domain’. The apical domain of MDCK epithelial cells thus behaves like one big ‘percolated raft’. Similarly, many plant membranes seem to contain floating microdomains enriched in sterols but in some cases, like the PM of plasmodesmata sleeves, the whole membrane seems to be enriched in sterols and has some ‘raft’-like properties (Raffaele et al., 2009). Similarly, the sterol-enriched domains at the apex of tip growing cells (Liu et al., 2009; Ovečka et al., 2010) and pathogen penetration sites (Bhat et al., 2005) could be formed by local enrichment of sterol-based microdomains.

Advanced optical microscopy in the analysis of PM dynamics

In recent years, TIRF/VAEM and spinning-disk imaging of Arabidopsis root cells has been applied to gain detailed insight into the spatial organization of membrane trafficking in plant cells. Most of the addressed process seems to be CME. Plant CME involves basic components previously described in the Opisthokont model systems [namely adaptor protein complex 2 (AP-2), clathrin triskelion, and dynamin-related proteins] driving endocytosis site selection, vesicle formation, and vesicle scission, respectively. Moreover, the recently described TPLATE complex drives endocytosis initiation in plant cells (Van Damme et al., 2011; Gadeyne et al., 2014). All of the basic CME machinery components localize into distinct puncta within the PM with some, although limited, lateral diffusion and a similar lifetime due to appearing and disappearing within the PM plane (Konopka and Bednarek, 2008; Gadeyne et al., 2014).

Despite rich evidence of the importance of minor phospholipid species in CME, plant cell biologists are only beginning to address this issue in the context of spatial organization within the membrane. Ischebeck et al. (2013) showed a decrease in CLC foci density and an increase in their size in pip5k1 pip5k2 double mutants compared with the situation in WT plants. Careful studies involving genetic and pharmacological tools and spinning-disk/TIRF/VAEM analysis of spatiotemporal phospholipid probes behaviour should be performed in future with regard to the role of minor phospholipids in the organization and kinetics of endocytosis and exocytosis.

Some novel methodical limitations might arise by addressing co-localization at the fine spatiotemporal level. For example, as pointed out by Heilmann and Heilmann (2014), interacting proteins can mask phospholipid species if they bind and make them inaccessible for phospholipid probes. Different probes may also bind particular phospholipids only in specific cellular contexts (Balla et al., 2000). Genetic and pharmacological alterations (also discussed below) might also alter the behaviour of proteins not typically interacting with affected phospholipid species. Localization and dynamics changes can be the result of altered membrane partitioning into microdomains due to compositional alterations. Studies on the widely used polarized trafficking models of PIN1 and PIN2 will also have to be supplemented more often with other membrane proteins (e.g. aquaporins, see also below), because apical and basal membranes, where most of the PIN2 and PIN1 population occurs, are not accessible for TIRF/VAEM studies and are difficult to access with spinning-disk microscopy.

Recently, R. Li et al. (2012) identified a clathrin-independent endocytosis pathway in plants, associated with flotillin 1 (Flot1). Both confocal and immunoelectron microscopy show that Flot1 is associated with the detergent-resistant fraction of the PM and does not co-localize with CLC. Knockdown of Flot1 results into a decrease of PM lipid order, so Flot1 could be required directly for the formation of sterol-enriched microdomains (Zhao et al., 2014). TIRF/VAEM-visualized Flot1 foci have limited lateral diffusion and mostly appear and disappear in the membrane plane. Flot1 and CLC foci display opposite sensitivity to methyl-β-cyclodextrin (mbC) and tyrphostin A23 (TyrA23) with CLC dynamics being altered by TyrA23 and being mbC insensitive, and with Flot1 foci dynamics being mbC sensitive. LatB and even more oyster treatment decreases the Flot1 foci diffusion coefficient (R. Li et al., 2012).

X. Li et al. (2011), Wang et al. (2013), and Hao et al. (2014) brought more insight into the spatial aspects of endocytosis, addressing both the role of CME and clathrin-independent endocytosis of selected ‘cargo’ proteins, namely PIP2,1 aquaporin, ammonium transporter AMT1;3, and reactive oxygen species regulator RbohD, respectively.

