Metabolic Cellular Communications: Feedback Mechanisms between Membrane Lipid Homeostasis and Plant Development

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Abstract:

Membrane lipids are often viewed as passive building block of the endomembrane system. However, mounting evidences suggest that sphingolipids, sterols and phospholipids are specifically targeted by developmental pathways, notably hormones, in a cell or tissue specific manner to regulate plant growth and development. Targeted modifications of lipid homeostasis may act as a way to execute a defined developmental program, for example by regulating other signaling pathways or participating in cell differentiation. Furthermore, these regulations often feedback on the very signaling pathway that initiated the lipid metabolic changes. Here, we review several recent examples highlighting the intricate feedbacks between membrane lipid homeostasis and plant development. In particular, these examples illustrate how all aspects of membrane lipid metabolic pathways are targeted by these feedback regulations. We propose that the time has come to consider membrane lipids and lipid metabolism as an integral part of the developmental program needed to build a plant.

Blurb: Boutté and Jaillais present a Review discussing the importance of membrane lipids in regulating plant growth and development. They consider the intricate feedbacks between membrane lipid homeostasis and plant development and propose that membrane lipid metabolism is an integral component of the developmental program needed to build a plant.

Introduction

Lipids are essential building blocks of all biological membranes. They have strong structural roles to maintain the integrity and function of these membranes. This includes for example the ability of membranes to act as impermeable barriers, but also the modulation of their physicochemical properties, including elasticity, fluidity, deformability or thickness (Bigay and Antonny, 2012; Boutte and Moreau, 2014; Holthuis and Menon, 2014). The endomembrane network is a structurally-linked membrane system in eukaryotic cells—either by direct membrane connections or vesicular trafficking. As such, the endomembrane system includes the endoplasmic reticulum and connected nuclear envelop, the Golgi apparatus and the *trans*-Golgi Network (TGN), endosomes, vacuoles/lysosomes and the plasma membrane. The endomembrane system is composed of three main lipid classes: sphingolipids, sterols and phospholipids (see Box 1). While the same three lipid classes are present in the endomembrane system of all eukaryotes, plants present some specificity, with for example the existence of phytosterols, specific headgroups in sphingolipids, or the incorporation of very-long-chain fatty acids (VLCFAs) in the phospholipid phosphatidylserine in addition to sphingolipids (Box 1) (Jaillais and Ott, 2020; Mamode Cassim et al., 2019; Murata et al., 1984). Additionally, there is a high degree of variability in the lipid composition of each compartment of the endomembrane network (Holthuis and Levine, 2005; Noack and Jaillais, 2017). This diversity is critical to establish the respective identity of each organelle, but also to ensure their specific function. For example, the lipids in the endoplasmic reticulum tends to be unsaturated and cone-shaped while the level of cholesterol is relatively low; this lipid composition creates a loose lipid packing (Bigay and Antonny, 2012; Boutte and Moreau, 2014; Holthuis and Menon, 2014). Oppositely, the plasma membrane contains more saturated cylinder-shaped lipids while the level of cholesterol is relatively high; this creates a tight lipid packing. Moreover, the ER is enriched in highly curved tubules which induce lipid packing defects favorable to translocation of proteins across the membrane, consistent with the function of this endomembrane compartment as a protein production center (Bigay and Antonny, 2012; Holthuis and Menon, 2014). By contrast, the membrane curvature of the plasma membrane is lower which limits lipid packing defects and creates a more impermeable membrane, consistent with the function of this endomembrane compartment as a barrier to the outside cellular world (Bigay and Antonny, 2012; Holthuis and Menon, 2014).

Lipid variability within membrane is enormous and comes not only from variations in the relative amount in each lipid class, sphingolipids, sterols and phospholipids but also from differential composition in their carbon chains and head groups. Indeed, they can harbor many different head groups with sometime drastically distinct properties, they can have acyl-chains of different sizes and with varied degree of unsaturation and hydroxylation (Box 1) (Holthuis and Menon, 2014; Mamode Cassim et al., 2019). Because of the importance of lipids for the

organization of the endomembrane system, changes in the equilibrium between, or within, lipid classes have strong impact on cellular functions. As such, the underlying metabolic pathways that drives the variations in membrane lipid composition are highly regulated. Importantly, these regulations are not only happening at the cell level but also within the context of an entire plant. Indeed, in addition to the lipid variability observed at the cellular scale, the membrane lipid composition also varies at the developmental scale, for example according to the organs, tissues and even cell types (Colin and Jaillais, 2019; Li et al., 2019; Stanislas et al., 2018). This suggest that membrane lipid metabolism is regulated by developmental pathways and that variations in membrane lipid homeostasis may impact plant development in turn. Here, we review the recent evidences that support the importance of membrane lipids in plant development with a focus on phytohormone responses. In particular, we will illustrate, using chosen examples mainly from Arabidopsis root, how developmental pathways target lipid metabolism to control growth and morphogenesis and in turn how modifications of membrane lipids feedback on these signaling pathways. These examples illustrate how all aspects of membrane lipid metabolic pathways are targeted by these feedback regulations, including: fatty acyl chain length and saturation degrees, lipid synthesis and breakdown, the role of metabolic intermediate or the rapid interconversion of lipid head groups.

Acyl-chain length of fatty acids is a membrane orchestrating factor of hormonal-induced development programs

Cell specification is a key process in plant development that occurs at defined spots of a tissue layer. It results in the regulation of cell proliferation, through modulation of cell division, and induction of cell pluripotency, an instrumental process in acquisition of founder cell identity and production of new organs. While transcriptional regulation networks have been well studied during *de novo* developmental processes primed by the phytohormone auxin, such as generation of new floral primordia in the shoot apical meristem or new lateral root primordia from the primary root, little attention has been given to how these transcriptional changes translate into cellular processes. Both the shoot apical meristem and lateral root primordia are excellent experimental systems to explore *de novo* generation of a new organ from founder cells. The shoot apical meristem is composed of several cell layers including the outermost epidermal L1 cell layer and more internal tissues involved in cell proliferation. In a genetic screen for *Arabidopsis* mutants displaying ectopic cell proliferation, the *pasticcino* (*pas*) mutants were first identified (Faure et al., 1998; Vittorioso et al., 1998). *PAS* genes are involved in VLCFAs synthesis. VLCFAs are included into a restricted number of membrane lipid classes, i.e. sphingolipids and phosphatidylserine (Box 1), or into extracellular lipids such as suberin and waxes (Delude et al., 2016a; Li-Beisson et al., 2013; Murata et al., 1984; Wattelet-Boyer et al., 2016). VLCFA are synthesized in the endoplasmic reticulum by the fatty acid elongase complex

