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REVIEW

The dynamics of plant plasma membrane proteins: PINs and beyond

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ABSTRACT

Plants are permanently situated in a fixed location and thus are well adapted to sense and respond to environmental stimuli and developmental cues. At the cellular level, several of these responses require delicate adjustments that affect the activity and steady-state levels of plasma membrane proteins. These adjustments involve both vesicular transport to the plasma membrane and protein internalization via endocytic sorting. A substantial part of our current knowledge of plant plasma membrane protein sorting is based on studies of PIN-FORMED (PIN) auxin transport proteins, which are found at distinct plasma membrane domains and have been implicated in directional efflux of the plant hormone auxin. Here, we discuss the mechanisms involved in establishing such polar protein distributions, focusing on PINs and other key plant plasma membrane proteins, and we highlight the pathways that allow for dynamic adjustments in protein distribution and turnover, which together constitute a versatile framework that underlies the remarkable capabilities of plants to adjust growth and development in their ever-changing environment.

KEY WORDS: *Arabidopsis*, PIN protein, Plasma membrane protein, Polarity, Protein sorting

Introduction

Plasma membrane proteins play key roles in mediating responses to the endogenous and environmental cues that regulate various developmental and metabolic events. Intrinsic membrane proteins have been implicated in various transport processes across membranes as well as in perception and further transmission of regulatory signals. Crosstalk at the plasma membrane interface is all the more important for sessile organisms such as higher plants, which are required to maintain a diverse spectrum of plasma membrane receptor and transport proteins in order to be able to properly sense and respond to fluctuations in their environment (Geldner and Robatzek, 2008; Otegui and Spitzer, 2008; Richter et al., 2009; Robinson et al., 2012; Korbei and Luschnig, 2013). In plants, as in any other eukaryote, this is achieved by a complex system of internal membranes that serves to transport proteins to their site of action and degradation. Exchange of material between these membranes is achieved by vesicles that bud off from one membrane, mature and fuse with another in a highly controlled fashion.

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Plasma membrane proteins are not static but exhibit remarkable mobility (Fig. 1). This involves lateral diffusion within the plasma membrane, which has been implicated as an important determinant for controlling protein-protein interactions and is suggested to influence rates of intracellular sorting of membrane proteins (Singer and Nicolson, 1972; Simons and Ikonen, 1997; Kleine-Vehn et al., 2011; Martiniere et al., 2012). Internalized plasma membrane proteins in general undergo endocytic sorting from the plasma membrane into sorting endosomes, which constitute components of the *trans*-Golgi network/early endosome (TGN/EE), followed by either recycling to domains at the plasma membrane or further sorting into late endosomes/multivesicular bodies (MVBs) for their ultimate degradation in the lytic vacuole (Murphy et al., 2005; Jürgens and Geldner, 2007; Schellmann and Pimpl, 2009; Zarsky and Potocky, 2010; Reyes et al., 2011; Robinson and Pimpl, 2013). Both protein recycling and degradation represent efficient means to modulate the localization, abundance and hence the activity of protein pools at the plasma membrane (Fig. 1).

Following the identification of *Arabidopsis* PIN-FORMED (PIN)-type auxin transport proteins some 15 years ago (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998), the analysis of the dynamics of plasma membrane proteins in plants experienced a great leap forward (Krecek et al., 2009; Grunewald and Friml, 2010; Löfke et al., 2013a). Auxin, as one of the 'classical' plant hormones, is central to the control of cell proliferation and morphogenesis and its activity has been linked to a wide variety of cellular events. Spatiotemporal variations in auxin distribution in particular act as an instructive signal for a multitude of developmental processes, ranging from polarity establishment during the earliest phases of plant embryogenesis to complex morphogenetic events, including the formation of entire organs (Finet and Jaillais, 2012; Jeong et al., 2012; Lau et al., 2012; Barbez and Kleine-Vehn, 2013; Pierre-Jerome et al., 2013). These variations in auxin distribution are the result of directional auxin transport, which promotes the establishment of auxin concentration gradients and has been linked to the asymmetric, polar distribution of PIN proteins at the plasma membrane (Fig. 2). Flexible control of both the directionality and the rate of auxin transport is achieved by a combination of dynamic readjustments in PIN distribution and levels at distinct plasma membrane sites (Wisniewska et al., 2006). This concept is supported by a large body of experimental evidence establishing PINs as key mediators of variations in auxin distribution and providing insights into the mechanisms that control their intracellular sorting. However, although PIN proteins are considered a major class of auxin transport proteins, it should be noted that it is still not resolved in mechanistic terms how PINs might facilitate such transport (see Box 1). In addition to PINs, studies of other plant plasma membrane proteins that also display distinct trafficking behaviors, such as the BRASSINOSTEROID INSENSITIVE 1 (BRI1) steroid hormone receptor, the FLAGELLIN-SENSITIVE 2 (FLS2)

