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Update

Attracted to membranes: lipid-binding domains in plants

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Abstract

Membranes are essential for cells and organelles to function. As membranes are impermeable to most polar and charged molecules, they provide electrochemical energy to transport molecules across and create compartmentalized microenvironments for specific enzymatic and cellular processes. Membranes are also responsible for guided transport of cargoes between organelles and during endo- and exocytosis. In addition, membranes play key roles in cell signaling by hosting receptors and signal transducers and as substrates and products of lipid second messengers. Anionic lipids and their specific interaction with target proteins play an essential role in these processes, which are facilitated by specific lipid-binding domains. Protein crystallography, lipid-binding studies, subcellular localization analyses, and computer modeling have greatly advanced our knowledge over the years of how these domains achieve precision binding and what their function is in signaling and membrane trafficking, as well as in plant development and stress acclimation.

Introduction

The membrane compartmentalization of eukaryotic cells distinguishes them from prokaryotic cells. Each eukaryotic cell compartment or organelle can maintain its own environment and membrane potential. Typically, membranes are composed of lipid bilayers (except lipid droplets) that consist of various lipids, including phospholipids, glycolipids, sphingolipids, and sterols (Deleu et al., 2014; Gronnier et al., 2018). In addition, membranes contain numerous proteins, which can be transmembrane (integral proteins) or membrane-associated (peripheral proteins), the latter including cytosolic proteins that bind membranes only temporally and spatially. Each of these proteins can have their own function, facilitating communication, transport, and trafficking between compartments and cells, and responding to local and environmental changes.

To facilitate the identification of different cellular compartments, membranes have evolved a small group of phospholipids with distinctive negatively charged head groups. These "anionic lipids" include phosphatidic acid (PA), diacylglycerol pyrophosphate (DGPP), phosphatidylserine (PS), and phosphatidylinositol (PI) with a range of phosphorylated forms, together called polyphosphoinositides (PPIs; Figure 1). PA is the simplest anionic phospholipid, consisting of a diacylglycerol (DAG) backbone attached to a phosphodiester group. To this phosphate, different groups can be attached, that is another phosphate for DGPP, a serine for PS, and a D-*myo* inositol for PI and its PPIs. The latter can be phosphorylated at the 3-, 4-, and/or 5-position

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ADVANCES

- Combined advances in genome sequencing and protein structure and computational analysis allow in-depth studies of LBDs in lipid–protein interactions. This has resulted in a better understanding of the requirements for phospholipid binding, which can be projected across species.
- PH and PX domains exhibit multiple LBD motifs that are able to bind multiple signaling lipids at once.
- Unravelling the protein structure of Arabidopsis PLDα1 revealed that the C2 domain, in addition to controlling membrane localization, also regulates catalytic activity. This has interesting implications for many other enzymes that contain a C2, including PLC.

of the inositol ring, giving rise to three distinct PI monophosphate [i.e. phosphatidylinositol 3-phosphate (PI3P), PI4P, and PI5P] and two PI bisphosphate [i.e. PI(3,5)P_2 and PI(4,5)P_2] isomers. This diversity of anionic phospholipids enables organelles, endosomes, and plasma membranes (PM) to exhibit distinct lipid signatures for specific signaling purposes (van Schooten et al., 2006; Testerink and Munnik, 2011; Heilmann, 2016; Gerth et al., 2017; Noack and Jaillais, 2017, 2020; Boutté and Jaillais, 2020; Jaillais, 2021). Metazoans also contain PI(3,4)P_2 and PI(3,4,5)P_3 and the specific enzymes to generate them, but these are typically lacking from plants (Meijer and Munnik, 2003; Munnik and Vermeer, 2010; Munnik and Nielsen, 2011).

To temporarily interact with membranes, which is different from permanent binding through transmembrane domains or lipid anchors (Ray et al., 2017), proteins have developed different strategies to bind lipids. Some just purely by charge, others grasping the head group completely and holding the membrane more tightly, sometimes even by secondary insertion. To achieve this, proteins have evolved various lipid-binding domains (LBDs) that recognize specific lipids (Hammond and Balla, 2015). In this way, proteins can identify specific compartments and respond to phospholipid signals generated in response to developmental and environmental cues (Gerth et al., 2017; Boutté and Jaillais, 2020; Jaillais and Ott, 2020; Noack and Jaillais, 2020), even though there are also proteins that bind lipids without defined LBD, such as dehydrins (Liu et al., 2017). Over the years, various lipid biosensors have been created by fusing LBDs to fluorescent proteins and expressing them in cell lines and whole organisms, including Arabidopsis (Várnai and Balla, 2006; Vermeer et al., 2006, 2009, 2017; van Leeuwen et al., 2007; Simon et al., 2014; Li et al., 2019; Platre et al., 2019). For the first time, we were able to see how dynamic and temporal these lipid signaling molecules actually are, and why they exhibit such high turnover rate compared to structural phospholipids (Mishkind et al., 2009; Zarza et al., 2020).

Earlier, we showed that the Arabidopsis genome encodes numerous proteins with PH (Pleckstrin Homology), FYVE (Fab1p, YOTB, Vac1p, EEA1), and PX (Phox-homology) LBDs (van Leeuwen et al., 2004). Over the years, several additional LBDs have been characterized (Silkov et al., 2011; Naughton et al., 2018; Chandra et al., 2019; Li et al., 2020). Here, we detail our current knowledge and highlight how signaling lipids and proteins use LBDs for communication in plant biology. Such interactions are typically analyzed in vitro by fat blot analysis and liposome-binding assays (Julkowska et al., 2013; Munnik and Wierzchowiecka, 2013), and in vivo by functionally characterizing the location of FP-tagged proteins, with or without introducing point mutations and functional complementation analyses of knockout (KO) phenotypes.

ENTH and ANTH domains

ENTH and ANTH domains have a similar LBD organization and are therefore discussed together. ENTH (*Epsin N-Terminal Homology*) is ~130- to 150-amion acid (aa) long and consists of eight α-helices (Figure 2A), forming a compact globular structure with an almost perfect superhelix (Zouhar and Sauer, 2014). Upon binding PI(4,5)P_2, the unstructured 14-aa N-terminal sequence becomes ordered and forms an amphipathic α-helix 0 (H_0) that inserts itself into the membrane, which facilitates curvature by pushing the lipid head groups apart (Ford et al., 2002). PI(4,5)P_2 binds to basic residues of H_0, H_1, H_3, and H_4, and the α1–α2 loop (Figure 3A).

ANTH (*AP180 N-Terminal Homology*) is structurally similar to ENTH, though not by sequence, and ANTH is much larger, with 250–300 aa forming 9–10 α-helices (Figure 2B). Analysis of numerous ANTH domains by Silkov et al. (2011) revealed multiple variations, both in PPI binding and H_0 formation. The "classic" PI(4,5)P_2-binding motif that binds PPIs via a short conserved K[X]$_9$[K/R][H/Y] motif between helices 1 and 2 (Figure 3A; Ford et al., 2001) can be "enhanced" or "super-enhanced" through addition of an extra basic aa adjacent to the basic patch, resulting in stronger PI(4,5)P_2 binding. ANTH proteins can also have a double PI(4,5)P_2-binding motif (termed N-ANTH), combining an "enhanced" motif with a PPI-binding motif in the position where ENTH would be. N-ANTH domains have an H_0 helix for membrane penetration, like ENTH domains. In the other ANTH domains, this H_0 helix is absent (Figure 3A; Silkov et al., 2011). Plants contain ANTH domains with the "classical" domain as well as those falling into the N-ANTH categories.

