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Phospholipids across scales: lipid patterns and plant development

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Abstract:

Phospholipids are major building blocks of cell membranes and as such they have a key structural role in maintaining their integrity as a hydrophobic barrier. However, phospholipids not only have structural but also regulatory functions that are involved in a myriad of signaling pathways. Integrative approaches in plants recently revealed that certain phospholipids have distinct patterns of accumulation at the tissue or organ scales, which turned out to be important cues in a developmental context. Using examples on different phospholipid classes, including phosphatidylinositol-4,5-bisphosphate, phosphatidylserine, phosphatidylcholine and phosphatidic acid, we review how spatio-temporal lipid patterns arise at the organismal level and what are their downstream consequences on plant development.

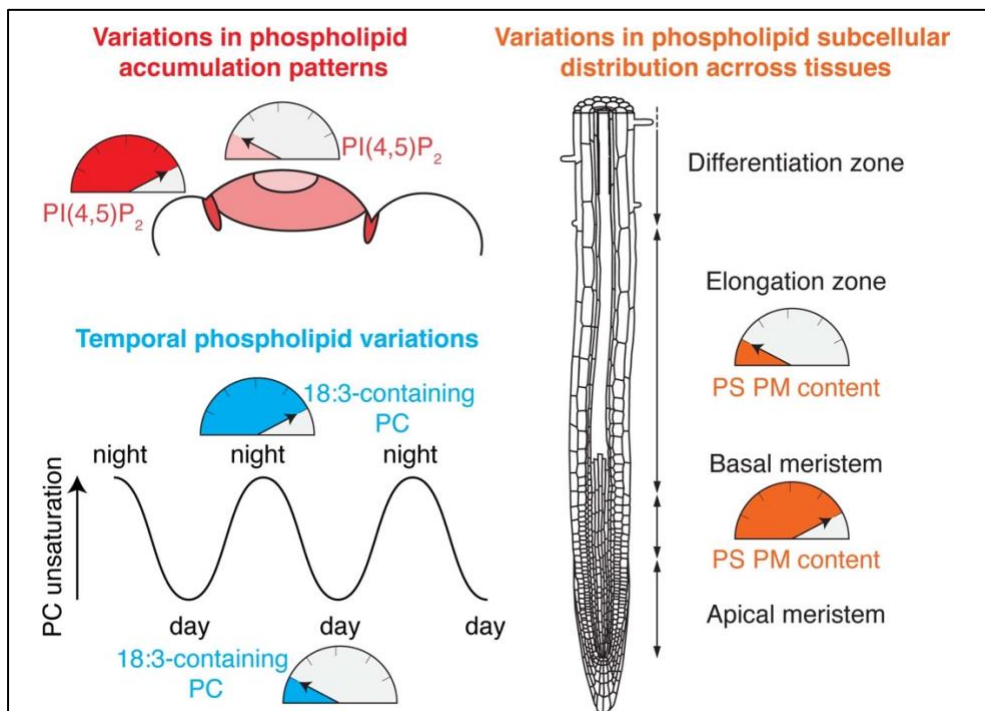
Abbreviations:

PI4P, phosphatidylinositol-4-phosphate; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate, PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol, DAG, diacylglycerol; PIPs, phosphoinositides; ER, endoplasmic reticulum; PM, plasma membrane; PH, pleckstrin homology; Lact, Lactaderin; PLC, Phospholipase C; PSS1, PHOSPHATIDYLSERINE SYNTHASE1; PIP5K, PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE; CVP, COTYLEDON VASCULAR PATTERN; CVL, CVP2-LIKE; ROP, RHO-OF-PLANTS; PECT, CTP:PHOSPHORYLETHANOLAMINE CYTIDYLYLTRANSFERASE; FT, FLOWERING LOCUS T; LHY, LONG ELONGATED HYPOCOTYL; CCA, CIRCADIAN CLOCK ASSOCIATED.

Highlights:

- PI(4,5)P₂ tissue-specific accumulation patterns control cell differentiation
- PI4P/PI4Kβs accumulate at the cell plate of dividing cells and regulate cytokinesis
- PS variations at the tissue scale regulate membrane activities at the nanoscale
- Diurnal phospholipid oscillations regulate flowering and the circadian clock

Graphical abstract:



Introduction

Phospholipids, also referred to as glycerophospholipids, are a class of lipids whose structure is based on a glycerol backbone that connects a hydrophobic tail - consisting of two long-chain fatty acids linked at the *sn-1* and *sn-2* position (diacylglycerol) - and a hydrophilic head group (Figure 1a). Because of their amphiphilic nature, phospholipids are key structural components of membrane bilayers, with the fatty acid chains aggregating together in the core of the bilayer and the hydrophilic heads on both sides. The simplest phospholipid class, with only a phosphate group at position *sn-3*, is phosphatidic acid (PA). The phosphate head group can be further modified to form the different classes of phospholipids, by addition of a choline, serine, ethanolamine, inositol or glycerol molecule, forming phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) or phosphatidylglycerol (PG), respectively (Figure 1a). PG main functions are in plastids and mitochondria and will not be discussed hereafter [1]. Phosphatidylinositol can be further phosphorylated on the 3rd, 4th and/or 5th position of the inositol head group to form phosphoinositides (PIPs, also referred to as polyphosphoinositides) such as phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) (Figure 1a) [2].