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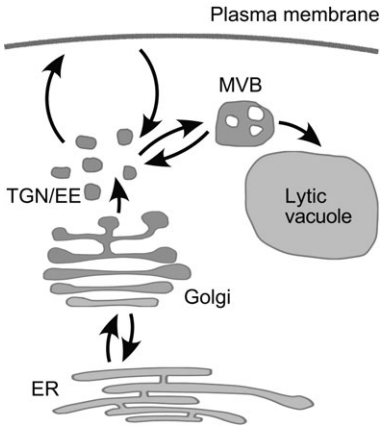

Fig. 1. Trafficking routes for plasma membrane proteins in plants. Following the translocation of *de novo* synthesized plasma membrane proteins into the endoplasmic reticulum (ER) lipid bilayer, sorting proceeds through the Golgi and further into the *trans*-Golgi network/early endosome (TGN/EE). Vesicular transport then relocates cargo either to the plasma membrane or, via the late endosomal multivesicular body (MVB) compartment, into the vacuole. Proteins that are endocytosed from the plasma membrane are either recycled via TGN/EE compartments or sorted for degradation to the lytic vacuole.

bacterial flagellin receptor, BORON TRANSPORTER 1 (BOR1), IRON-REGULATED TRANSPORTER 1 (IRT1) and the PLASMA MEMBRANE INTRINSIC PROTEIN 2 (PIP2) water channel, have shed light on the trafficking mechanisms used in plants.

In this Review, we provide an overview of the mechanistic basis of plant plasma membrane protein dynamics and highlight how studies of PINs have contributed to our understanding of vesicular trafficking in the context of plant growth, development and adaptation. We also draw on examples of other plant plasma membrane receptors and transporters that have provided key insights into the control of vesicular trafficking and its regulation by ever-changing environmental conditions.

Determinants of plasma membrane protein exocytosis

Regardless of its specific function, any plasma membrane protein needs to be sorted from its site of synthesis to its destination at the plasma membrane. This is mediated by sophisticated sorting steps that are essentially conserved throughout the eukaryotic kingdom. Below, we first provide an overview of how newly synthesized plasma membrane proteins are inserted into the endoplasmic reticulum (ER), sorted from the ER to the Golgi, and then transferred to the plasma membrane of plant cells, highlighting the various factors that can modulate each of these steps.