Arabidopsis has 8 ENTH- and 18 ANTH-containing proteins (Figure 3B–D). Phylogenetic analysis shows that the clade with ENTH proteins, although falling into distinct classes, is nested within the clade of ANTH proteins, suggesting a common origin (Figure 3D; de Craene et al., 2012). Sequence analyses show that for the ENTH proteins, only EPSIN1-3 have a well-defined H_0 helix and PPI-binding motif. For the other five proteins, this is less conserved. For the

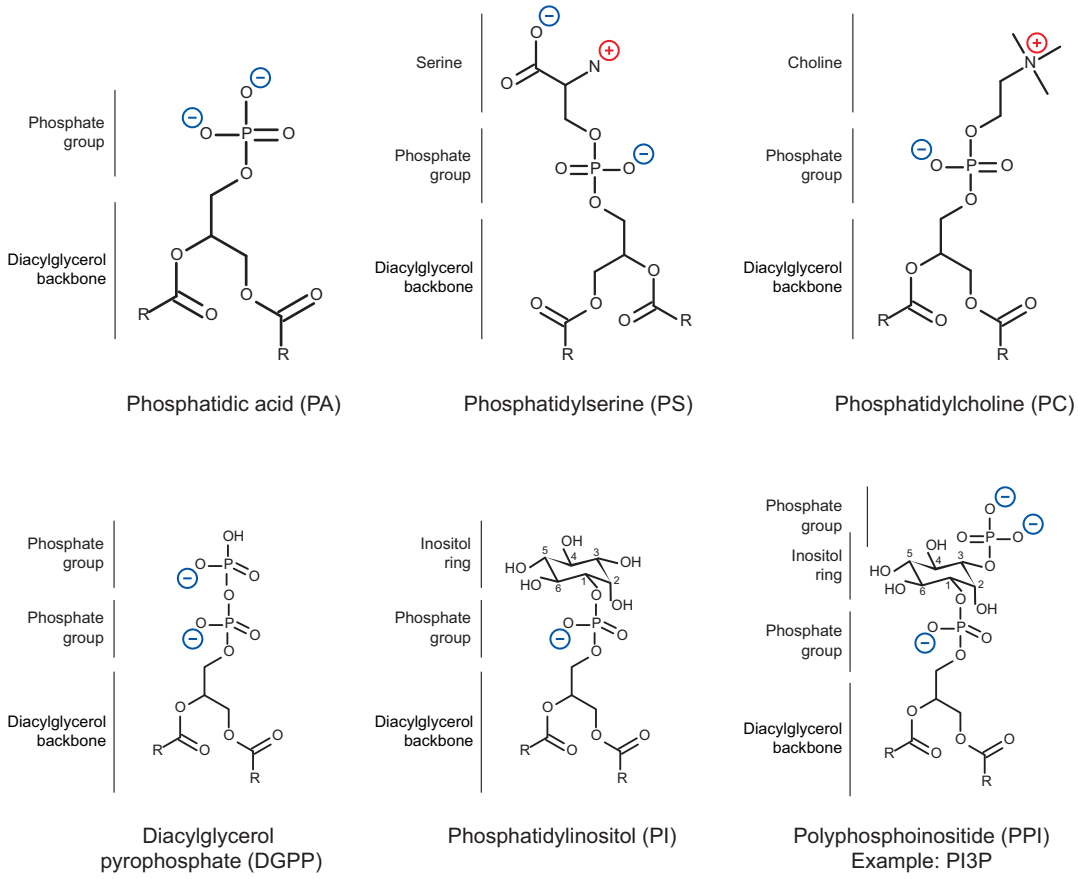

Figure 1 Structure and charge distribution of some important phospholipids. These include PA, DGPP, PS, PC, PI, and the PPI, PI3P. The basal structure of a phospholipid is composed of a diacylglycerol backbone with a phosphate group attached, giving PA. DGPP is formed by phosphorylation of PA forming a pyrophosphate. The others are formed by attaching an alcohol group to the PA backbone via a phosphodiester linkage, that is serine, choline, or inositol, forming PS, PC, and PI, respectively. PPIs are formed by phosphorylating PI at the D-3, D-4, and/or D-5 position of the inositol ring, giving rise to PI3P, PI4P, PI5P, phosphatidylinositol 3,4-bisphosphate [$PI(3,4)P_2$], $PI(3,5)P_2$, $PI(4,5)P_2$, and phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$]. The spatial representation of inositol and PI3P illustrates how phosphorylation of inositol results in unique 3D structures. Charge is indicated in blue (−) and red (+).

ANTH proteins, five contain an N-ANTH domain with a classic PPI-binding motif (N-C, N-ANTH-classic, in Figure 3C), which makes them likely $PI(4,5)P_2$ targets. Five others contain an N-ANTH domain with a less conserved PPI-binding motif (N-A, N-ANTH-alternative, in Figure 3C), suggesting they likely bind other PPI species than $PI(4,5)P_2$. Similarly, there are 8 "classical" ANTH proteins with a less conserved PPI-binding motif, allowing them to bind other PPIs.

Lipid-binding specificity has only been addressed for the ENTH protein EPSIN2 that binds PI3P (Figure 3D; Lee et al., 2007) and for the ANTH proteins Epsin-like Clathrin Adaptor 1 (ECA1) and ECA2 that can bind $PI(4,5)P_2$, $PI(3,4,5)P_3$, PA, and DGPP (Figure 3D; Silkov et al., 2011; McLoughlin et al., 2013; Kaneda et al., 2019; Putta et al., 2020). Differences in lipid-binding preference of ANTH domains are indirectly supported by the work of Song et al. (2012), who analyzed the subcellular localization of ECAs in vivo. ECA1, which has an enhanced classic $PI(4,5)P_2$-binding motif (Silkov et al., 2011), is mainly localized to the PM, whereas ECA2 and ECA4, having a less conserved classic $PI(4,5)P_2$-binding motif, are not. ECA2 is mainly present in the cytosol, with weak signals at PM and endosomes, whereas ECA4 is predominantly bound to endosomes (Song et al., 2012). Interestingly, all studies on ENTH and ANTH proteins revealed participation in clathrin-coated vesicle formation (Barth and Holstein, 2004; Song et al., 2006, 2012; Lee et al., 2007; Zhao et al., 2010; Sauer et al., 2013; Adamowski et al., 2018; Li et al., 2018; Muro et al., 2018; Kaneda et al., 2019).

Recent studies in yeast and mammalian systems showed that ENTH/ANTH domains are involved in assembling huge protein complexes (12-mer and 16-mer), both homomeric and heteromeric, and binding up to 24 PIP_2 molecules (Garcia-Alai et al., 2018; Heidemann et al., 2020). The formation of these complexes is PIP_2 dependent and likely bind membranes much stronger than monomers (Heidemann et al., 2020). In fact, ENTH/ANTH complex formation might be one of the drivers for membrane bending and scission required for vesicle formation.