There are different ways to classify phospholipids based on for example: i) their relative abundance (abundant vs minor lipids, i.e. less than 5% of total phospholipids), ii) their net charges (neutral vs anionic), iii) their metabolic fluxes (relatively stable concentration vs acute production/interconversion and fast turn-over), iv) their site of production (produced at the site of action or exported), v) their shape (conical, straight or inverted conical) or vi) their fatty acid chains (number of carbons:number of unsaturation) (Figure 1a and b). Overall, abundant phospholipids (e.g. PC, PE) are often considered as “structural” lipids in the sense that they are essential to establish a hydrophobic barrier. These phospholipids are produced by biosynthetic enzymes mainly in the endoplasmic reticulum (Figure 1b and c). Low abundant lipids (e.g. PA and PIPs) are labelled as “signaling” lipids in the sense that they mostly have regulatory effects on membrane functions. These signaling lipids are negatively charged, which enable specific interactions with proteins. In addition, they have fast turn-over and can be produced in an acute manner by lipid kinases, phosphatases or phospholipases at their site of action outside of the endoplasmic reticulum (Figure 1b and c) [2].

However, the frontiers between the “structural” and “signaling” lipids are not always clear-cut. Indeed, these categories mostly arise from considerations at the cellular scale, initially drawn from studies using single cell systems such as yeast or in vitro cell culture. As such,

phospholipid biosynthesis and localization are often depicted in a single cell. However, recent evidence suggests that specific pattern of phospholipid accumulation arise at the tissue or organ level in plants, particularly for classical “signaling” lipids but also for “structural” lipids. These patterns have downstream consequences on cell behaviors ultimately impacting plant development. Here, we review three examples in which spatiotemporal phospholipid pattern impacts plant development in different ways, highlighting the importance of integrative approaches to study phospholipid biology.

Phosphatidylinositol-4,5-bisphosphate patterns and their roles in tissue differentiation

Phosphoinositides are minor phospholipids, making less than 1% of total phospholipids (Figure 1a). They have specific accumulation patterns in the different compartments of the cell and as such they determine organelle identity [2,3]. Furthermore, because of their respective charges and shape, they are key determinants of membrane physicochemical parameters (Figure 1b) [2]. Some of these parameters appears to be invariant between different tissues. For example, in all cell types analyzed so far, the plasma membrane is the most electronegative compartment, a feature that specifies its identity [4]. The strong electrostatic field of the plasma membrane is powered by the accumulation of PI4P in this membrane, the most abundant phosphoinositide species in plants (Figure 1a) [4]. PI4P accumulation at the plasma membrane appears to be invariant when analyzing different tissue and organ [4]. However, during cell division, PI4P strongly accumulates at the cell plate, which is therefore highly electrostatics [4][5]. The accumulation of PI4P at the cell plate correlates with the localization of PI4K β 1 in this organelle and the double mutant *pi4k β 1 β 2* is defective in cytokinesis and phragmoplast establishment [6,7]. By contrast, PI(4,5)P₂ is not only patterned at the level of the cell but also at the tissue/organ scales.

Evidence for PI(4,5)P₂ patterning at the tissue scale comes from quantitative imaging studies of PI(4,5)P₂ sensors lines [8]. These transgenic lines stably express PI(4,5)P₂ binding domains fused to a fluorescent protein as an indirect way to localize this phospholipid [9-12]. Indeed, such fusion proteins are recruited to membranes that accumulate this lipid [12]. By contrast, in the absence of its target lipid, the sensor accumulates in the cytoplasm as a default localization [12]. In some cases, the chronic absence of the biosensor cognate lipid induces its subsequent destabilization and degradation. For example, the C2 domain of the bovine Lacthaderin protein (C2_{Lact}), a PS-binding domain, is unstable and degraded when expressed in a mutant background that lacks PS biosynthesis [13]. Quantitative 3-D imaging revealed that the PI(4,5)P₂-binding Plekstrin Homology (PH) domain of Phospholipase C (PH_{PLC}) accumulates