that catalyzes rounds of 2-carbon elongation in a 4-step mechanism. The elongase complex is made of a 3-KETOACYL-COA SYNTHASE (KCS), a 3-KETOACYL-COA REDUCTASE (KCR), a 3-HYDROXYACYL-COA DEHYDRATASE (HACD), and a TRANS-2,3-ENOYL-COA REDUCTASE (ECR) (Haslam and Kunst, 2013). PASTICCINO2 (PAS2) is a HACD while PASTICCINO1 (PAS1) physically interacts with the elongase complex core members KCR1, PAS2 and ECR/CER10 to favor VLCFAs synthesis (Roudier et al., 2010). Hypertrophy of apical parts and uncoordinated cell division in the *pas* mutants were originally correlated to hypersensitivity to the phytohormone cytokinin (Faure et al., 1998; Vittorioso et al., 1998). Later on, it was shown that expression of *PAS2* is restricted to the outermost L1 epidermal cell layer of the shoot apical meristem (Nobusawa et al., 2013). Importantly, the *pas2* mutant causes decreased VLCFAs synthesis in the epidermal cell layer of the shoot meristem, increased synthesis of cytokinin and overproliferation of cells in internal tissues of the shoot apical meristem (Nobusawa et al., 2013). Hence, VLCFAs regulate auxin-induced shoot apical meristem development through repression of cytokinin synthesis in a non-cell autonomous manner. In *Arabidopsis* root, *PAS2* expression is restricted to the internal endodermis cell layer while expression of a second HACD, the *PROTEIN TYROSINE PHOSPHATASE-LIKE* (*PTPLA*), is confined to root vascular tissue and pericycle cells (Morineau et al., 2016). Interestingly, PAS2-dependent fatty acyl elongation is enhanced in absence of PTPLA which suggests that a non-cell autonomous signal transit between the pericycle and the endodermis, similarly to what was observed in the shoot apical meristem (Morineau et al., 2016). The physiological function of this pericycle/endodermal communication through VLCFAs has been elegantly addressed during induction of cell pluripotency, which is essential for new organ generation, especially during formation of lateral root primordia which originates from auxin-induced initiation site in the pericycle (Figure 1). Interestingly, on the one hand, the condensing enzymes KCS2, KCS20 and KCS1, important for >C20 fatty acyl elongation, are specifically expressed in root endodermis (Lee et al., 2009; Shang et al., 2016). On the other hand, KCS1-mediated VLCFAs production has been shown to restrict pericycle cell proliferation and lateral root primordia development (Figure 1) (Shang et al., 2016; Trinh et al., 2019). It is thought that restriction of lateral root primordia development in the pericycle by VLCFAs production in the endodermis partly relies on the nuclear protein ARABIDOPSIS ABERRANT LATERAL ROOT FORMATION (ALF4), a regulator of cullin-RING E3 ligases, whose expression is restricted to the pericycle (Bagchi et al., 2018; Shang et al., 2016).

However, the production of VLCFAs has to be precisely controlled both in time and space to fit the lateral root primordia pattern observed along the main root and thus avoid anarchical lateral root development. The AP2/EREBP transcription factor *PUCHI* controls cell proliferation during the early stages of lateral root formation (Hirota et al., 2007). Interestingly, *PUCHI* targets the spatial expression of genes involved in VLCFA synthesis such as *KCS1*, *KCS20*, *KCR1*, *PAS2* and *ECR/CER10* during lateral root primordia development (Figure 1) (Trinh et al., 2019). VLCFA mutants display lateral root formation defects and *puchi* mutant displays altered VLCFA

content with a reduction of C22 fatty alcohols and C24 sphingolipids (Trinh et al., 2019). At later stages of lateral root development, *PUCHI* expression disappears from the center of lateral root primordia and becomes confined to their base and flanks (Figure 1) (Trinh et al., 2019). Consistently with a role of *PUCHI* in restricting cell proliferation in boundaries, the distance between two lateral root primordia is reduced in the *puchi* mutant compared to the wild-type (Trinh et al., 2019). Lateral inhibition of lateral root primordia development by *PUCHI* is further supported by the finding that *PUCHI* is a downstream element of the peptide hormone TARGET OF LBD SIXTEEN 2 (TOLS2) which is induced by auxin, through the transcription factor LOB DOMAIN-CONTAINING PROTEIN 16 (LBD16), and which is perceived by the RECEPTOR-LIKE KINASE7 (RLK7) (Toyokura et al., 2019). TOLS2 is induced by auxin in lateral root founder cells and has the ability to move nearby to repress the initiation of other lateral roots in close proximity of the developing primordia (Figure 1) (Toyokura et al., 2019).

VLCFA-containing membrane lipids as executive producer of the hormonal transcriptional script

Auxin induces cell proliferation and pluripotency in new lateral root primordia and at the same time separately activates the production of a mobile peptide that will trigger the TOLS2/RLK7/PUCHI pathway in neighboring cells to restrict cell proliferation through the induction of VLCFA synthesis (Figure 1). Hence, the precise induction of VLCFA synthesis delineates a zone of the root where cell proliferation induced by auxin is controlled and this will define a periodic pattern along the main root. However, an important question to address is how do VLCFAs execute the hormone-induced developmental program? VLCFAs are found mainly in the sphingolipids where they constitute more than 80% of sphingolipids in plants (Wattelet-Boyer et al., 2016). VLCFAs are additionally found in phosphatidylcholine in mammals, phosphatidylinositol in yeast and in phosphatidylserine in plants (Murata et al., 1984; Poulos, 1995; Schneiter et al., 2004)

Beside membrane lipids, extracellular lipids such as the cuticle and suberin contain VLCFAs. The cuticle is a hydrophobic barrier at the surface of epidermal cells and is composed from cutin, a polymer of C16 and C18 fatty acids, and cuticular waxes, a polymer of VLCFA-derived compounds (Bernard and Joubes, 2013; Delude et al., 2016b; Kunst and Samuels, 2003). Suberin is a polymer made of VLC ω-hydroxyacids, dicarboxylic acid and fatty alcohols (Delude et al., 2016b; Franke et al., 2005). VLCFAs presence in extracellular lipids could be of particular importance for targeted development programs since unusual cutin-like compounds were found to be enriched in a cell layer overlaying the lateral root primordia and play a role in lateral root emergence (Berhin et al., 2019). Moreover, the LONG-CHAIN FATTY ACID SYNTHETASE 2 (LACS2) enzyme is required for cutin synthesis and lateral root development

(Macgregor et al., 2008). Hence, modulation of cuticle lipids plays a role in lateral root formation most probably by regulating the permeability of the cuticle to ions and osmotic solutes (Berhin et al., 2019; Macgregor et al., 2008). However, targeted auxin/TOLS2/PUCHI-mediated lateral root primordia formation is unlikely to occur through cuticle as unusual cutin-like compounds detected in developing lateral root primordia are not VLCFAs and the level of typical suberin and cutin compounds was found to be similar between wild-type and *puchi* mutant (Trinh et al., 2019).

Aside from extracellular lipids and membrane sphingolipids, the acyl-chain length of phosphatidylserine and the quantity of phosphatidylserine at the plasma membrane vary during plant development (Li et al., 2014; Platre et al., 2019). Phosphatidylserine is required for auxin-mediated meristem maintenance of the shoot apex (Liu et al., 2013). Moreover, phosphatidylserine is a crucial lipid which stabilizes auxin-induced nano-clustering of Rho of Plants 6 (ROP6), a small guanosine triphosphatase (GTPase) involved in auxin signaling (Platre et al., 2019). Interestingly, in animal cells, phosphatidylserine is located in the cytoplasmic leaflet of the plasma membrane (Yeung et al., 2008) and has the ability to induce the nanoclustering of GPI-anchored proteins located at the outer leaflet, through trans-bilayer coupling (Raghupathy et al., 2015). This coupling requires long acyl-chains of 18 carbons for both GPI and phosphatidylserine (e.g. PS 18:0/18:1) (Raghupathy et al., 2015; Skotland and Sandvig, 2019). Hence, phosphatidylserine does not necessarily require VLCFA to induce nano-clustering of proteins in animal cells. However, whether VLCFA of phosphatidylserine is required to target specific developmental programs is currently unknown in plant cells.