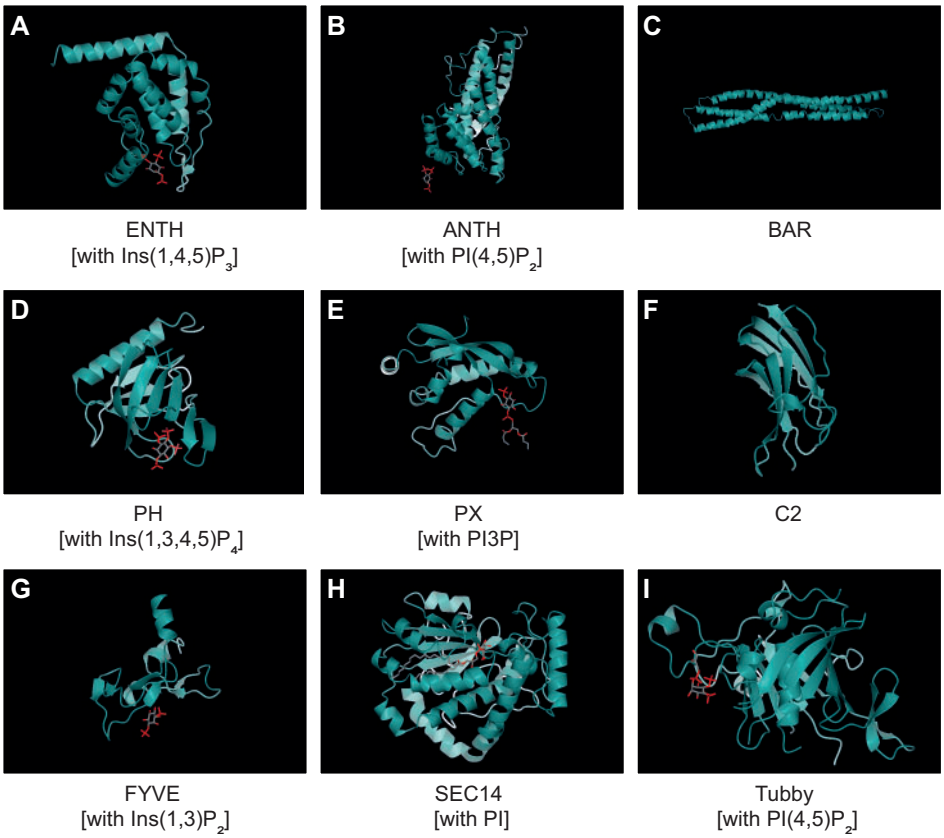

Figure 2 Structures of some phospholipid-binding domains. Additional info can be obtained via the Protein Data Bank codes, that is (A) ENTH: 1H0A; (B) ANTH: 1HFA; (C) BAR: 4ATM; (D) PH: 1FGY; (E) PX: 1H6H; (F) C2: 6KZ8; (G) FYVE: 1JOC; (H) SEC14: 3B7N; (I) Tubby: 1I7E. Some structures were resolved in complex with a ligand (i.e. phospholipid or water-soluble InsP head group), which is shown in red (A, B, D, E, G–I).

Plant N-ANTH and EPSIN proteins have the required H_0 helix to make such multi complexes in plant cells, though this remains to be shown.

BAR domains

Proteins that have a Bin/Amphiphysin/Rvs (BAR) domain form a large superfamily, most of which contain additional domains (i.e. PX, PH, or SH3; Qualmann et al., 2011; Salzer et al., 2017). Three main types can be distinguished, N-BAR, F-BAR, and I-BAR, which differ in their intrinsic curvature and lipid-binding properties. BAR domains consist of two coiled-coils, formed by three long helices (Figure 2C). Through dimerization, it creates a curved or crescent shape, with clusters of acidic residues at the concave side that are involved in lipid binding (Madsen et al., 2010; Qualmann et al., 2011; Salzer et al., 2017). Most BAR domains prefer membranes containing PS or a combination of PS and PPIs, like $PI(4,5)P_2$ (Yoon et al., 2012; Salzer et al., 2017), whereby lipid specificity is fine-tuned through coincidence detection by other LBDs present (Salzer et al., 2017).

N-BAR domains typically have an amphipathic helix (H_0) at the N-terminus, which upon binding phospholipids inserts itself into the membrane like a wedge, causing the membrane to bend. In general, N-BAR domains create a high degree of curvature. F-BAR domains lack this H_0 and exhibit a much lower degree of intrinsic curvature. I-BAR domains are more zeppelin-shaped, with an inverse curvature (Salzer et al., 2017).

At low concentrations, BAR proteins are thought to function in membrane curvature sensing, whereas at high concentrations, their role in vesicle formation emerges (Madsen et al., 2010; Simunovic et al., 2015; Salzer et al., 2017). The latter is achieved through their scaffolding function, whereby the binding of the intrinsically shaped BAR domain to the membrane forces it to adopt a similar shape. Subsequent formation of lattices through inter-dimer interactions molds the membrane into tubules or vesicles (Qualmann et al., 2011; Salzer et al., 2017).

Plants possess only a small number of BAR proteins, with Arabidopsis containing 13 (Supplemental Figure S1). Strikingly, they all belong to the N-BAR type. They can be divided into four groups on the basis of additional domains, including sorting nexin (SNX), ARF-GAP (AGD), SH3 (SH3P), and a group of uncharacterized proteins (Supplemental Figure S1). Phospholipid-binding properties of plant BAR domains have only been studied for two SH3Ps, which bind PA and PPIs (Text Box 1). SNX1-BAR likely binds PA (Lin et al., 2020). Whereas they form vesicles, SNX proteins also regulate cargo sorting and vesicle tethering (Heucken and