to a low extent at the center of the shoot apical meristem in Arabidopsis, while it is stabilized in the boundary region (Figure 2a) [8]. By analogy to the C2_{Lact} sensor, one may speculate that destabilization of PH_{PLC} occurs when PI(4,5)P₂ levels are low. This hypothesis was recently substantiated in cotyledons, as PH_{PLC} accumulates about four fold when seedling are treated with a high concentration of NaCl (a condition known to induce PI(4,5)P₂ synthesis [10]) or U-73122 (which inhibits PI(4,5)P₂ hydrolysis by phosphoinositide-specific-PLC (PI-PLC) and thereby promotes its accumulation) [14]. Although the exact role of the PI(4,5)P₂ pattern in the shoot apical meristem needs to be defined, it could be involved in the two main functions of the meristem: stem cell maintenance and organogenesis. For instance, in the *clv3-17* mutant, which displays a larger central zone with more stem cells, the PI(4,5)P₂ pattern tends to disappear, suggesting that destabilization of the PH_{PLC} sensor correlates with stem cell maintenance [8]. The shoot apical meristem is also characterized by a typical mechanical stress pattern, with strong and highly anisotropic tensions in boundaries compared to the central zone (Figure 2a) [15]. A positive correlation between the accumulation of PI(4,5)P₂ sensors and mechanical stresses in the boundary zones suggests that the PI(4,5)P₂ pattern could be linked with tissue mechanics and mechanical signal transduction [8]. Future work will reveal how this PI(4,5)P₂ pattern is established and what its function is in shoot meristem biology.

Lipid sensors are not the only clues pointing at the importance of specific PI(4,5)P₂ patterns at the tissue level. Indeed, PI4P 5-kinases (PIP5K), PI(4,5)P₂ 5-phosphatases and PI-PLCs have tissue specific expression patterns [16-21]. For example, *PIP5K1*, *PIP5K2*, *PLC3* and *PLC5* are expressed in the root meristem and in vascular tissues and *PIP5K2* expression is regulated by the phytohormone auxin [16-18][19,20]. Furthermore, the plasma membrane-localized 5-phosphatase *COTYLEDON VASCULAR PATTERN2* (*CVP2*) is expressed in developing vascular cells in various organs and displays exquisite expression specificity for the protophloem in the root [22-25]. *CVP2* and its homolog *CVP2-LIKE1* (*CVL1*), as well as *PIP5K1* and *PIP5K2* are involved in venation pattern establishment in cotyledons, while *plc2*, *plc3* and *plc5* mutants have root architecture phenotypes [17,19,20,22]. The *cvp2 cvl1* double mutant root protophloem fails to fully differentiate, as revealed by the presence of undifferentiated “gap” cells within protophloem strands (Figure 2b) [25]. Surprisingly, *CVP2* overexpression, which promotes PI(4,5)P₂ processing into PI4P, also induces protophloem gap cells and phenocopies the *cvp2 cvl1* double mutant, which accumulates PI(4,5)P₂ (Figure 2b) [25]. Furthermore, inducible overexpression at the plasma membrane of a highly processive PIP5K, which boosts PI(4,5)P₂ production at the expense of the PI4P pool, also induces gap cells in developing protophloem (Figure 2b) [24]. Therefore, it appears that the PI(4,5)P₂/PI4P

balance, specifically in protophloem cells, rather than the absolute amount of PI(4,5)P₂ is critical for the correct differentiation of this tissue (Figure 2b).

Cellular analyses revealed a correlation between increased vacuolar trafficking upon the alteration of the PI(4,5)P₂/PI4P ratio and protophloem differentiation, suggesting that vacuole biogenesis and/or related endocytic trafficking regulated by these phosphoinositides could be linked with phloem development [24]. Furthermore, PIP5K1 and PIP5K2 are not only localized at the plasma membrane but also in the nucleus [17,18]. Nuclear targeting is actively regulated, at least in the case of PIP5K2 [26]. These localizations suggest that PI(4,5)P₂ could also have a direct role in gene expression regulation, which has been reported in animal systems [27,28]. Overall, these data indicate that the establishment of specific PI(4,5)P₂ accumulation pattern at the tissue level are hallmarks of tissue differentiation, at least in some cases. Some of the future challenges will be to sort which of the pleiotropic functions of this signaling lipids could be instrumental to regulate tissue differentiation.

Phosphatidylserine variations at the tissue level regulate plasma membrane organization at the nanoscale

PS shares characteristics with both above-mentioned phospholipid categories. Similar to “structural” phospholipids, it is synthesized in the endoplasmic reticulum by a single enzyme called PHOSPHATIDYLSERINE SYNTHASE 1 (PSS1) in Arabidopsis and PS is not acutely produced in compartments outside of the endoplasmic reticulum (Figure 1b and c) [29]. However, it is acidic, interacts with specific proteins and is present at low levels (i.e. from 1 to 5% of total phospholipids depending on the tissue [29]), which is a hallmark of “signaling” lipids (Figure 1a and b) [2]. PS is produced in the luminal leaflet of the endoplasmic reticulum and is then actively orientated by flippases in the cytoplasmic membrane leaflet [30]. This regulation is organelle specific, which allows the accumulation of PS in the cytoplasmic leaflet of post-Golgi compartments [30]. Localization studies using PS specific biosensors revealed that it accumulates at the plasma membrane and all along the endocytic pathway in Arabidopsis [4,13]. This accumulation gradient is critical to define an “electrostatic” membrane territory within the cell, which specifies the identity of plasma membrane-derived organelles [13].