Contrastingly, VLCFAs of sphingolipids are known to be instrumental in executing hormone-induced developmental program. α-hydroxylated VLCFAs, specific of sphingolipids (Box 1), were found to be a target of the auxin/PUCHI-mediated lateral root primordia formation and this is consistent with the role of the ceramide synthases LONGEVITY ASSURANCE GENE 1 HOMOLOGUE (LOH) in lateral root development (Bach et al., 2011). In *loh* mutant, the auxin influx carrier AUX1 and the auxin efflux carrier PIN1, both required for lateral root formation, are mis-localized in endosomal compartments, which indicates an involvement of sphingolipids in endomembrane trafficking of auxin carriers (Bach et al., 2011). Interestingly, pharmacological reduction of acyl-chain length of sphingolipids, which does not alter the total quantity of sphingolipids, inhibits secretory sorting of the auxin efflux carrier PIN2 at a specific sub-domain of the TGN, a major post-Golgi sorting station (Wattelet-Boyer et al., 2016). Immuno-isolation of intact TGN compartments revealed that the TGN sub-domain involved in PIN2 secretory sorting is enriched in α-hydroxylated VLCFAs, the specific hallmark of sphingolipids, similarly to what was found in TGN-derived vesicles in yeast (Klemm et al., 2009; Wattelet-Boyer et al., 2016). Mis-sorting of PIN2 at the TGN upon the reduction of the acyl-chain length of sphingolipids resulted in the alteration of the capacity of plants to orient their root growth

towards the gravity vector (Wattelet-Boyer et al., 2016). Hence, the acyl-chain length of sphingolipids is not only involved in developmental programs but also in the perception and response of plants to the environment. Identifying the mechanisms through which sphingolipids act in protein sorting at the TGN is a future challenge shared in all communities working on sphingolipids.

In summary, auxin triggers cell proliferation and pluripotency, which create a niche of stem cells. In parallel, auxin activates signaling pathways that will restrict this zone in space, as well as activation of VLCFAs biosynthesis, which will feedback on auxin transport to inhibit cell proliferation and organize coordinated growth of the new organ.

VLCFAs are regulating cell-to-cell transport during defined developmental programs

Cell-to-cell transport is known to partly rely on plasmodesmata which are membrane-lined channels connecting the cytoplasm between adjacent cells. Lipid characterization of isolated plasmodesmata revealed that they are significantly enriched in sterols and sphingolipids containing α-hydroxylated VLCFAs and phytosphinganine (tri-hydroxylated long chain base t18:0) (Grison et al., 2015; Liu et al., 2020). Phytosphinganine binds plasmodesmata-located protein 5 (PDLP5) which regulates plasmodesmata cell-to-cell connectivity (Liu et al., 2020; Sager et al., 2020). Furthermore, the sphingolipid biosynthesis *phloem unloading modulator* (*plm*) mutant has reduced level of VLCFA-ceramides and displays enhanced cell-to-cell trafficking between the pericycle and the endodermis (Yan et al., 2019). VLCFA-mediated restriction of cell-to-cell trafficking between the pericycle and the endodermis is particularly relevant when considering the restriction of lateral root primordium development at the pericycle by VLCFAs production in the endodermis (see discussion in previous section; (Shang et al., 2016; Trinh et al., 2019)). Indeed, during lateral root initiation, the plasmodesmata-mediated connectivity between the initial auxin-primed pericycle cell and the neighboring cells is reduced as soon as the first pericycle cell division occurs (Benitez-Alfonso et al., 2013). Auxin is a small molecule that can likely diffuse through plasmodesmata, as shown previously during the tropic growth of *Arabidopsis* hypocotyl in response to unilateral light (Han et al., 2014), in the root meristem (Mellor et al., 2020) or in leaves (Gao et al., 2020). An advantage of reducing cell-to-cell connectivity in the early stages of lateral root primordia development could be to concentrate auxin at a restricted area of the pericycle to trigger cell proliferation and regulate the spatial patterning of lateral root primordia along the main root. Sphingolipids-mediated regulation of cell-to-cell connectivity at plasmodesmata occurs through both the external deposition of callose (a β-(1,3)-glucan polymer), induced by the recruitment of PDLP5, and the internal modulation of plasmodesmata membrane structure (Liu et al., 2020; Yan et al., 2019). Additionally, a non-negligible number of receptor-like kinases have been identified at

plasmodesmata such as CLAVATA1 (CLV1) acting in stem cell specification, and its receptor kinase interactor CRINKLY4 (Stahl et al., 2013). Another plasmodesmata-located receptor, the Qiān Shǒu kinase (QSK1), associates more strongly to plasmodesmata when the acyl-chain length of fatty acids was reduced by pharmacological treatment (Grison et al., 2019). Interestingly, QSK1 is necessary for callose deposition and lateral root development (Grison et al., 2019). Hence, VLCFA-modulation of plasmodesmata occurs through several mechanisms to regulate cell-to-cell connectivity during defined developmental programs.

Membrane sterols participate to non-cell autonomous auxin-mediated tissue patterning

It is known from a long time that membrane sterols are crucial for plant development as the first genetic screens performed in *Arabidopsis* identified from the start several sterol-biosynthesis genes as master regulator of cell division, embryogenesis, body patterning and growth axes polarity (Mayer et al., 1991). More precisely, the ratio between 24-ethyl sterols (β-sitosterol and stigmasterol, see Box 1) and 24-methyl sterols (campesterol, see Box 1) has been found to be critical during general plant development (Schaeffer et al., 2001). This is important since this ratio is controlled by the last steps of sterol-biosynthesis and could be a way for plants to finely tune morphogenesis in a given tissue. The 24-ethyl/24-methyl sterols ratio is balanced by the C24-methylation of 24-methylene Iophenol which is synthesized by the HYDRA1 enzyme. Interestingly, while *hydra1* mutant displays root patterning defects in the epidermis, columella and vascular tissues, tissue-specific expression of *HYDRA1* in the epidermis significantly rescued the cell patterning defects in all tissues (Figure 2) (Short et al., 2018; Souter et al., 2002). Contrastingly, expression of *HYDRA1* in the pericycle, endodermis or vascular tissues had limited effects on the rescue of root cell patterning defects of the *hydra1* mutant (Short et al., 2018). Thus, sterols are involved in the transit of a non-cell autonomous signal from the epidermis to the other tissues of the root. Campesterol is the precursor of the plant hormone brassinosteroids and could be a mobile signal; nonetheless, exogenous application of brassinosteroids does not rescue any of the cell patterning defects of the *hydra1* mutant (Short et al., 2018). Interestingly, *Arabidopsis* mutants in sterol-biosynthesis steps upstream the 24-ethyl/24-methyl sterols checkpoint all display defects in the localization of PIN auxin efflux carriers and altered tissue distribution of auxin (Men et al., 2008; Pan et al., 2009; Souter et al., 2002; Willemsen et al., 2003). Moreover, specific expression of *HYDRA1* in epidermis rescues PIN localization defects in the *hydra1* mutant (Figure 2) (Short et al., 2018). Hence, it is very tempting to hypothesize that auxin would be a non-cell autonomous signal controlled by membrane sterols.