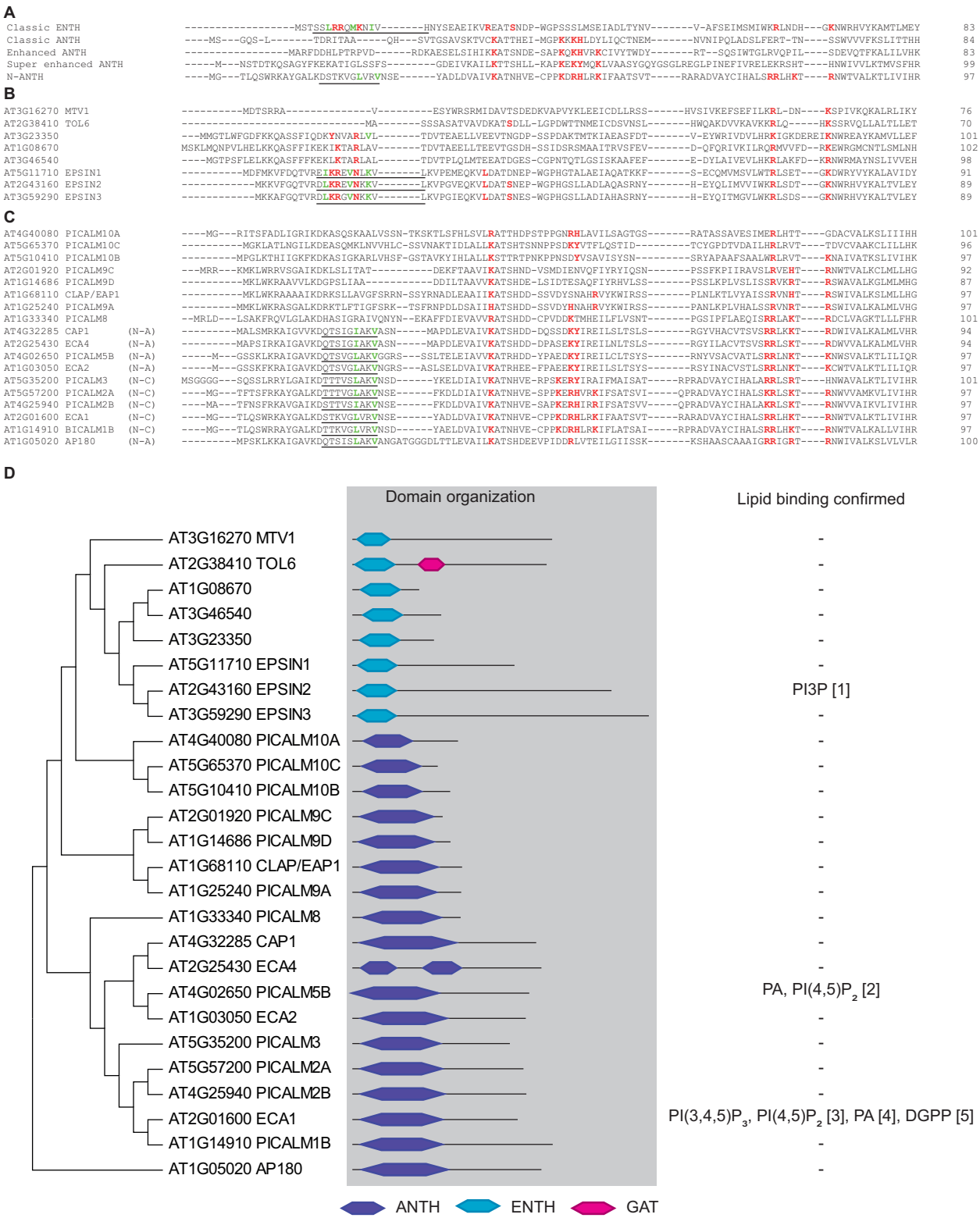

Figure 3 ENTH and ANTH domains, PPI-binding, and phylogenetic analysis. (A) Aligned sequences with different types of predicted PPI-binding sites (Silkov et al., 2011): Classic ENTH (1H0A); Classic ANTH (1HFA); Enhanced ANTH (EAK81231); Super-enhanced ANTH (CAJ03889); N-ANTH (AT2G01600, ECA1). B, Alignment of ENTH domains from Arabidopsis proteins. C, Alignment of ANTH domains of Arabidopsis proteins. Proteins predicted to have an N-ANTH domain with classic PI(4,5)P_2-binding properties are indicated by N-C (N-ANTH-classic); proteins predicted to have an alternative N-ANTH domain and likely bind other PPIs are indicated by N-A (N-ANTH-alternative). A–C, Sequences were aligned using Clustal Omega at ebi.ac.uk. H_0 in classic ENTH and putative H_0 in Arabidopsis ENTH and ANTH proteins are underlined, with structurally equivalent hydrophobic residues in green. Residues predicted to be involved in PPI binding (Silkov et al., 2011) are shown in red. (D) Phylogenetic representation of Arabidopsis ENTH and ANTH proteins with a schematic overview of all domains found through InterPro and experimentally tested lipid preferences: (1) (Lee et al., 2007); (2) (Kaneda et al., 2019); (3) (Silkov et al., 2011); (4) (McLoughlin et al., 2013); and (5) (Putta et al., 2020). Phylogenetic analysis was performed using MEGA X (Kumar et al., 2018).

Box 1 RAISING THE BAR FOR SH3P

Cited articles: Lam et al., 2001, 2002; Zhuang et al., 2013; Gao et al., 2015; Kolb et al., 2015; Ahn et al., 2017; Nagel et al., 2017; Baquero Forero and Cvrčková, 2019.

SH3P proteins interact with membranes via their BAR domain and use the SH3 domain for protein–protein interactions. Arabidopsis has three SH3P proteins. AtSH3P1 localizes to the PM, TGN vesicles, and ER (Lam et al., 2001), whereas AtSH3P2 localizes to the PM, early and late endosomes/MVBs, autophagosomes, and to the cell plate in dividing cells (Ahn et al., 2017); both AtSH3P1 and AtSH3P2 colocalize with clathrin. Lipid-binding analysis showed that SH3P1-BAR preferably binds PA, PI4P, and PI(4,5)P_2, whereas SH3P2-BAR prefers PI(4,5)P_2 and PI(3,4,5)P_3 (Lam et al., 2001; Ahn et al., 2017).

SH3P2 represents the only plant protein for which BAR-domain activity has been analyzed, showing in vitro tubulation activity on vesicles containing PI(4,5)P_2 or PI(3,4,5)P_3, confirming that SH3P2-BAR has membrane bending capacity similar to its mammalian and yeast counterparts (Ahn et al., 2017). Since PIP_3 is absent from plants, this could mean it is a PI(4,5)P_2-specific phenomenon. This was confirmed in planta, where SH3P2 was shown to be involved in tubulation at the actively growing region of the cell plate, possibly together with dynamin-related protein 1A (Ahn et al., 2017). During autophagy, SH3P2 is detected on the tubular-forming structures of developing autophagosomes (Zhuang et al., 2013). During CME, SH3P2 is enriching ubiquitinated cargos and, through interaction with VPS23, passes these on to the endosomal sorting complex required for transport (ESCRT) machinery for sorting (Nagel et al., 2017). SH3P2 has also been shown to interact with the de-ubiquitylating enzyme-associated molecule with the SH3 domain of STAM3 (AMSH3), whose activity was influenced by this interaction (Nagel et al., 2017), and with the PI3P-binding protein, FREE1/FYVE1 (Gao et al., 2015; Kolb et al., 2015) and formin, FH5 (Baquero Forero and Cvrčková, 2019).

Whereas less is known about SH3P1 and SH3P3, it is clear that they are also involved in CME. SH3P1 slightly inhibits the activity of the clathrin-uncoating factor auxilin-like (Lam et al., 2001). SH3P3 inhibits GTPase activity of dynamin-like protein ADL6 (Lam et al., 2002).

Ivanov, 2018; see Supplemental Text T2). AGDs regulate vesicle trafficking for the ARF subfamily of small G proteins (Naramoto et al., 2016; see Supplemental Text T2).

PX domains

The Phox-homology (PX) domain is a PPI-binding module of ~110–140 aa, consisting of three antiparallel β-strands (β1–β3), followed by three α-helices (α1–α3; Figure 2E). H_1 and H_2 are connected by an extended sequence, termed the PPK loop. In most PX domains, this loop contains a conserved ΨPxxPxK motif (Ψ = large aliphatic aa V, I, L, or M; green in Figure 4A). PX domains have two potential PPI-binding sites. The canonical (or classic) PPI-binding site prefers PI3P and has the consensus motif, R[Y/F]X_{23-30}KX_{13-23}R (blue in Figure 4A), where X stands for any aa residue. The first arginine (R) is found at the end of the β3-strand and the lysine (K) is part of the ΨPxxPxK motif (Teasdale and Collins, 2012; Jia et al., 2014; Mas et al., 2014; Chandra et al., 2019). Further modeling showed that for PI3P binding to be stable, the β1–β2 loop first needs to be inserted into the membrane, facilitating the entry of PI3P into the binding pocket (Jia et al., 2014).

A recent study by Chandra et al. (2019) led to the discovery of an alternative, secondary PPI-binding site, formed by the semi-conserved His/Tyr side chain in H_1 (7 aa from the conserved R[Y/F] of the canonical binding pocket) and the positively charged basic side chains located between His/Tyr and the ΨPxxPxK motif (indicated in red in Figure 4A), binding to PIP_2 and PIP_3. As both binding sites can be occupied simultaneously, it will help the PX protein to localize to membranes more effectively and specifically (Chandra et al., 2019).

Arabidopsis contains 11 PX proteins (van Leeuwen et al., 2004; Agudelo-Romero et al., 2020; Figure 4). These include six Sorting Nexin proteins (SNX1, SNX2a, SNX2b, and SNX3-5), two phospholipase Ds (PLDζ1 and 2), and three EREX (Endosomal Rab Effector with PX domain; EREX, EREL1, and EREL2) proteins (see Supplemental Text T2). The latter act as effectors of the small G protein, RAB5, mediating vacuolar trafficking (Sakurai et al., 2016; see Supplemental file). PLDs produce PA and are typically involved in lipid signaling during plant development and (a)biotic stress responses (Hou et al., 2016; Li and Wang, 2019). SNX proteins are part of a retromer-like protein complex, which is involved in endosomal trafficking of important membrane transporters, such as PIN2 (auxin), BOR1 (boron), IRT1 (iron), or receptors (e.g. BRI1 for brassinosteroids; Jaillais et al., 2006; Kleine-Vehn et al., 2008; Pourcher et al., 2010; Ivanov et al., 2014; Simon et al., 2016; Hirano et al., 2017; Yoshinari et al., 2018; VanDamme, 2021).