Interestingly, the relative accumulation of PS biosensors at the plasma membrane or endosomes varies during root epidermis differentiation (Figure 3a) [31]. Indeed, PS biosensors mostly accumulate at the plasma membrane in meristematic cells, while they partition more evenly between the cell surface and endosomes in differentiating epidermal cells (Figure 3a). Experimental manipulation of the PS content by knock-out, knock-down or overexpression of

the Arabidopsis *PSSI* gene leads to the graded activation of plasma membrane auxin signaling pathway, which is regulated by the small GTPase RHO-OF-PLANTS6 (ROP6) [31,32]. Interestingly, PS is dispensable for the proper targeting of ROP6 to the plasma membrane [31]. However, super resolution microscopy and single molecule imaging revealed that PS is required for the auxin-induced clustering and stabilization of ROP6 in plasma membrane nanodomains (Figure 3b and c). ROP6 stabilization in nanoclusters is required for downstream auxin signaling. This effect is dose-dependent, with no stabilization of ROP6 in nanodomains in the absence of PS and therefore no downstream signaling, even when ROP6 is constitutively active (Figure 3c). By contrast, ROP6 is hyper stabilized in nanodomains in response to auxin in *PSSI* overexpressing plants (Figure 3c). Accordingly, these plants have the same phenotypes as plants expressing a constitutive active version of ROP6, including the chronic inhibition of endocytosis and a hypergravitropic root response [31].

One of the future challenges will be to decipher how is the PS pattern developmentally-controlled during cell differentiation, a process which could involve lipid flippases or regulation of PS transfer at membrane contact sites, which are close (below 30nm) and stable apposition of two membranes involves, among other functions, in lipid exchanges [30,33-36]. Furthermore, PS itself is present in plasma membrane nanodomains, and single molecule tracking experiments revealed that these PS molecules are slow moving within the plane of the plasma membrane [31]. This raises the question of how a phospholipid may be immobilized within a membrane and whether this process could also be regulated at the tissue scale.

Temporal phospholipid patterns

The last example is perhaps the least expected as it entails PC, an archetype “structural” lipid. Indeed, transgenic lines with imbalanced PE/PC ratio, obtained by inducible knock-down or overexpression of CTP:PHOSPHORYLETHANOLAMINE CYTIDYLYLTRANSFERASE1 (PECT1, a rate limiting enzyme in PE biosynthesis), revealed that PC levels at the shoot apex correlate with flowering time: lines with heightened PC content flower early, while plants with reduced PC levels are late flowering (Figure 4a) [37,38]. The early flowering time phenotype observed in plants with heightened PC content is dependent upon expression of FLOWERING LOCUS T (FT), a master regulator of flowering time [39]. Interestingly, FT directly binds to PC but not PE in vitro [37]. Furthermore, PC but not PE oscillate depending of the time of day, with specific molecular species of PC showing various temporal pattern of accumulation (Figure 4b). For example, PC species containing less unsaturated fatty acids are predominant

during the day, while PC species with linolenic acid (18:3) increase during the night (Figure 4b). In vitro, PC species with linolenic acid bind poorly to FT by contrast to less unsaturated PC species [37]. Transgenic plants that overexpress FATTY ACID DESATURASE3 (FAD3) show high content in PC species with 18:3 fatty acids and are late flowering (Figure 4b) [37,40]. Together these data suggest that FT preferentially interacts with the PC species that accumulate during the day to promote flowering (Figure 4b) [37]. However, it remains unclear how PC contributes to FT activity at the molecular level and how FT discriminates the different PC species based on their degree of unsaturation.

Of note, PC is not the only phospholipid showing temporal pattern of accumulation; and some molecular species of PA or PS also cycle diurnally [41]. These cycling are not just regulated by the light/dark cycle but they are under direct circadian regulation and are abolished in clock mutants [42]. Interestingly, PA itself regulates the core clock components LONG ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED1 (CCA1). PA directly binds to both LHY and CCA1, which inhibits their DNA binding capacity [42]. Together these results suggest that the interaction of PA with LHY/CCA1 may function in a negative feedback loop to integrate the circadian clock with lipid metabolism (Figure 4c).

Concluding remarks and future perspectives

The examples highlighted in this review show that phospholipid patterns are multiscales, from membrane nanodomains up to the organismal scale, with specific phospholipid patterns arising according to the cell type, the tissue differentiation status or the time of day. Tissue-specific control of lipid patterns is an emerging concept, but its exploration is somewhat limited by our ability to monitor their dynamics and crosstalks. By design, genetically encoded phospholipid biosensors reveal lipid subcellular localization, but not their respective accumulation [12]. As such, these biosensors are suited to detect changes in phospholipid localization, as exemplified for PS [31]. However, if the levels of phospholipids vary but not their subcellular localization, the current tools are not quantitative enough to measure these variations with spatial resolution. In some cases, the stability of the lipid sensor is dependent on the quantity of this lipid within the cell. However, we do not understand the mechanistic bases for such lipid-induced stabilization/destabilization. We therefore urgently need new methods to image and quantify phospholipid accumulation and turnover with tissue and cellular resolution. Such methods could include for example the design of new quantitative lipid sensors, the use of lipid immunolocalization or mass spectrometry imaging of lipid species. The latter is particularly promising since it can not only discriminate different phospholipids but also their various

unsaturation degree [43-46]. However, this technique is still limited to very abundant phospholipids, such as PC. In addition, the best resolution reported in plant samples to date is ~10 μm , which is sufficient for analyses at the organ/tissue scales but not cellular/subcellular levels [43-46].