Using *Arabidopsis* root hairs, which develop as a projection from trichoblast epidermal cell, auxin was found to act as a non-cell autonomous signal controlling the positioning of the root

hair in the plane of epidermal cells (Figure 2) (Boutte and Grebe, 2009; Fischer et al., 2004; Ikeda et al., 2009). Interestingly, at the root hair initiation site, sterols accumulate with ROP GTPases, ROP2 and ROP6 and are necessary for correct ROP positioning. Moreover, sterols are required for the polar localization at early hair bulging sites of the AGC kinases of the D6PK family, which are involved in auxin transport (Figure 2) (Armengot et al., 2016; Barbosa et al., 2014; Stanislas et al., 2015; Stanislas and Jaillais, 2019; Zourelidou et al., 2014). Hence, sterols feed back on auxin transport during auxin-regulated cell polarization.

Phosphatidylserine gradients regulate Rho-mediated auxin signaling at the tissue level.

Phosphatidylserine is produced in the ER by the PHOSPHATIDYLSERINE SYNTHASE1 (PSS1) enzyme (Yamaoka et al., 2011). However, in the ER, phosphatidylserine is oriented toward the lumen and is not accessible to cytosolic proteins (Platre and Jaillais, 2016, 2017). Analysis of phosphatidylserine biosensors revealed that instead, phosphatidylserine is present on the cytosolic leaflets of the plasma membrane and endosomal compartments (Platre et al., 2018; Simon et al., 2016). Quantitatively, phosphatidylserine appears to form a concentration gradient from the cell surface to the late endocytic pathway, including the vacuolar membrane (i.e. the tonoplast) (Figure 3A) (Platre et al., 2018). Interestingly, this gradient is not homogeneous but appears to be developmentally controlled (Platre et al., 2019). Indeed, in Arabidopsis root epidermis, meristematic cells have a very sharp concentration gradient of phosphatidylserine from the plasma membrane to endosomal compartments (Figure 3B). By contrast, elongating epidermal cells have a more balanced phosphatidylserine cellular gradients with less phosphatidylserine sensor localized at the plasma membrane and proportionally more at the surface of endosomes/TGN (Figure 3B) (Platre et al., 2019). Hence, there is not only a cellular phosphatidylserine gradient within the endomembrane system but also a tissue-level phosphatidylserine gradient (Figure 3A-B) (Colin and Jaillais, 2019). The variations of phosphatidylserine accumulation at the plasma membrane at the tissue scale appears to be functionally relevant as it impacts the signaling activity of the Rho GTPase ROP6 (Platre et al., 2019). ROP6 coordinates cytoskeleton dynamics and intracellular trafficking during the root response to gravity and is activated in response to auxin (Armengot et al., 2016; Chen et al., 2012; Lin et al., 2012). Phosphatidylserine is required to stabilize ROP6 into plasma membrane nanoclusters, which is itself needed for downstream signaling activity (Platre et al., 2019). Importantly, phosphatidylserine is not only required but is rate limiting for this process. Indeed, a decreased phosphatidylserine synthesis leads to attenuated ROP6 nanoclustering and response, while an increased phosphatidylserine production leads to constitutive ROP6 nanoclustering and downstream signaling (Platre et al., 2019). Therefore, variations of phosphatidylserine accumulation at the plasma membrane during cell differentiation impact the cellular sensitivity of root cells to auxin (Figure 3C) (Colin and Jaillais, 2019). Accordingly,

elongating epidermal cells, which have less PS at the plasma membrane, have a weaker response to ROP6-mediated auxin signaling than root meristematic cells. Interestingly, auxin exogenous application promotes phosphatidylserine accumulation at the plasma membrane (Platre et al., 2019). Therefore, phosphatidylserine appears to be at the heart of a feedback regulatory loop, by which auxin impacts on the subcellular accumulation of phosphatidylserine to regulate ROP6 signaling and hence a subset of the auxin responses (Figure 3C) (Jaillais and Ott, 2020; Platre et al., 2019). There are many unknown components in this regulatory loop, including which of the many auxin pathways (e.g. ROP6 signaling, TIR/AFB-mediated nuclear auxin signaling, non-canonical TIR/AFB signaling) feedbacks on phosphatidylserine subcellular accumulation and what are the phosphatidylserine metabolic pathways that are targeted by the auxin signal (e.g. biosynthesis, flipping from luminal to cytosolic leaflet, transport). Although these metabolic intermediates are currently unknown, this example nicely illustrates how a membrane lipid, such as phosphatidylserine, may regulate a given signaling output, while at the same time being targeted for regulation by that signaling pathway.

Opposites attract: membrane targeting by electrostatics in the regulation of hormone signaling.

Phosphatidylserine, together with phosphoinositides (e.g. PI4P, PI(4,5)P_2) and phosphatidic acid are anionic lipids (Colin and Jaillais, 2019). As such, they impact a key physicochemical property of biological membranes: their electrostatic field (Noack and Jaillais, 2017; Platre and Jaillais, 2017). The extensive use of lipid and electrostatic biosensors revealed that the endomembrane system of plant cells may be roughly divided in two cellular territories (Platre et al., 2018; Simon et al., 2016). The first one includes membrane that are not electrostatics (i.e. no accumulation of anionic lipids on their cytosolic leaflet) and that corresponds to ER-derived membranes. The second is an electrostatic territory and is composed of post-Golgi compartments, including the TGN, the plasma membrane, endosomes and the tonoplast (Platre et al., 2018). The separation of the endomembrane system into two distinct membrane territories has a strong impact on protein localization, including both peripheral proteins that may be recruited to their target membrane through interactions with anionic lipids, but also for integral transmembrane proteins, which can be stabilized or destabilized by membrane electrostatics.

Phosphatidylserine is the only anionic lipid that accumulates in all post-Golgi compartments and thus can be seen as a hallmark of the electrostatic territory in plants (Platre et al., 2018). By contrast, PI4P and phosphatidic acid strongly accumulate at the plasma membrane and together they power a very strong electrostatic field which is a key determinant of the identity of this membrane (Figure 3D) (Platre and Jaillais, 2017; Platre et al., 2018; Simon et al., 2016). Consequently, proteins that contains a polybasic region engage electrostatic interactions with

anionic lipids and may be targeted specifically to the plasma membrane. This is for example the case of several key regulators of hormonal pathways, including the AGC kinases PINOID and D6PKs, which are master regulators of PIN-mediated auxin transport (Barbosa et al., 2016; Lee et al., 2018; Marhava et al., 2020; Simon et al., 2016; Stanislas et al., 2015). Mutants versions of PINOID or D6PKs that are not able to interact with anionic lipids, fail to interact with the plasma membrane and are not functional (Barbosa et al., 2016; Simon et al., 2016). Another example of proteins regulated by plasma membrane electrostatics is the BRI1 KINASE INHIBITOR1 (BKI1) (Simon et al., 2016). BKI1 is an inhibitor of the BRASSINOSTEROID INSENSITIVE1 (BRI1) receptor kinase (Jaillais and Vert, 2016; Wang and Chory, 2006). It is an intrinsically disordered protein, which binds to the plasma membrane via a polybasic membrane hook (Jaillais et al., 2011). Interestingly, the localization and thereby function of BKI1 is not only regulated by anionic lipids but also by brassinosteroid signaling itself (Figure 3D) (Jaillais et al., 2011; Wang and Chory, 2006). Here, brassinosteroid does not target the metabolic production of anionic lipids, but directly the BKI1 proteins (Jaillais et al., 2011; Wang and Chory, 2006). Indeed, upon brassinosteroid perception, BRI1 phosphorylates BKI1 on a conserved tyrosine within its polycationic membrane hook (Jaillais et al., 2011). This tyrosine phosphorylation triggers the rapid dissociation of BKI1 from the plasma membrane and allows further downstream BRI1 signaling. A likely mechanistic explanation for this membrane release is that the phosphorylation, which is negatively charged, creates a local electrostatic clash with the highly anionic plasma membrane, thereby releasing BKI1 into the cytosol (Jaillais and Vert, 2016). In favor of this hypothesis, a BKI1 mutant in which the tyrosine residue within the membrane hook is substituted for a negatively charged aspartic acid, is unable to bind to the plasma membrane (Jaillais et al., 2011). This example shows that hormonal pathways may not only target lipid metabolism to modulate their signaling activity, but may also directly manipulate protein-lipid interactions, for example via protein phosphorylation.