In silico analysis of the PPI-binding sites in Arabidopsis PX proteins (Figure 4, A) predicts that SNX1, SNX2a, and SNX2b, and EREL1 and EREL2 most likely bind PI3P via the canonical site and may bind other PPIs via the alternative site. Experimentally, SNX1 was shown to bind PI3P and PI(3,5)P_2 (Hirano et al., 2015). For SNX2b, only PI3P binding was observed (Phan et al., 2008). EREX and SNX3 and SNX4 are predicted to bind PPIs via both sites, although a lipid overlay assay for EREX only revealed PI3P binding (Sakurai

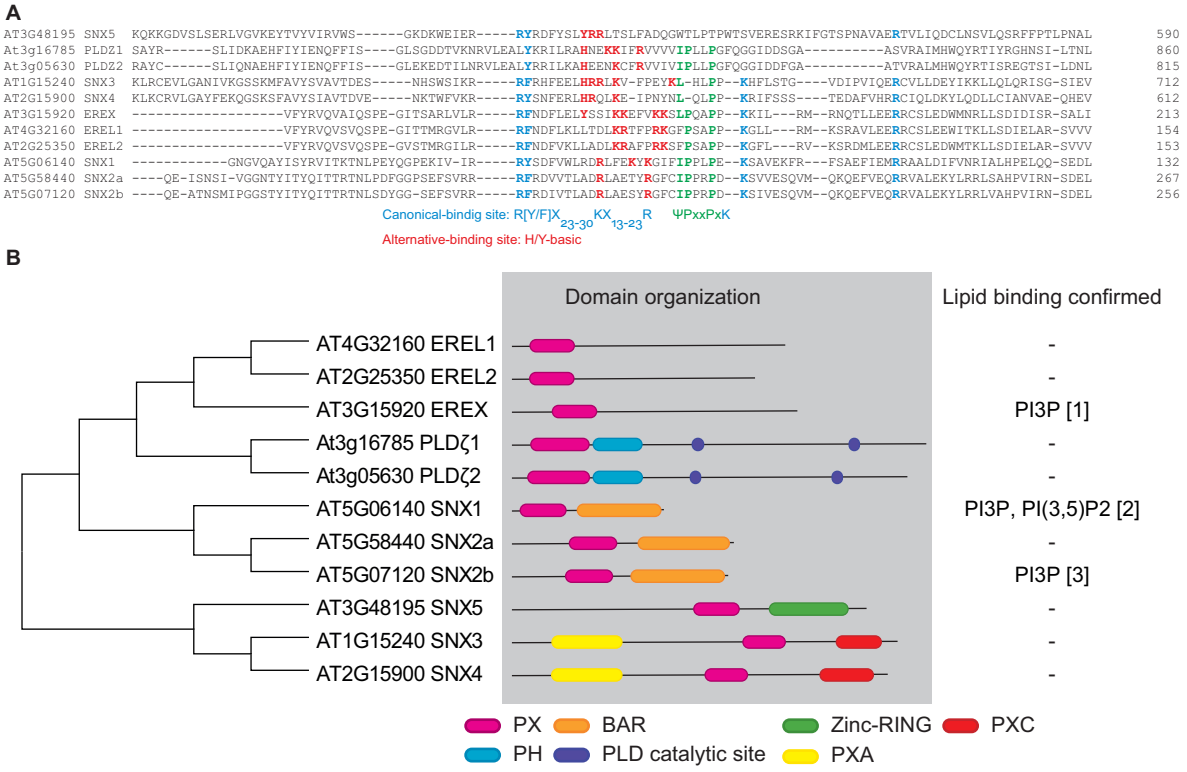

Figure 4 Arabidopsis PX domains, phylogenetic analysis, and lipid binding. A, Alignment of PX domains of Arabidopsis PX domain containing proteins. Sequences were aligned using Clustal Omega at ebi.ac.uk. The ΨPxxPxK motif (Ψ = large aliphatic aa V, I, L, or M) residues are in green, the residues of the canonical (R[Y/F]X_{23-30}KX_{13-23}R) PPI- (PI3P-) binding site are in blue, and the residues of the alternative PPI-binding site (H/Y-basic) are in red, according to Chandra et al. (2019). B, Phylogenetic representation of Arabidopsis PX proteins. A schematic overview of all domains found through InterPro. References for phospholipid binding of PX: (1) (Sakurai et al., 2016); (2) (Hirano et al., 2015); and (3) (Phan et al., 2008). Phylogenetic analysis was performed using MEGA X (Kumar et al., 2018).

et al., 2016). Based on sequence criteria (Figure 4A), it is unlikely that the PX of PLDζ1 or PLDζ2 will bind PI3P via the canonical site. SNX5 contains two half binding sites (Figure 4A) and may likely not bind PPIs at all.

FYVE domains

With 60–80 aa, FYVE (Fab1p, YOTB, Vac1p, EEA1; the first proteins where it was discovered; Stenmark et al., 2002) represents one of the smallest LBDs. Structurally, it has two long hydrophobic loops at the N-terminus, followed by two small double-stranded antiparallel β-sheets and a C-terminal α-helix (Figure 2G). The domain is stabilized by two zinc-binding clusters in the hydrophobic loops and is highly specific for PI3P. Many FYVE proteins are involved in endosomal and vacuolar trafficking and autophagy (Stenmark et al., 2002; Lystad and Simonsen, 2016; Chung 2019).

Binding PI3P is a three-step process: First, close proximity to the membrane is obtained via the hydrophobic loops, then detection and specific binding of PI3P is achieved, which reduces the hydrophobicity of residues surrounding the binding pocket, thus allowing the third step, membrane penetration and anchoring (Kutateladze and Overduin, 2001; Diraviyam et al., 2003). The PI3P-binding pocket is formed by the conserved WxxD, (R/K)(R/K)HHCR, and RVC motifs at the end of the β1-strand, whereby the lipid's head group ends up parallel to the β1 strand. Due to its small size, PPI specificity is mostly indirect, with only PI3P or PI5P fitting inside the FYVE pocket, with PI3P being preferred (Kutateladze and Overduin, 2001; Agudelo-Romero et al., 2020). Membrane localization is further positively influenced by coincidence detection, either via homodimerization, additional lipid-binding motifs, or protein–protein interaction (Lystad and Simonsen, 2016).

Arabidopsis has 15 proteins with a FYVE domain. They all contain the conserved Wxx(D/G), (R/K)(R/K)H(H/N)C(R/Y), and RVC motifs (Supplemental Figure S2; van Leeuwen et al., 2004; Wywial and Singh, 2010; Agudelo-Romero et al., 2020). Lipid binding was analyzed for four of them, including FREE1/FYVE1, a protein that is involved in complex regulation in endosomal trafficking, autophagy, and vacuolar biogenesis as well as ABA signaling and indeed binds PI3P (Barberon et al., 2014; Gao et al., 2014; Kolb et al., 2015; Garcia-Leon et al., 2019; Li et al., 2019; Zhao et al., 2019). CELL DEATH RELATED ENDOSOMAL FYVE/SYLF PROTEIN 1 (CFS1) also binds PI3P and interacts with ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT 1 (ESCRT-1; Sutipatanasomboon et al., 2017). The two other proteins belong to the uncharacterized PRAF family (for PH domain, RCC1 and FYVE domain), of which Arabidopsis contains nine members. For PRAF1 (At1G76950), FYVE was

shown to bind various D3-PPIs, whereas PH binds PI(4,5)P_2 (Jensen et al., 2001). The second, which confusingly is called PARF-1 (At1G65920), was shown to bind PI3P and PI5P (Heras and Drøbak, 2002). Functionally, little is known about PRAF proteins, though a recent study in *Medicago truncatula* showed a role in root and nodule development (Hopkins et al., 2014).