While we begin to uncover some of the enzymes involved in lipid patterning at the tissue scale, the next challenge will be to decipher what are the downstream targets that respond to these patterns, and what are the developmental consequences. This is a particularly daunting task since phospholipids have pleiotropic targets and are therefore expected to affect the localization or activity of many effector proteins. Pinpointing relevant interactions will therefore require in depth biochemical knowledge of the protein/lipid binding interfaces in order to modulate these interactions both from the lipid but also protein sides. At the same time, such efforts will require integrative biology approaches to blend deep biochemical knowledge into their cellular and developmental contexts.

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This work shows that PIP5K2 is not only localized at the plasma membrane but also in the nucleus. PIP5K2 is actively addressed to the nucleus by a functional nuclear localization signal, and interacts with alpha-importin. Furthermore, using immunolocalization, this study shows that both PI4P and PI(4,5)P₂, which are the substrate and product of PIP5K2, respectively, are present in the nucleus, more specifically the nucleoplasm. Together, this work opens the possibility that some of the phenotypes attributed to PI(4,5)P₂ may be due to gene expression regulation rather than membrane-related events.

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Figures

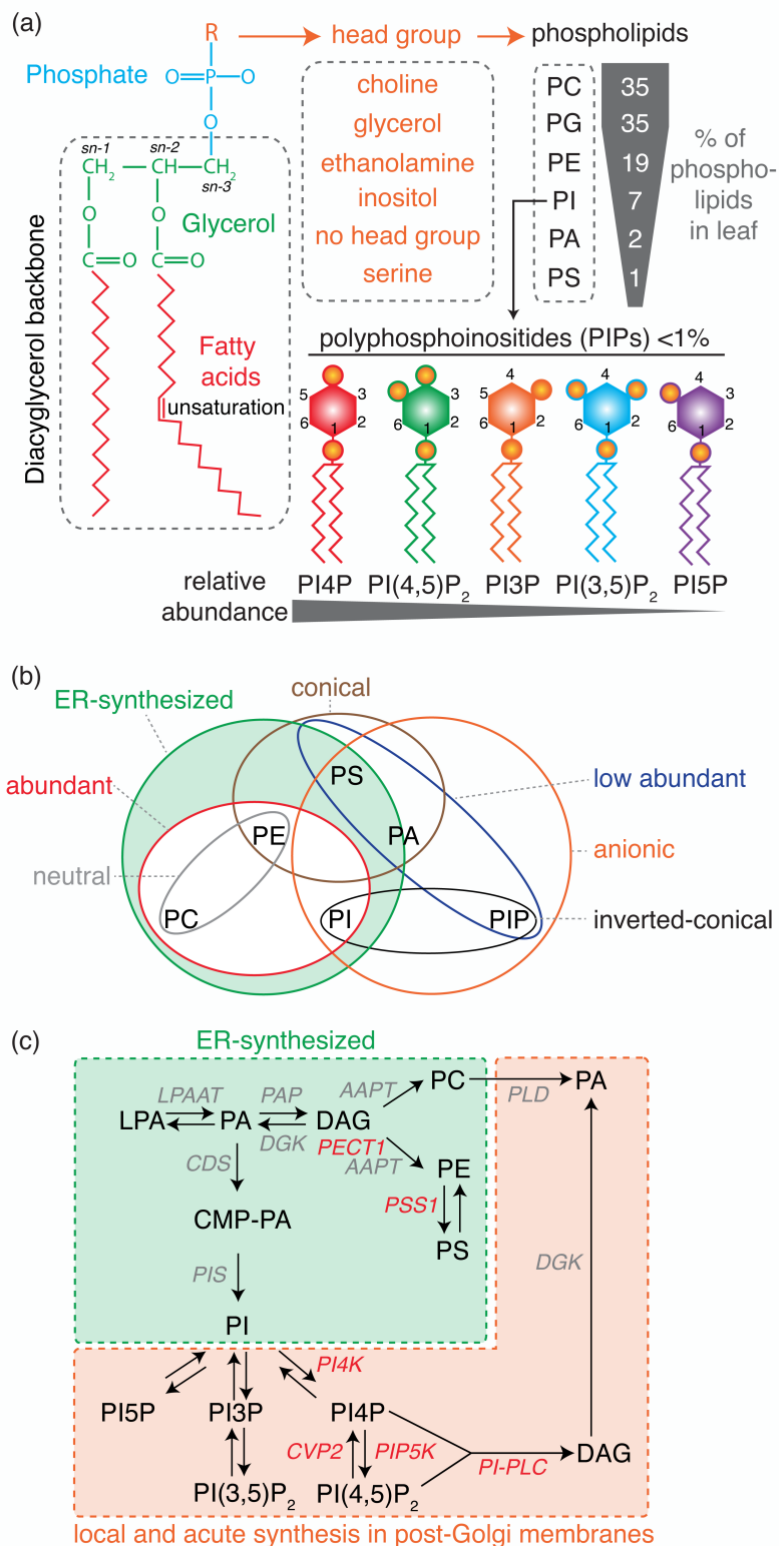


Figure 1: Phospholipids properties and biosynthetic pathways. (a) Schematic representation of phospholipid general structure, with the relative amount of each phospholipid species depicted as measured in Arabidopsis leaf (data compiled from [29,47]). (b) Euler diagram illustrating phospholipid properties. (c) Phospholipid biosynthesis pathway. Note that phospholipid synthesis pathway in plastid/mitochondria are not depicted. The main rate-

limiting enzymes involved in phospholipid interconversion are indicated (enzymes in red are discussed in this review). Abbreviations: DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PI3P, phosphatidylinositol-3-phosphate; PI4P, phosphatidylinositol-4-phosphate; PI5P, phosphatidylinositol-5-phosphate; PI(3,5)P₂, phosphatidylinositol-(3,5)-bisphosphate; PI(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; LPAAT, LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE; PAP, PA PHOSPHATASE; AAPT, AMINOALCOHOL AMINOPHOSPHOTRANSFERASE; DGK, DAG KINASE; PECT, CYTIDINE TRIPHOSPHATE:PHOSPHORYLETHANOLAMINE CYTIDYLTRANSFERASE; PSS, PS SYNTHASE; CDS, CYTIDINE DIPHOSPHATE-DAG SYNTHASE; CMP-PA, CYTIDINE MONOPHOSPHATE PA; PIS, PI SYNTHASE; PI4K, PI4-KINASE; PIP5K, PI4P 5-KINASE; PLC, PHOSPHOLIPASE C; PLD, PHOSPHOLIPASE D.