Dual-colour TIRF/VAEM observations showed that part of RbohD foci co-localize with CLC and part of the foci co-localize with Flot1 (Hao et al., 2014). NaCl treatment induces rapid RbohD internalization, and a combination of fluorescence cross-correlation spectroscopy (with CLC or Flot1) and pharmacology indicates that CME is the major pathway for internalization, but a substantial part of RbohD is also internalized via a clathrin-independent, Flot1-associated pathway. Inhibition of either pathway also increases the amount of foci at the membrane and decreases their diffusion rate under normal osmotic conditions.

AMT1;3 NH₄⁺ carrier availability at the PM is regulated by NH₄⁺ accessibility (Wang et al., 2013). A high ammonium concentration results in AMT1;3-selective endocytosis, which is preceded by aggregation of transporters into clusters. CLC- and Flot1 co-localization experiments and pharmacological (TyrA23 and mbC) treatments have demonstrated the utilization of both clathrin-dependent and
clathrin-independent pathways in AMT1;3 internalization, with the clathrin-dependent pathway being prevalent.

X. Li et al. (2011) carefully analysed single-molecule (or up to a tetrameric complex) behaviour of PIP2:1 aquaporin. Disruption of sterol-rich microdomains by mbC causes aggregation of the transporters. The sterol synthesis inhibitor fenpropimorph and sphingolipid synthesis inhibitor PPMP have a similar effect. While endocytosis accompanying constitutive PIP2:1 cycling is driven solely by the clathrin pathway, NaCl-induced endocytosis involves both CME and Flot1 pathways with the clathrin-dependent pathway being prevalent (X. Li et al., 2011).

As described above, the proteins clearly partition into sterol-dependent microdomains but also localize to other PM fractions. The universal implication suggesting protein restriction into rafts due to its presence in the detergent-resistant membrane extract might thus be an overinterpretation. It is also noteworthy that fenpropimorph treatment can influence the dynamics of CME components (Konopka and Bednarek, 2008; Konopka et al., 2008). The previously mentioned studies relied mostly on specific disruption of CME or clathrin-independent endocytosis by TyrA23 or sterol microdomain-disrupting agents, respectively. However, if sterol microdomain disruption affects CME as well, single-molecule studies of protein partitioning and endocytosis will be more difficult to interpret than is currently assumed.

Recently, Fendrych et al. (2013) performed a pioneering study of plant exocytosis spatial details, which addressed the EXO70A1, EXO84b, SEC8, and SEC6 subunits of the vesicle-tethering complex exocyst. Individual subunits co-localized in distinct highly dynamic spots different from the endocytotic puncta labelled by DRP1C. These spots did not move within the optical plane but appeared and disappeared in the plane. Most of the exocytotic foci thus do not correspond to vesicles but contain dynamic exocyst particles or membrane microdomains where these particles are targeted. The foci decrease in density and partly cluster into clumps after prolonged actin disruption. Similar localization and dynamics were also shown for the SEC3a exocyst subunit (Zhang et al., 2013). As both SEC3 and EXO70 subunits of the exocyst directly bind PI(4,5)P₂ in Opisthokont models (He et al., 2007; Liu et al., 2007; Zhang et al., 2008), PI(4,5)P₂-enriched microdomains could be important in exocyst recruitment. Detailed localization studies of exocytotic machinery components together with genetic and pharmacological disruptions of selected lipid species formation will be of outstanding importance in order to understand the role of membrane microdomains in spatial exocytosis organization.

It is also important to note that when studied only by diffraction-limited microscopy without additional methods, like single fluorophore quantification and bleaching, ‘microdomain localization’ can represent different phenomena. Diffraction-limited spots can harbour clusters of proteins (that may localize together due to partitioning into a lipid islet) but also single protein–protein complexes. Some of the patches described by Spira et al. (2012) and Jarsch et al. (2014) might thus represent randomly distributed proteins at certain density.

A dance with molecules: molecular dynamics (MD) simulations as the ‘computational microscope’

To describe the role of protein–protein interplay in cell polarity, it is important to understand the mechanistic details of lipid–lipid, lipid–protein, and protein–protein interactions. Although we have experienced major progress in experimental techniques, their resolution is still limited for the description of events that underlie processes in the biological membranes (Manna et al., 2014). Over the last 20 years, molecular simulations and especially MD have become a valuable tool to study membrane and protein systems. Together with the increase in computational power and better algorithms, MD results have reached time and length scales directly comparable with experiments and they thus represent a complementary tool to the experimental methods. Moreover, they enable us to probe the system of interest at the single-molecule level (Bennett and Tieleman, 2013).