PH domains

The pleckstrin homology (PH) domain consists of ~120 aa, which form an antiparallel β-sandwich that is closed off at one end by one or two amphipathic α-helices. At the open end of the β-sandwich, there are three hypervariable loops (β1/β2, β3/β4, and β5/β6) that can bind PPIs (Figure 2D). PH domains have two potential PPI-binding sites, a canonical C-site and an alternative A-site (Lemmon, 2008; Kahn and Lambright, 2015; Naughton et al., 2018; Yamamoto et al., 2020). The C-site has a binding pocket formed by the positively charged aa of the β1/β2 and β3/β4 loops. This binding pocket is further characterized by a KXn(K/R)XR motif in the β1/β2 loop (Lemmon, 2008; Naughton et al., 2018). The A-site is located between the β1/β2 and β5/β6 loops, with the binding pocket formed by the (K/R)-X-W motif in the β1/β2 loop and positively charged residues at the β5/β6 loop (Naughton et al., 2018). Initially, it was assumed that the PH domain used either pocket to bind PPIs but work on ARF-GAP protein; ASAP1 revealed that both pockets were simultaneously used to bind the membrane (Jian et al., 2015). Modeling suggests that the use of both binding pockets is widely spread (Naughton et al., 2018; Yamamoto et al., 2020). The PH domain of GRP1 was recently shown to bind up to five PIP_3 molecules simultaneously, allowing a very tight binding to the PM (Yamamoto et al., 2020).

PPI preference of the PH domains is determined by aa in the three hypervariable loops at the open end of the β-sandwich, especially by the KXn(K/R)XR and/or (K/R)XW motifs. Many of the analyzed PH domains show specificity for PI(4,5)P_2 and/or PI(3,4,5)P_3; however, preference for other PPI isomers has also been observed (Naughton et al., 2018).

The PH domain is, like in other eukaryotes, one of the most common LBDs in plants. Arabidopsis has 59 proteins with a clearly defined PH domain (Supplemental Figure S3). In contrast to earlier reports (Stevenson et al., 1998; Stevenson-Paulik et al., 2003), and according to the UniProt and InterPro databases, Arabidopsis PI4-kinase (PI4K) does not have a PH domain. After studying the original reports and comparing them with current domain annotation, we believe that the region labeled by Stevenson-Paulik et al. (2003) as PH domain is currently designated as phosphoinositide 3-kinase family accessory domain (PIK domain; InterPro). The function remains unclear, but it is likely involved in substrate presentation. Of the 59 Arabidopsis PH proteins, lipid binding has only been studied for 12 (Supplemental Figure S3), which has revealed a wide variety of PPI and PA preferences. As discussed for PDK1 in Text Box 2, PPI preference is not always clear-cut.

C2 domains

The C2 domain consists of ~130 aa that form an eight-stranded, antiparallel β-sandwich (Figure 2F). Whereas the β-sandwich core is highly conserved, the loops connecting them are variable in sequence and conformation (Corbalan-Garcia and Gomez-Fernandez, 2014; Stahelin et al., 2014), which allows variation in responsiveness to Ca^{2+} and lipids. Membrane binding can be Ca^{2+} dependent as well as independent. The Ca^{2+}-dependent binding takes place as follows: 2–3 Ca^{2+} ions bind the calcium-binding region (CBR) located at loops at the top side of the domain. Once bound, the C2 domain can bind anionic lipids like PA and PS or cationic lipids like PE and PC. The Ca^{2+}-independent binding of C2 binds PPIs, like PI(4,5)P_2, through aa located in the β3 and β4 strands that form a β-groove. Depending on the C2 domain, it uses either one or both ways to bind membranes (Corbalan-Garcia and Gomez-Fernandez, 2014; Stahelin et al., 2014). There are 123 Arabidopsis proteins that contain a C2 domain (Supplemental Figure S4). For the majority, lipid-binding analyses are lacking. An exception forms PLDα1, of which a crystal structure, including the C2, was recently resolved (Li et al., 2020; see Text Box 3). C2 proteins that have been functionally characterized include enzymes, like PLC and PLD whereby the C2 domain has a role in positioning the enzyme to access its substrate (Otterhag et al., 2001; Jiménez et al., 2003; van Wijk et al., 2018; Li and Wang, 2019; Li et al., 2020; see Supplemental Text T2), as well as structural proteins, like the membrane tethers synaptotagmins (SYTs) and Multiple C2 Domains and Trans-Membrane Region Proteins (MCTPs), which are connected to the ER via a transmembrane domain and the PM via their C2 domains, regulating the distance between ER and PM (Jiménez et al., 2003; Pérez-Sancho et al., 2016; Brault et al., 2019; Ishikawa et al., 2020; Bayer and Rosado, 2021). See also discussion in Supplemental Text T2.

Tubby domains

The Tubby domain consists of ~270 aa, folded into a slightly oblong 12-stranded β-barrel that is surrounded by a central hydrophobic helix at the C-terminus (Figure 2I). The domain has a highly positively charged groove around one half of the barrel and a smaller negatively charged patch on the other side (Boggon et al., 1999). The domain binds mainly PI(4,5)P_2 on one end of the positively charged groove, characterized by a conserved KxR motif that binds the 4- and 5-phosphates of PIP_2. The lipid is further held in place by an Arg located ~30-aa upstream (Santagata et al., 2001). In vivo, the Tubby domain shows a strong preference for PI(4,5)P_2, and in vitro it also binds PI(3,4)P_2 and PI(3,4,5)P_3 (Santagata et al., 2001; Szentpetery et al., 2009; Simon et al., 2014). The positively charged groove of Tubby is also able to bind dsDNA, likely in a sequence-specific manner (Boggon et al., 1999).

Initially, Tubby proteins were discovered in metazoans as transcription factors bound to the PM via PI(4,5)P_2, for which receptor-mediated activation of PLCβ triggers their

Box 2 PDK1

Cited articles: Deak et al., 1999; Anthony et al., 2004; Rentel et al., 2004; Vermeer et al., 2006, 2009; Vermeer and Munnik, 2010; Rodriguez-Villalon et al., 2015; Gujas et al., 2017; Li et al., 2019; Scholz et al., 2019; Doumane et al., 2020; Tan et al., 2020; Xiao and Offringa, 2020.

PDK1 (3-phosphoinositide-dependent kinase 1) is an AGC protein kinase family member functioning as a master regulator of downstream AGC kinases, such as UCN, OXI1, PAX, and D6PK. PDK1 has a C-terminal PH domain that binds PA and PPIs, and an N-terminal protein kinase domain that activates downstream AGC kinases. In Arabidopsis, two PDK1s exist, PDK1.1 and PDK1.2 (Deak et al., 1999; Anthony et al., 2004; Scholz et al., 2019; Tan et al., 2020; Xiao and Offringa, 2020).

In metazoans, PDK1 requires PIP_3 binding for activation (Anthony et al., 2004; Scholz et al., 2019; Tan et al., 2020; Xiao and Offringa, 2020). Plants lack PIP_3, but PA and $PI(4,5)P_2$ activate Arabidopsis PDK1 in vitro, suggesting they fulfill this role (Anthony et al., 2004). Recent analysis on the PDK1's PH domain showed binding of PI3P and PI4P both in vitro and in vivo (Tan et al., 2020). So it remains unclear what the preferred lipid of the intact PDK1 is. Nevertheless, there is consensus about its PM localization. With PI4P, $PI(4,5)P_2$, and PA present at the PM, each of these lipids is a likely in planta target since PI3P is never on the PM (Vermeer et al., 2006).

To identify PDK1's true target lipid, multiple strategies could be used. (1) Liposome studies for assessing PDK1s ability to bind predicted lipids rather than lipid–protein overlay assays. (2) FP-tagged PDK1 to monitor membrane-binding behavior, using conditions when these lipids are specifically formed, together with the respective lipid biosensors (Vermeer et al., 2006, 2009; Vermeer and Munnik, 2010; Li et al., 2019). (3) Combining this with the inducible production (Rodriguez-Villalon et al., 2015; Gujas et al., 2017) or removal of these lipids e.g. iDePP lines (Doumane et al., 2020). (4) The ability of PDK1 to phosphorylate downstream targets under such conditions will also be highly valuable, for example D6PK (Tan et al., 2020), PAX (Xiao and Offringa, 2020), or OXI1/AGC2 (Anthony et al., 2004; Rentel et al., 2004).