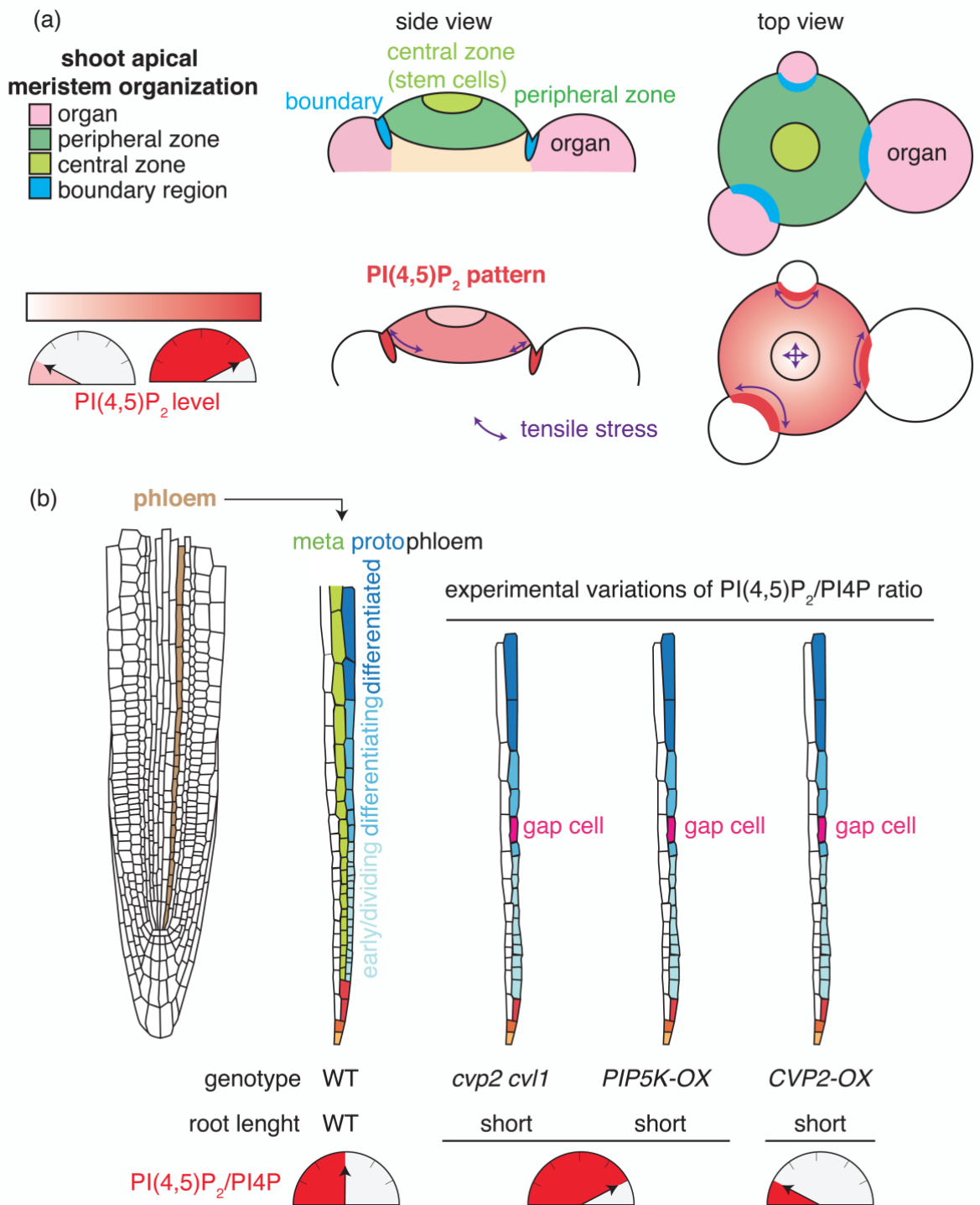


Figure 2: PI(4,5)P₂ patterning in meristematic tissues and impact of imbalanced PI(4,5)P₂/PI4P ratio on protophloem differentiation. (a) Schematic representation of the shoot apical meristem organization (top) and PI(4,5)P₂ accumulation pattern together with the predicted tensile stress pattern (bottom). (b) Schematic representation of protophloem differentiation in Arabidopsis root meristem and phenotype associated with experimental variations in the PI(4,5)P₂/PI4P balance. Root schematic was modified from B. Peret: https://figshare.com/articles/Primary_and_lateral_root_ai/5143987