In MD simulations, the system is represented as an ensemble of particles that follow the laws of classical physics, and time evolution of the system is obtained by the numerical solution of Newtonian equations (Manna et al., 2014). A crucial part of MD simulations is the force field, which describes interactions between parts of the simulated systems in terms of a potential energy function, which is composed of two types of interaction, bonded and non-bonded (Manna et al., 2014). Typical simulated times for all-atom simulations are hundreds of nanoseconds, but simulations reaching microseconds have been also reported (Deleu et al., 2014). The simulation times can be further increased to tens of microseconds and even to milliseconds using a coarse-grained (CG) force field, in which groups of atoms are represented as a one particle (Ingólfsson et al., 2014a).

Biophysical principles of membrane domain formation elucidated by MD simulations

CG-MD simulations have been used to address the formation of lipid nanodomains or ‘rafts’ (reviewed by Bennett and Tieleman, 2013). Several studies have shown phase separation of ternary mixtures composed of various lipid species with results matching experimental observations (Risselada and Marrink, 2008; Schäfer and Marrink, 2010; Perlmutter and Sachs, 2011). Recently, the model of the mammalian PM containing 63 different lipid species was published showing the transient occurrence of lipid domains on a microsecond scale (Ingólfsson et al., 2014b). Interestingly, the authors observed clustering of phosphoinositides in the inner leaflet of the PM. The combination of an experimental and computational approach was used successfully to study the effect of polyunsaturated lipids on mammalian endocytosis (Pinot et al., 2014). The authors found that polyunsaturated lipids increased the ability of dynamin and endophilin to deform and vesiculate membranes by reducing the energetic cost of membrane bending and fission.

The raft hypothesis suggests that the formation of lipid nanodomains would influence the distribution and activity of
membrane proteins. It was shown that ionic proteins indeed cluster with P(4,5)P2 molecules, using CG-MD and STED microscopy (van den Bogaart et al., 2011). Different small GTPases were shown to be partitioned into the specific lipid domains based on the type of their lipid anchor (Janosi and Gorfe, 2010; Z. Li et al., 2012; de Jong et al., 2013). The mechanism of membrane targeting of diverse peripheral membrane proteins or their domains has been revealed using both all-atom MD and CG-MD simulations (Stansfeld and Sansom, 2011; Pleskot et al., 2012b). Several recent papers have demonstrated the effect of protein on the distribution of specific lipids in the lipid bilayer (Kalli et al., 2013; Charlier et al., 2014; Jefferys et al., 2014). Moreover, proteins such as the BAR domain or EXO70p can induce the membrane curvature, as was shown both computationally and experimentally (Arkhipov et al., 2008; Zhao et al., 2013).

Many peripheral membrane proteins are not specific towards one lipid species but instead recognize multiple targets in the membrane leading to conditional binding (Moravecic et al., 2012). It was shown that phosphatidyethanolamine enhances protein–PA interactions by increasing the negative curvature of the membrane and thus facilitating the insertion of hydrophobic amino acid residues into the lipid bilayer (Kooijman et al., 2007). Lipid distribution is also affected by specific interactions with ions: P(4,5)P2 molecules were shown to form clusters via their interaction with divalent cations, particularly Ca2+ (reviewed by Wang et al., 2014).

A dream of exocytosis: concluding remarks

Although a substantial amount of data has been acquired about the organization, dynamics, and general importance of lipids at the macromolecular level, we still know surprisingly little about the identity and role of lipids in constituting distinct microdomains in plant cells. Over the last decade, the existence of membrane microdomains in plant cells has been firmly established, and it has been shown that there is substantial diversity in their composition, mobility, and stability. With the general availability of advanced microscopic methods and the wealth of mutants affecting polar membrane trafficking, it is necessary to cross the barrier to this other level of protein–membrane interactions studies. At the same time, recent advances in chemical biology provide us with a number of specific drugs interfering with the proteins and lipids involved in cell polarity. To this end, it will be extremely beneficial to study the effect of both established and novel chemical inhibitors at the microdomain level in living cells.

Both exocytosis and endocytosis have been shown to require specific lipid production in selected cases. It is noteworthy that most studies analysing the role of PPIs or sterols in membrane traffic are endocytosis-biased. It is often impossible to distinguish exocytosis-related defects, endocytosis-related defects, or a combination of both from each other without specific experiments. Many cell polarity phenotypes associated with altered lipid levels are thus probably the result not only of endocytosis but also of exocytosis alteration.

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