Whereas the current dogma is that lipid binding by the PH domain of PDK1 is required for PM localization and activation, recent research (Xiao and Offringa, 2020) showed that PDK1.1 has alternative splice variants, including one without a PH domain. Using this PDK1.1(-PH) variant, PAX was shown to be both phosphorylated in the cytosol and also partly rescue the *pdk1.1 pdk1.2* loss-of-function mutant, indicating that at least part of PDK1's regulation is, or can be, lipid independent.

release and subsequent translocation into the nucleus where they act as transcriptional regulators (Boggon et al., 1999; Cho and Stahelin, 2005). However, due to a highly variable N-terminus, including WD40, suppressor of cytokine signaling (SOCS), F-box, and transcription–modulation segments, Tubby-like proteins (TLPs in plants, TUBs in metazoans) turn out to have various functions (Boggon et al., 1999; Mukhopadhyay and Jackson, 2011; Lai et al., 2012).

Plants tend to contain more TLPs than metazoans but exhibit less variation in the N-terminus (Wang et al., 2018). Arabidopsis has 11 TLPs that all contain an F-box at its N-terminus, except TLP8 (Supplemental Figure S5). They are localized to the PM with the exception of TLP4, a likely pseudogene, and TLP8 (Lai et al., 2004; Mukhopadhyay and Jackson, 2011; Bao et al., 2014). TLP3 was shown to bind $PI(4,5)P_2$ and in vivo to detach from the PM in response to salt, mannitol, or H_2O_2 treatment (Reitz et al., 2012; Bao et al., 2014). Yeast two-hybrid assays showed that TLPs, via the F-box domain, interact with one or more Arabidopsis Skp1-like proteins (Bao et al., 2014), implying that when released from the PM, these TLPs function as part of the SCF complex in the proteasomal degradation pathway. How plant TLPs are released from the PM and which proteins they recruit for degradation remains unknown.

In a recent study, TLP2 was suggested to function as a transcription factor (Wang et al., 2019). Using protoplasts, it was shown that co-transfection of TLP2 with Nuclear Facto-Y subunit C (NF-YC) resulted in the activation of UDP-glucose epimerase1 (UGE1) transcription. From mammalian work, we know that Tubby can bind dsDNA, but the transcriptional activation is regulated by transcription–modulation segments at the N-terminus (Boggon et al., 1999) that TLP2 lacks. Perhaps TLP2 promotes transcription factor activity in a protein degradation-dependent way, as was shown for the F-box protein UFO (Stefanowicz et al., 2015).

SEC14 domains

SEC14 belongs to unique category of LBDs that completely engulf a lipophilic molecule, with the hydrophobic tail oriented toward the middle of the protein and the hydrophilic head group oriented outward. The domain is named after the yeast phosphatidylinositol transfer protein (PITP), Sec14p, which was isolated in a genetic screen for secretory defects (Bankaitis et al., 1989). SEC14 consists of ~185 aa that fold into a two-lobed globular structure, formed by four antiparallel β-strands, bordered by two-long α-helices (Figure 2H; Grabon et al., 2019). A hydrophobic binding pocket is located at the larger of the two lobes. The whole

Box 3 PLD

Cited articles: Hong et al., 2016; Li et al., 2020.

Recent analysis of the crystal structure of Arabidopsis PLDα1 beautifully illustrates the importance of protein folding and how individual domains interact (Li et al., 2020).

PLD hydrolyzes structural phospholipids at the phosphodiester bond, producing PA and the head group of the lipid. Arabidopsis contains 12 PLDs, grouped into PLDα (1-3), PLDβ (1,2), PLDγ (1-3), PLDδ, PLDε, and PLDζ (1,2). Except for the latter, they all contain an N-terminal C2 domain; PLDζs have an N-terminal PX and PH domain. All PLDs contain two His-x-Lys-(x)(4)-Asp (HKD) motifs that form the catalytic site of the enzyme. Most PLDs prefer PC, PE, and PG as substrate. In vitro, the enzymatic activity of C2-PLDs typically depend on mM Ca^{2+} concentrations, and some are enhanced by $PI(4,5)P_2$ or detergent (Hong et al., 2016). The Ca^{2+} dependency was earlier assumed to rely on the Ca^{2+}-dependent phospholipid-binding capacity of the C2 domain. However, recent analysis of crystal structure of PLDα1 revealed otherwise. Li et al. (2020) showed that the two HKD motifs are packed against each other in a saddle-like conformation, sharing the substrate-binding pocket, and is closed off by a lid when PLD is inactive. The catalytic site is located at the bottom of the binding pocket. A novel Ca^{2+}-binding site was discovered nearby the catalytic site. The idea is that when a C2-PLD binds the membrane, and Ca^{2+} binds the novel Ca^{2+}-binding site near the catalytic site, the lid of the lipid-binding pocket opens, enabling phospholipid binding and subsequent hydrolysis. Although the main function of C2 appears to target PLD to the membrane, its binding to the catalytic domain is essential for PLD's activity. The PLD is targeted to the membrane using positively charged surface patches at the C2 domain, but also between C2 and the catalytic domain. After activation of the PLD and binding of the substrate, the C2 domain twists away from the catalytic domain (Li et al., 2020).

These results show that C2 domains can play an active role in regulating enzyme activity, not only by facilitating membrane interaction.

structure resembles a closed fist, holding the lipid, with the thumb forming the small N-terminal lobe that closes off the pocket (Saito et al., 2007; de Campos and Schaaf, 2017).

Whereas initially characterized as PITP, evidence that they indeed transfer lipids in vivo is lacking. Currently, there are two working hypotheses how SEC14 might use its phospholipid-binding capacity. The first model is based on the original hypothesis, but instead of the protein freely traveling though the cytosol, it is tethered to a donor and accepting membrane, with the SEC14 domain being able to move between the two (Kim et al., 2013). The second so-called nanoreactor model proposes that SEC14 regulates PPI metabolism by presenting PI to PI4K, thereby boosting PIP synthesis. So far, the nanoreactor model is only supported for Sec14p- and Sec14-like proteins in yeast (Grabon et al., 2019).

In Arabidopsis, 35 SEC14-containing proteins are present (Supplemental Figure S6). These can be separated in three subgroups: (1) free-standing Sec14-like proteins; (2) SEC14-nodulin proteins; and (3) PATELLINs (PATL), SEC14-GOLD proteins (Huang et al., 2016; Montag et al., 2020). The only free-standing Sec14-like protein analyzed for its lipid binding is PITP7/CPSFL1, which binds PA-containing liposomes and colocalizes with PA and PI4P in vivo (Hertle et al., 2020). PITP7/CPSFL1 is required for vesicle formation at the inner envelope membrane of the chloroplast for photoautotrophic growth (Hertle et al., 2020; Schroda 2020). For the PATELLINs, it was shown that PATL1 binds most PPIs, with highest preference for PI5P and $PI(4,5)P_2$ (Peterman et al., 2004). PATL2 binds all PPIs too, with highest affinity for the three PIP isomers, and interestingly, when the protein is phosphorylated, this shifts toward the PIP_2s (Suzuki et al., 2016). Recent elegant analysis of PATL2 by Montag et al. (2020) revealed PI4P and PI(4,5)P2 binding, but surprisingly also cardiolipin, a typical phospholipid found in mitochondria and plastids consisting of two PG molecules fused via the head group, and of which the two phoshatidyl moieties can be located in two different membranes. PATL3 preferentially binds PI4P and $PI(4,5)P_2$ over PA and PI3P (Wu et al., 2017). All PATLs are involved in cell plate formation and are likely involved in recycling of membrane proteins (Peterman et al., 2004; Zhou et al., 2018). For the SFHs, no direct phospholipid-binding analysis has been reported but SEC14 domains of various SFHs are able to rescue the yeast Sec14p mutant (Vincent et al., 2005). SFH1 is involved in the tip-directed $PI(4,5)P_2$ gradient required for polar root hair growth (Vincent et al., 2005; Ghosh et al., 2015).

Additional lipid-binding domains

Besides the main LBDs discussed above, some additional are summarized in Table 1 and briefly discussed in the Supplemental Text T1.