Reinforcing cell polarity: a regulatory feedback loop between auxin and phosphoinositide metabolism.

PROTEIN KINASE ASSOCIATED WITH BRX (PAX) is an AGC kinase involved in root protophloem differentiation (Marhava et al., 2018). PAX is specifically expressed in root protophloem and localized on the rootward pole of these cells (Marhava et al., 2018). PAX directly phosphorylates PIN proteins in protophloem to enhance polar auxin efflux, which decreases the intracellular auxin level (Marhava et al., 2018) (Figure 4A). PAX activity is counterbalanced by its protein partner BREVIS RADIX (BRX), which negatively regulates PAX, thereby decreasing auxin efflux and increasing intracellular auxin concentration (Marhava et al., 2018; Rodriguez-Villalon et al., 2015). Heightened intracellular auxin dissociates BRX from the membrane into the

cytosol and as such promotes PAX activity and auxin efflux, creating a regulatory feedback loop akin to a molecular rheostat (Marhava et al., 2018; Scacchi et al., 2009) (Figure 4A-B).

Interestingly, like PINOID or D6PKs, PAX is targeted to the plasma membrane via electrostatic interactions with anionic phospholipids (Barbosa et al., 2016) and its localization is dependent on two PI4P kinases PIP5K1/PIP5K2, which produce $PI(4,5)P_2$ (Figure 4C) (Marhava et al., 2020). PIP5Ks are coimmunoprecipated by both BRX and PAX and the *pip5k1k2* double mutant shows a protophloem differentiation phenotype similar to *pax* and *brx* mutants. PIP5K1/2, PAX and BRX are all localized on the rootward pole of protophloem cells in a plasma membrane domain at the center of the cell and referred to as a muffin-like pattern. This muffin-like membrane patch is complementary to PIN1 localization, which accumulates in a donut-like membrane domain surrounding PIP5Ks/PAX and BRX (Figure 4D-F) (Marhava et al., 2020). The PIN1 donut-like localization is altered in *pip5k1k2*, *pax* and *brx* mutants suggesting that the activity of the complex is involved in PIN1 localization. Thus, PIN1 localization in donut appears to be important for protophloem differentiation. However, the exact function of this annular pattern, by opposition to a localization on the entire face of the cell's rootward pole, is unclear.

Because PIP5K1/2 are localized in the muffin-like patch, it is tempting to speculate that their lipid product, $PI(4,5)P_2$, is also enriched in this domain. A local increase in $PI(4,5)P_2$ may create a high local electrostatic field, which could contribute to the polar recruitment of PAX specifically in the muffin-like patch (Figure 4D). In animals, $PI(4,5)P_2$ is essential for endocytosis as it mediates the recruitment of many key regulators of clathrin-mediated endocytosis (Posor et al., 2015). A similar scenario is likely in plants, since most of these core regulators of endocytosis are conserved and $PI(4,5)P_2$ is known to positively contribute to endocytosis (Ischebeck et al., 2013). Interestingly, DYNAMIN-RELATED PROTEIN 1A (DRP1A), a dynamin-like protein involved in clathrin-mediated endocytosis in plants, localizes in the muffin-like domain (Marhava et al., 2020). This suggests that DRP1A may participate in an endocytic process, which locally removes PIN1, thereby promoting its donut-like localization (Figure 4D-E). Thus, $PI(4,5)P_2$ and their biosynthetic enzymes, PIP5Ks, are part of a self-organizing polarity module (Marhava et al., 2020; Weijers, 2020). On the one hand, they contribute to localizing PAX/BRX to the muffin-like polar patch and on the other hand, they are critical to establish the PIN1 donut domain (Figure 4F). Because it is a rate limiting component in cellular auxin efflux, PIN1 determines intracellular auxin levels, which itself impacts on BRX localization (Marhava et al., 2018; Scacchi et al., 2009), but also *PIP5K* and *BRX* expression (Mei et al., 2012; Mouchel et al., 2006; Tejos et al., 2014). This example highlights how lipids participate in self-organizing systems to obtain robust polarity maintenance, which in this case is required for the differentiation of the root vasculature. Interestingly, auxin regulates this polarity module in several ways, including the control of BRX localization, by a so far uncharacterized mechanism, and gene expression. The fact that auxin acts through multiple pathways on lipid metabolism is a concept that will likely

be extended to many other developmental outputs, starting with the ROP6/phosphatidylserine module (Figure 3C). Indeed, each auxin pathways will likely function at different time scales and/or at different concentrations, thereby expanding the regulatory repertoire that membrane lipids have to offer to control plant development.

Conclusion

In the examples described above, it is clear that most membrane lipid classes have key roles in the hormonal control of plant development. Their metabolism is directly regulated by these pathways, notably in response to auxin. The modification of membrane lipid homeostasis is therefore part of the downstream cellular toolkit, which executes given developmental responses. In addition, developmentally-controlled modifications of lipid metabolism often feedback on those signaling pathways to either downregulate or activate them, providing negative feedback regulations or self-reinforcing loops. In the case of auxin, which have several paralleled signaling pathways, membrane lipids appear as key factors involved in the crosstalk between the different arms of the auxin responses. However, the importance of lipid metabolism in plant development is only beginning to be uncovered as we still do not fully appreciate to what extent phytohormones impact membrane lipids, notably at the single cell level. In addition, there are still few cases in which we understand the downstream impacts of targeted membrane lipid alterations on the development of growing tissues and organs. The cell wall is now recognized as a major target of developmental regulators to control plant morphogenesis (Echevin et al., 2019; Eng and Sampathkumar, 2018; Kierzkowski and Routier-Kierzkowska, 2019; Landrein and Ingram, 2019; Zhao et al., 2018). We propose that membrane lipids, which build the very interface between the cytoplasm and the cell wall (Ackermann and Stanislas, 2020), should also be considered on an equal footing with cell wall modifying processes when considering plant morphogenesis.

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Declaration of interest:

The authors declare no competing interests.