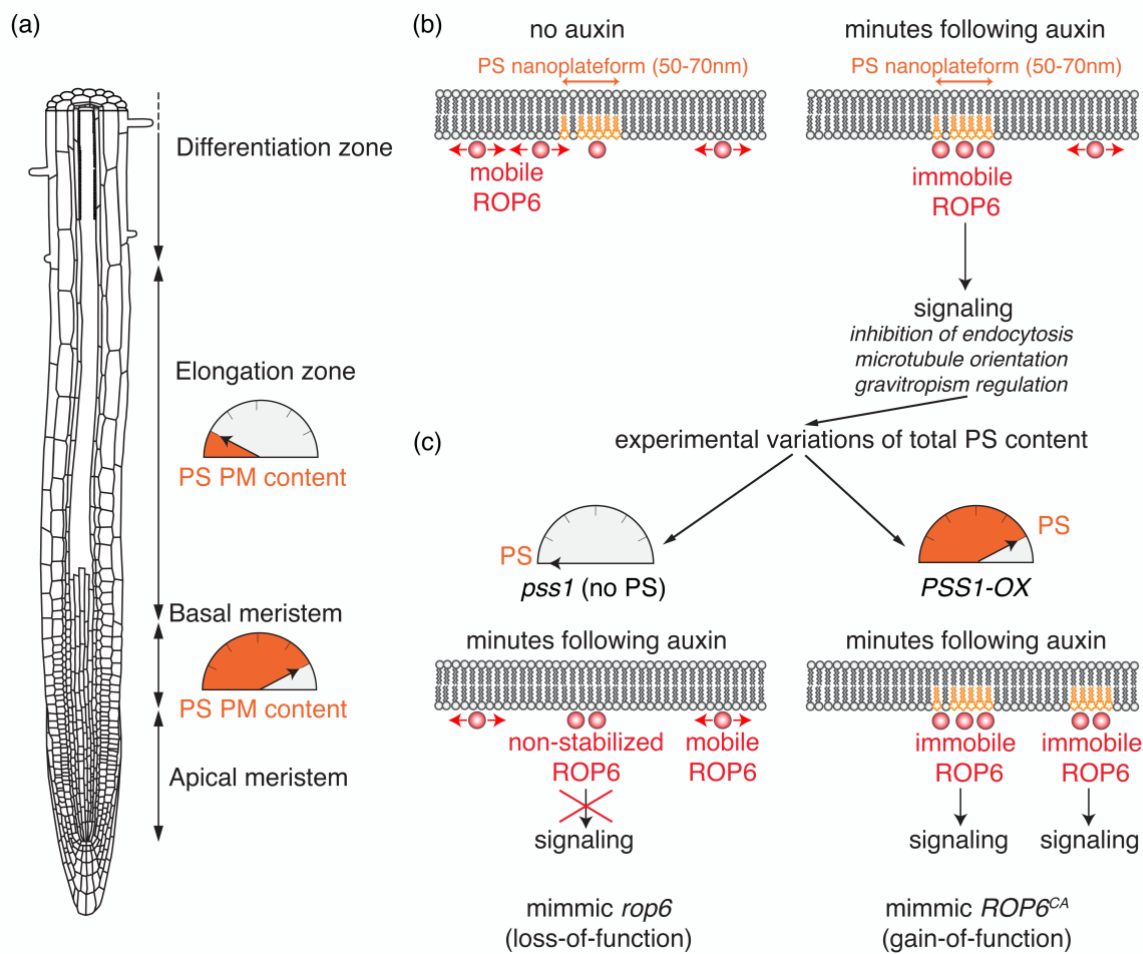


Figure 3: Impact of PS variations on ROP6-mediated auxin signaling. (a) Variation of PS content at the plasma membrane during root differentiation. (b) Stabilization of ROP6 into PS-containing nanodomains upon auxin treatment and (c) impact of experimental variations of total PS content on ROP6 nanoclustering and downstream auxin signaling. Root schematic was modified from B. Peret: https://figshare.com/articles/Primary_and_lateral_root_ai/5143987