Future perspectives

It is clear that LBDs form an integral part of many plant proteins. Whereas they facilitate the spatiotemporal interaction of proteins with membranes, they can also be involved in activation mechanisms of enzymes. The lipids they typically bind (e.g. PPIs, PA) provide identity to organelles and

Table 1 Overview of lipid-binding domains in plants.

Domain	Arabidopsis proteins	Average size (amino acids)	Preferred lipid	Example protein	References
ENTH	8	~130–150	$PI(4,5)P_2$	AT5G11710 EPSIN1	(Ford et al., 2002)
ANTH	18	~250–300	$PI(4,5)P_2$	AT1G05020 AP180	(Silkov et al., 2011)
BAR	13	~240	anionic phospholipids	At1g31440 SH3P1	(Salzer et al., 2017)
PX	11	~110–140	PI3P, PIP_2 and PIP_3	AT5G06140 SNX1	(Chandra et al., 2019)
FYVE	15	~60–80	PI3P	AT1G20110 FREE1/ FYVE1	(Wywial and Singh, 2010)
PH	59	~120	PPIs; specificity and affinity varies	AT5G04510 PDK1.1	(Naughton et al., 2018; Yamamoto et al., 2020)
C2	123	~130	anionic and cationic lipids; PPIs	AT3G15730 PLDα1	(Corbalan-Garcia and Gomez-Fernandez, 2014; Stahelin et al., 2014)
Tubby	11	~270	$PI(4,5)P_2$	At2G47900 TLP3	(Mukhopadhyay and Jackson, 2011; Wang et al., 2018)
SEC14	35	~280	PS, PA, PI, and PPIs	AT1G72150 PATL1	(Grabon et al., 2019)
C1	137	~30	DAG	AT5G07920 DGK1	(Colón-González and Kazanietz, 2006; Rahman and Das, 2015)
FERM	1	~300	PI4P, $PI(4,5)P_2$	AT5G65930 KCBP/ ZWICHEL	(Hamada et al., 2000; Buschmann et al., 2015)
Annexins	8	~310	anionic phospholipids	AT1G35720 ANN1	(Clark et al., 2012; Yadav et al., 2018)
PDZ	16	~90	$PI(4,5)P_2$	AT1G55480 ZKT	(Gardiner et al., 2011; Chen et al., 2012)
Nlj16-like nodulin	11	~120	$PI(4,5)P_2$	AT4G34580 SFH1	(Ghosh et al., 2015)
GRAM	15	~70	PPIs, PA, PS	AT2G22475 GEM	(Choudhury et al., 2006; Mauri et al., 2016; Sandhu et al., 2018
SYLF	2	~220	PI3P	At3g43230 CFS1	(Hasegawa et al., 2011)

endosomal compartments and are typically involved in signaling as well as membrane fission and fusion (Testerink and Munnik, 2011; Gerth et al., 2017; Noack and Jaillais, 2017, 2020; Colin and Jaillais, 2020; Bayer and Rosado, 2021; Jaillais, 2021; Liua et al., 2021) .

Filling in the gaps

As the overall understanding of LBDs becomes clearer, the effect of individual LBDs on a protein's function, and hence, its cellular function, remains largely unknown. It will be important to characterize lipid binding much more extensively and in particular in vivo to grasp this bigger picture (see Outstanding Questions). In vitro, isolated LBDs may bind differently from intact proteins, purely by, for example, folding, lack of interacting proteins, pH, or posttranslational modification. For in vivo analyses, one could make use of the many KO mutants that have become available over the years for lipid kinases, phosphatases, and phospholipases (e.g. Munnik and Testerink, 2009; Gerth et al., 2017; Zarza et al., 2019, 2020). Colocalization- and fluorescence resonance energy transfer (FRET)-type analyses using genetically encoded lipid biosensors (Vermeer and Munnik, 2010; Simon et al., 2014; Li et al., 2019; Platre et al., 2019) should be used to validate the in vivo interactions of lipid and LBDs and LBD proteins. In addition, novel genetic tools that enable the inducible removal of specific lipids from membranes (iDePP; Doumane et al., 2020) will provide excellent means for more accurate studies of LBD's function for proteins in live cells. Protein crystallography and introduction of crucial mutations will provide details to understand this mechanistically and maybe even evolutionarily.

Precision signaling

The last few years showed that anionic lipids and LBD proteins play a major role in signaling in various developmental and stress response processes. With seemingly identical lipid responses for a wide variety of stress signals, for example an increase of PIP_2 is observed for salt, osmotic, and heat stress (van Leeuwen et al., 2007; Mishkind et al., 2009; Zhang et al., 2018), distinct cellular responses need to be triggered to deal with these stresses. Like for Ca^{2+} signaling (Johns et al., 2021), it is largely unknown how this specificity is achieved (see Outstanding Questions), although the answer is likely that a combination of signals in time and space will create such specificity. The recent discovery of a link between PLDζ2--derived PA- and SNX1--dependent vacuolar degradation of PIN2 during root hair development under phosphate limitation (Lin et al., 2020) offers a glimpse of how such specificity might be achieved. PLDζ2 and SNX1 are both multi-LBD proteins, with PLDζ2 having a PX and a PH domain and SNX1 a PX and BAR domain, allowing for coincidence detection. Having PLDζ2 recruited to specific membranes enriched in particular PPIs via its PX and PH domains (currently unknown which) ensures that the PA produced by PLDζ2 accumulates in an environment favorable for SNX1 binding (and activity). It will be exciting to find how

OUTSTANDING QUESTIONS

- How is lipid-binding specificity achieved?
- How does sequence variation in the lipid-binding motif contribute to this specificity?
- What effect has lipid binding on the protein's structure, activity, and binding partners, as well as on the cellular process the protein is involved in?
- How are ENTH, ANTH, and BAR proteins involved in facilitating faster clathrin-mediated endocytosis and with larger vesicles?
- How are anionic phospholipids involved in precision signaling, and how does this relate to other universal second messengers, like Ca^{2+}?
- How are the enzymes that generate these signaling lipids regulated? Which receptors or sensors are acting upstream?

precision signaling of other parts of endo- and exocytosis is regulated. Precision signaling is also required for the release of TLPs from the PM. Currently, it is still unknown whether this involves PLC and how specific TLPs are identified.

How do plant LBD proteins enable larger and faster clathrin-coated vesicle formation?

Recently, clathrin-mediated endocytosis (CME) was visualized for the first time in plant cells (Narasimhan et al., 2020). Whereas they build larger vesicles in plants than in mammalian and yeast cells, they also do this in a shorter time. BAR, ENTH, and ANTH proteins all take part in vesicle formation, and with the recent discovery that some ENTH/ANTH proteins can form lattices (Garcia-Alai et al., 2018; Heidemann et al., 2020), it will be interesting to analyze if lattices of plant ENTH and N-ANTH proteins are stronger and enable larger and faster vesicle formation and also to determine the specific roles of PA and PPIs (see Outstanding Questions).

The past decades have clearly shown that anionic lipids and their interacting LBD proteins play a crucial role in plant development and stress signaling. The next decade, we are sure, will tell us more as to how.

Supplemental data

Supplemental Text T1. Additional lipid-binding domains.

Supplemental Text T2. Interesting LBD-containing proteins.

Supplemental Figure S1. Arabidopsis BAR proteins.

Supplemental Figure S2. Phylogenetic representation of Arabidopsis FYVE proteins with additional InterPro domains and lipid binding.

Supplemental Figure S3. Arabidopsis PH proteins. Phylogenetic representation and schematic overview of all domains found through InterPro and lipid binding.

Supplemental Figure S4. Phylogenetic representation, domain overview, and lipid binding of Arabidopsis C2 proteins.

Supplemental Figure S5. Arabidopsis Tubby proteins.

Supplemental Figure S6. Phylogenetic representation of Arabidopsis SEC14 proteins.

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