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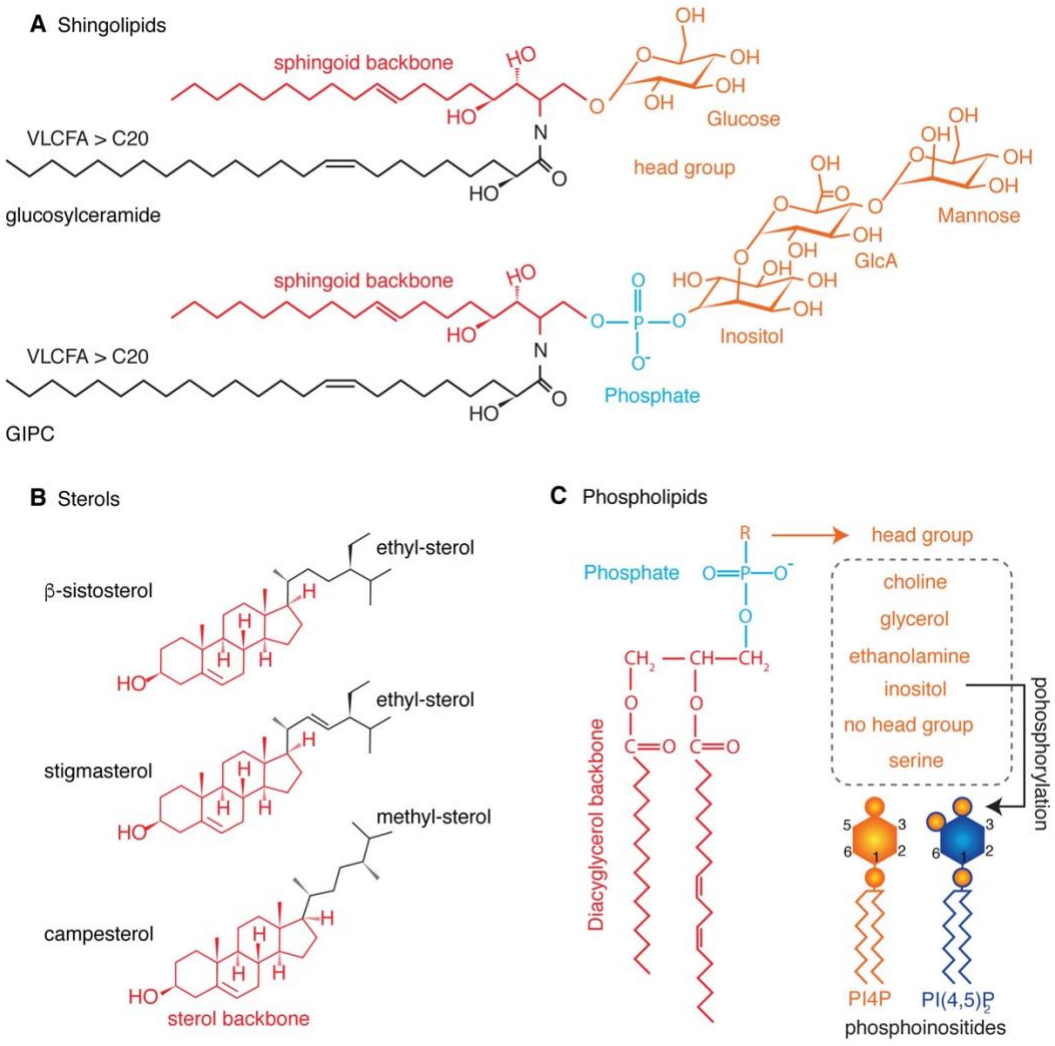


Box 1: Typical structures of the three-main lipids of the Arabidopsis endomembrane system. Membrane lipids in the plant endomembrane system are made of three main lipid classes, sphingolipids, sterols and phospholipids. **A)** Sphingolipids are lipids with a sphingoid backbone grafted with a Very-Long Chain Fatty Acid (VLCFA) and are abundant in plants (up to 10% of total lipids) (Mamode Cassim et al., 2019). The main sphingolipids in plants are glucosylceramide (~35%) and glycosyl inositol phosphoryl ceramide (GIPC, ~65%) (Mamode Cassim et al., 2019). They are mostly located in the outer leaflets of the plasma membrane but also in the lumen of the *trans*-Golgi Network (TGN), endosomes and the vacuolar membrane, where they can represent about 10 to 20% of total lipids (Jaillais and Ott, 2020; Mamode Cassim et al., 2019). **B)** Sterols are membrane lipids constituted from four rings and a lateral chain (Boutte and Grebe, 2009). This basic structure of free sterols can be further modified by addition of a fatty acid, producing steryl esters, a sugar, producing steryl glucoside, or both, resulting in acyl steryl glycosides. Plants produce three major sterols amongst which β-sitosterol and stigmasterol are both 24-ethyl sterols, and campesterol, a 24-methyl sterol acting also as a precursor of brassinosteroids, a class of phytohormones involved in multiple plant developmental processes (Belkhadir and Jaillais, 2015; Mamode Cassim et al., 2019). **C)** Phospholipids are glycerolipids made of a glycerol backbone linking two long chain fatty acids to a hydrophilic head group with a phosphate group (Colin and Jaillais, 2019). They can have diverse head groups, which determine the phospholipid class. Some phospholipids are relatively abundant, such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Colin and Jaillais, 2019). Low abundant phospholipids are also important functionally and often acts as lipid signal in membranes. These low abundant phospholipids are anionic and include phosphatidic acid, phosphatidylserine and the phosphorylated derivative of phosphatidylinositol, the phosphoinositides, such as phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) (Colin and Jaillais, 2019).

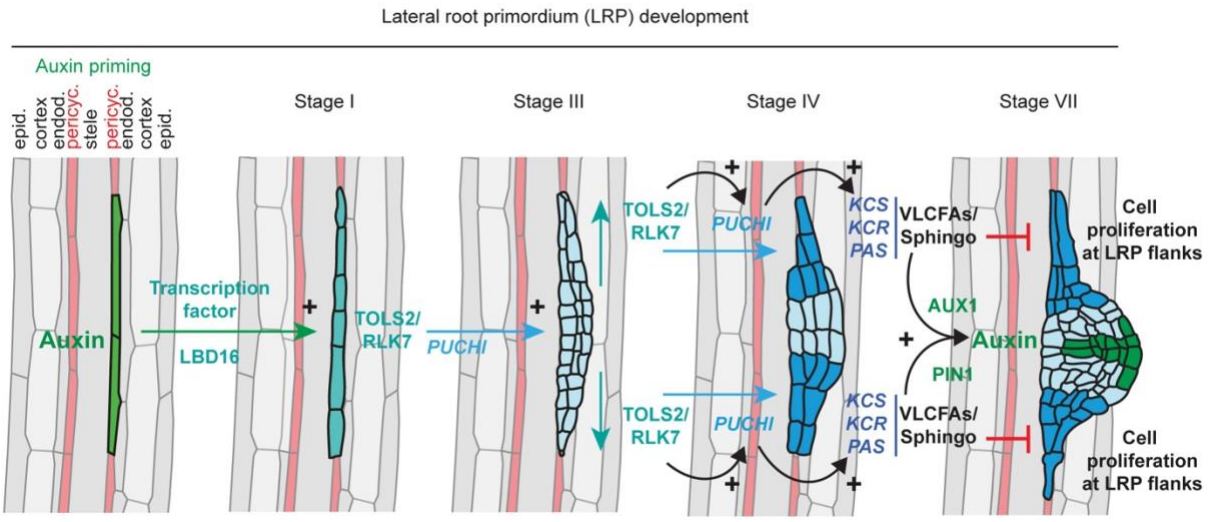
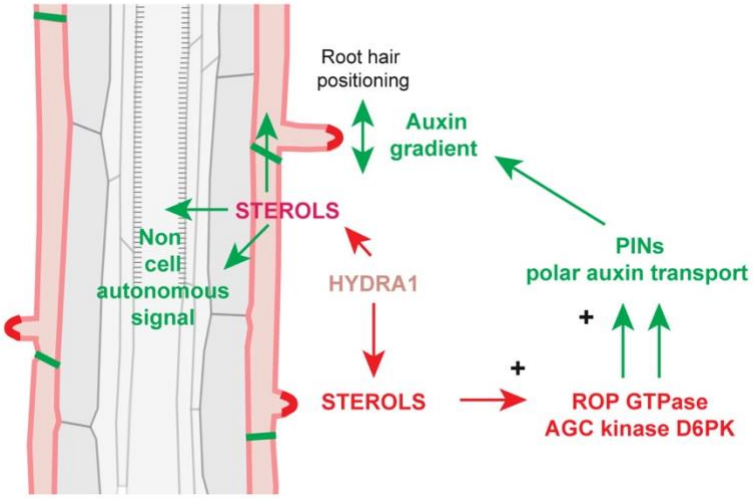