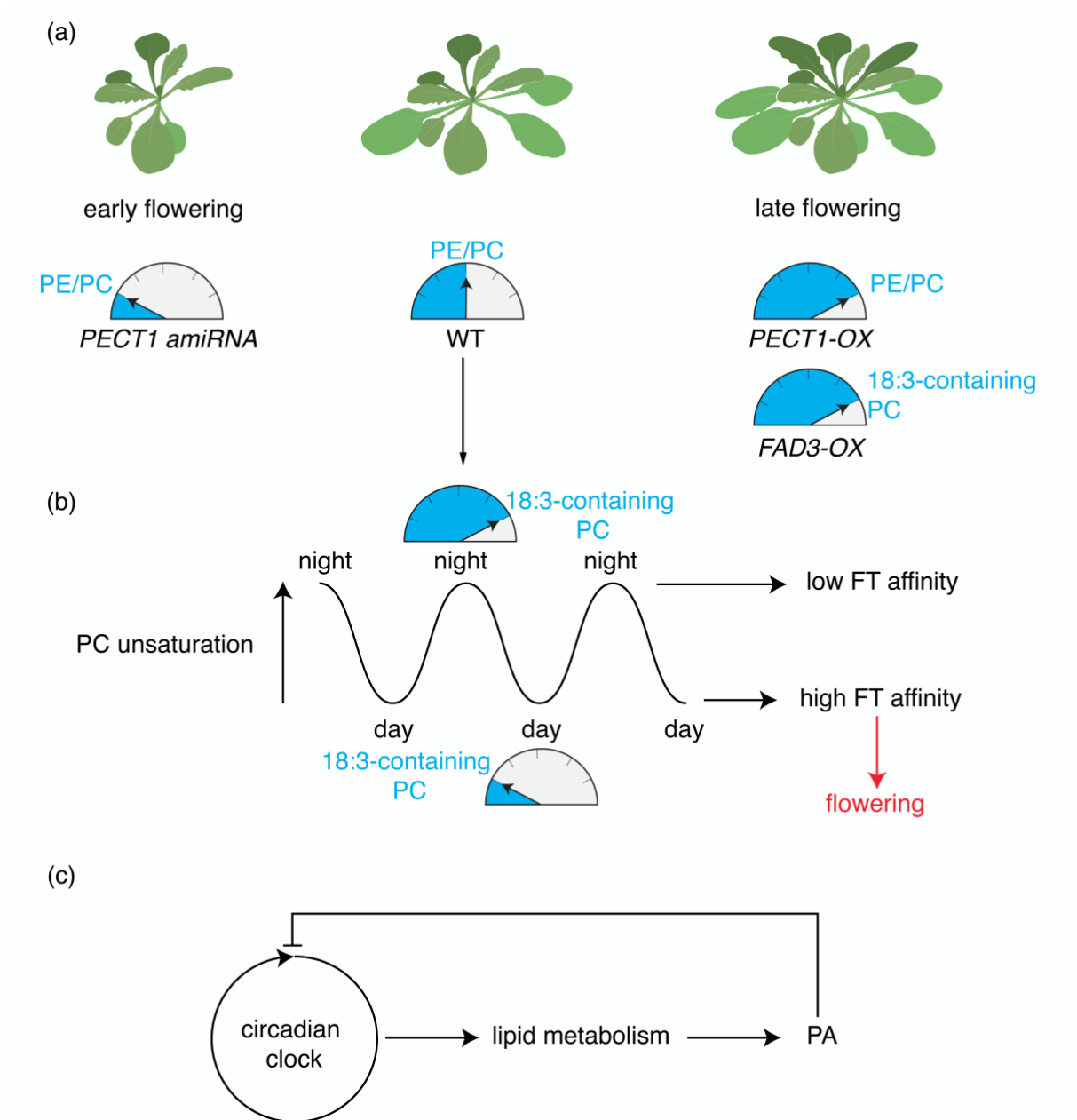


Figure 4: Impact of PC variations on flowering time and of PA on circadian clock. (a) Impact of experimental variations of the PE/PC ratio on flowering time and (b) diurnal variations of polyunsaturated 18:3-containing PC. (c) Feedback regulations between the circadian clock and PA. Arabidopsis rosette schematic was modified from F. Bouché: https://figshare.com/articles/Arabidopsis_-_Rosette_drawing_steps/4688839