Figure 1: Feedbacks between auxin and VLCFAs during development of lateral root primordium. Schematic representation of auxin-triggered VLCFAs feedbacks on cell proliferation and auxin transport. In pericycle (light red), an auxin maximum (green) triggers the synthesis of several transcription factor, including LBD16 (LOB DOMAIN-CONTAINING PROTEIN 16) which will activate the synthesis of the peptide TOLS2 (TARGET OF LBD SIXTEEN 2, sea green). TOLS2 induces cell signaling through the RECEPTOR-LIKE KINASE 7 RLK7, and triggers the synthesis of the transcription factor PUCHI (light blue). The TOLS2 peptide is able to move to neighboring cells and reinforce the synthesis of PUCHI (blue) which targets transcription of genes involved in the synthesis of Very Long Chain Fatty Acids (VLCFAs) such as KCS (3-KETOACYL-COA SYNTHASE), KCR (3-KETOACYL-COA REDUCTASE) and PAS (PASTICCINO). Within membrane lipids, VLCFAs are mainly contain in sphingolipids (Sphingo) that repress cell proliferation at lateral root primordium flanks and regulate polar auxin transport, thereby it will positively feedback on the establishment of an auxin maximum in the new developing root. AUX1: AUXIN TRANSPORTER1 (auxin-influx carrier), PIN1: PIN-FORMED1 (auxin-efflux carrier), VLCFA: very-long-chain fatty acids, epid.: epidermis, endod.: endodermis, pericyc.: pericycle. Figure based on https://doi.org/10.6084/m9.figshare.5143987.v4 and https://doi.org/10.6084/m9.figshare.4786357.v1



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762 **Figure 2: Non-cell autonomous sterol function in root hair positioning through ROP GTPase, AGC kinase and**
763 **polar auxin transport**. In epidermis (red), HYDRA1 controls the ratio between 24-ethyl sterols (β-sitosterol and
764 stigmasterol) and 24-methyl sterols (campesterol) in a non-cell autonomous manner. Sterols concentrate at the root hair
765 initiation site and are involved in polar positioning of RHO-OF-PLANTS (ROP) GTPase and the D6P-Kinase, which
766 activate polar auxin transport, create an auxin gradient and define the position of the root hair initiation site at epidermal
767 cells. Sterols mediate the patterning of tissues adjacent to epidermis through a non-cell autonomous signal which could
768 be auxin. Figure based on https://doi.org/10.6084/m9.figshare.5143987.v4

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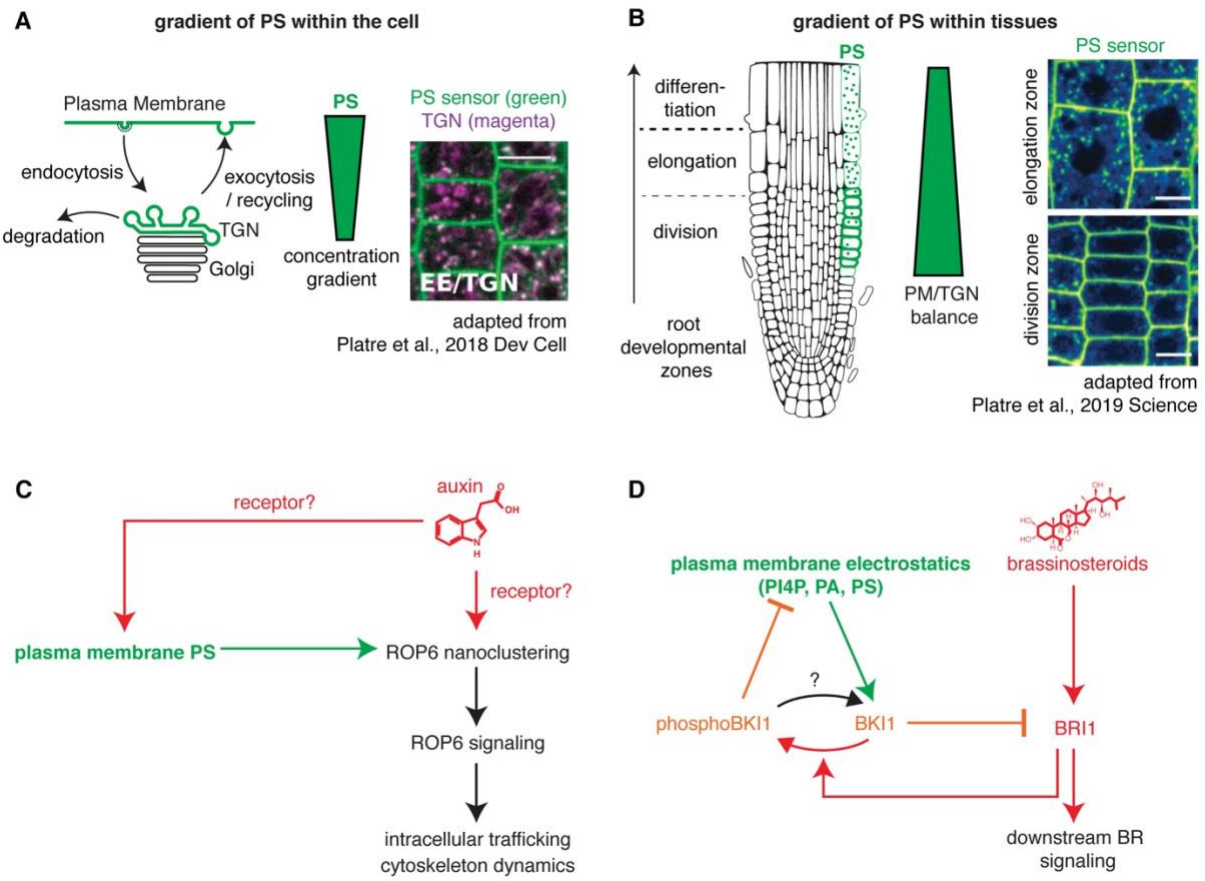
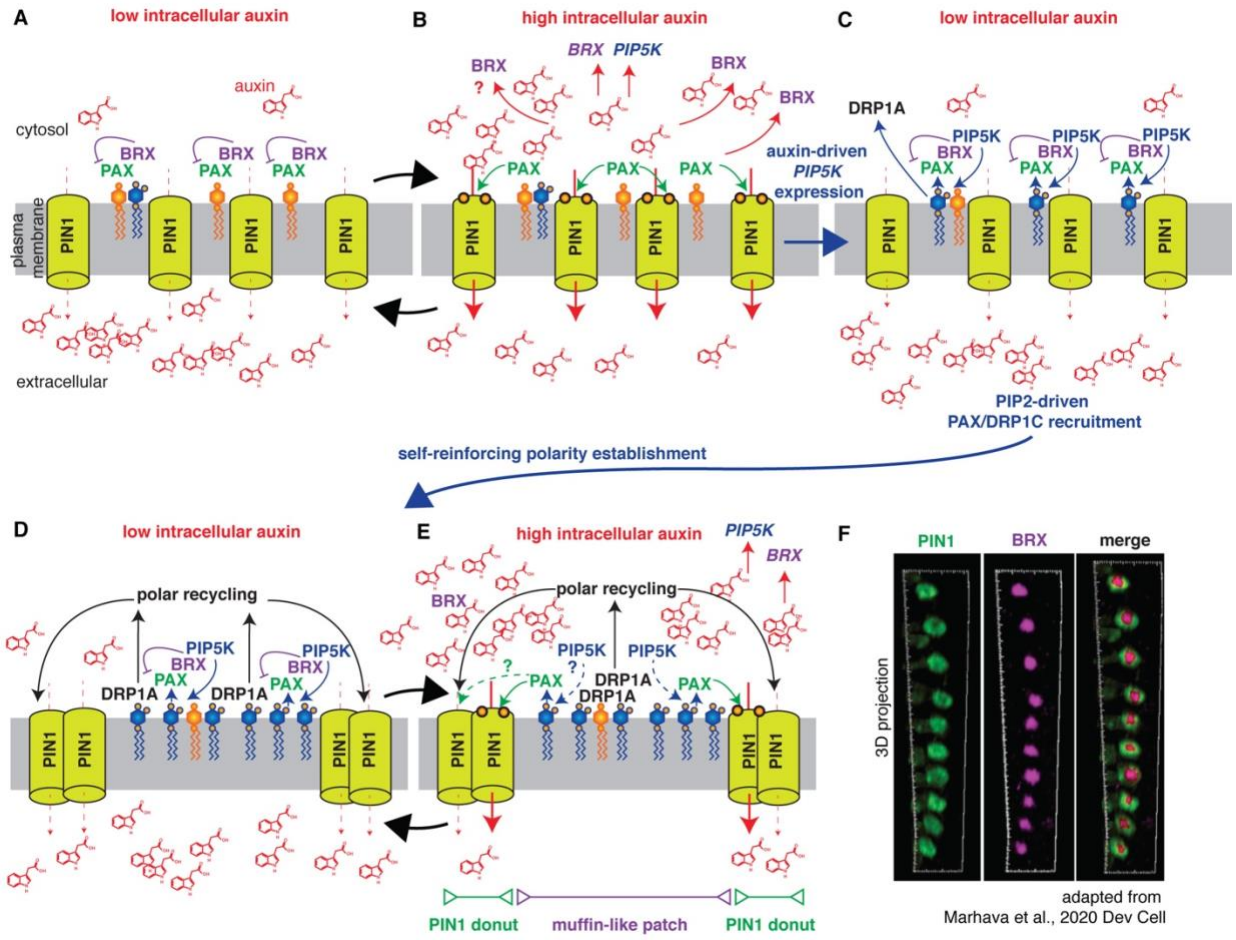


Figure 3: Feedback regulations between anionic phospholipids and auxin/brassinosteroid signaling. A) Schematic representation of the phosphatidylserine accumulation gradient at the cell level (left), and example of colocalization between a phosphatidylserine sensor and a *trans*-Golgi Network (TGN) marker (right, image adapted from Platre et al., 2018). B) Schematic representation of the phosphatidylserine accumulation gradient at the tissue level (left), and example of confocal images showing the localization of a phosphatidylserine sensor in the Arabidopsis root epidermis elongation zone (top right) and meristem (bottom right, images adapted from Platre et al., 2019). C) Auxin triggers RHO-OF-PLANTS6 (ROP6) nanoclustering, which is phosphatidylserine-dependent, and subsequent downstream signaling, while at the same time auxin also controls phosphatidylserine subcellular accumulation. Note that the receptor that perceives auxin upstream of ROP6 is unknown. Similarly, the auxin signaling pathway involved in regulating the phosphatidylserine gradients at the cell and tissue scales are unknown, but could involve the ROP6 pathway itself. C) Anionic lipids antagonize brassinosteroid signaling by recruiting BRI1 KINASE INHIBITOR1 (BKI1) to the plasma membrane, which inhibits BRASSINOSTEROID INSENSITIVE1 (BRI1). Brassinosteroid signaling, via BRI1 kinase activity, releases this inhibition by phosphorylating BKI1, thereby blocking the interaction between BKI1 and anionic lipids and removing BKI1 from the plasma membrane. BR: brassinosteroid, PS: phosphatidylserine, PA: phosphatidic acid.



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788 **Figure 4: Feedback mechanisms between auxin and PI(4,5)P_2 in the regulation of polar auxin transport in**
789 **protophloem**. A) PROTEIN KINASE ASSOCIATED WITH BRX (PAX) is recruited to the plasma membrane via
790 electrostatic interactions with anionic phospholipids. When the intracellular auxin concentration is low, BREVIS
791 RADIX (BRX) interacts with PAX and negatively regulates its activity. PIN-FORMED1 (PIN1) is not phosphorylated
792 by PAX thus has a low auxin efflux activity. B) As a result of low PIN1 activity, the intracellular auxin concentration
793 rises, which triggers the release of BRX from the plasma membrane (by an unknown mechanism). In the absence of
794 BRX, PAX phosphorylates PIN1, which activates polar auxin efflux. The original molecular rheostat hypothesis
795 proposed that PIN1 activation allows the system to come back to the situation depicted in A (Marhava et al., 2018).
796 However, high intracellular auxin triggers the expression of *BRX* and *PI4P-5-Kinases* (*PIP5Ks*), which can further lead to
797 the situation depicted in C. C) Once intracellular auxin levels are lowered, BRX and PIP5Ks are recruited to the plasma
798 membrane by PAX. PIP5K phosphorylates PI4P into PI(4,5)P_2 (PIP2), which in turn enhances PAX plasma membrane
799 interaction. PIP2 also recruits components of the clathrin-mediated endocytosis machinery, including DYNAMIN-
800 RELATED PROTEIN 1A (DRP1A). D) The local increase in PIP2 increases PAX targeting to the plasma membrane,
801 which further lead to enhanced recruitment of PIP5Ks to the plasma membrane, leading to a self-amplifying loop, which
802 fixes PAX/BRX/PIP5K polarity at the center of the cell (muffin-like patch). Increased PIN1 endocytosis in this domain
803 could exclude it from the muffin-like patch and participate in the donut-like localization of PIN1. E) Revised version of
804 the molecular rheostat model, with two distinct polar domains for PIN1 and PAX/BRX/PIP5K/DRP1A (Marhava et
805 al., 2020). F) 3D reconstruction of confocal pictures illustrating the complementary localization of PIN1 (green) and
806 BRX (purple) in root protophloem cells (adapted from Marhava et al., 2020). From panel A to E, only the rootward pole
807 of root protophloem cell is depicted.

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