General aspects of early secretory sorting events

In eukaryotes, *de novo* synthesized membrane proteins require translocation into the ER lipid bilayer for further sorting; thus, the translation of membrane proteins in general occurs in tight association with the ER. Prior to entering the ER, a stretch of ~20-30 amino acids at the N-terminus of the nascent protein is co-translationally recognized by the signal recognition particle, which relocates the translating ribosome to the SEC61 translocon, thereby funneling the new protein into the ER lumen. Several components of this machinery are evolutionarily conserved and have been identified in plants (Lindstrom et al., 1993; Toikkanen et al., 1996; Wang et al., 2008). Membrane protein insertion is also carried out by conserved factors, which assist in proper folding and protein quality control (Ellgaard and Helenius, 2003). For example, plant orthologs of binding immunoglobulin protein (BiP) together with its associated factors appear to function as chaperones primarily in the maturation of non-glycosylated proteins, whereas calreticulin (CRT) controls the fate of glycosylated membrane proteins (Fontes et al., 1991; Denecke et al., 1995; Gupta and Tuteja, 2011). Evidence for the significance of these basic control mechanisms in the regulation of plasma membrane protein fate in plants is accumulating. For example, certain mutant alleles of the brassinosteroid receptor *BRI1* encode proteins that are retained in the ER instead of being sorted to the plasma membrane, which interferes with brassinosteroid-controlled plant development, presumably as a consequence of deficiencies in hormone perception (Su et al., 2011). Such protein retention is likely to be the result of an altered conformation, which appears to be recognized by conserved surveillance mechanisms (Hong et al., 2012). Proteins that fail to achieve the proper conformation become substrates for ER-associated protein degradation (ERAD), a ubiquitin-mediated process in which misfolded or damaged ER proteins are retrotranslocated into the cytosol and degraded by the proteasome (Meusser et al., 2005). Homologs of yeast and mammalian ERAD components have been identified in the *Arabidopsis* genome and include E3 ubiquitin ligases that label substrate proteins for degradation and accessory proteins such as the ATPase Cdc48 and the chaperone Hrd3 (Müller et al., 2005).

Anterograde sorting from the ER to the Golgi in plant cells follows conventional trails mediated by coat protein II (COPII)-coated vesicles. Conserved di-acidic and di-hydrophobic motifs found in specific transmembrane proteins are required for their selection into COPII-coated vesicles and have been characterized in plants (Sieben

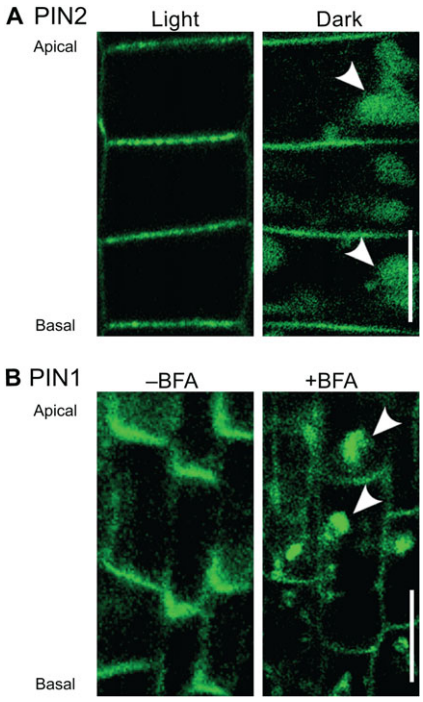

Fig. 2. The polar distribution of PIN proteins. PIN proteins are well-established readouts for studying distinct steps of membrane protein sorting. (A) Under normal conditions, PIN2 exhibits polar apical (shootward) localization in root epidermis cells (left). Environmental and intrinsic stimuli, such as incubation in darkness, promote its relocation to the vacuole (right, arrowheads). (B) PIN1, by contrast, accumulates at the basal (rootward) plasma membrane domain of stele cells in root meristems (left), facilitating directional auxin transport from the shoot to the root tip. The control of such polar localization involves protein recycling via endosomal compartments, a process that can be visualized by BFA treatment, which interferes with the exocytic sorting of endocytosed PIN1 (right, arrowheads). Scale bars: 10 μm.

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et al., 2008; Zelazny et al., 2009; Sorieul et al., 2011). SECRETION-ASSOCIATED AND RAS-RELATED 1 (SAR1), a Ras-like small GTP-binding protein, also appears to function in COPII vesicle assembly in plants, and plant orthologs of further constituents of COPII-coated vesicles, selective vesicle budding and cargo export from the ER have been identified (Bar-Peled and Raikhel, 1997; Osterrieder et al., 2010; Montesinos et al., 2012; Takagi et al., 2013). This is also true for elements of COPI-mediated retrograde transport, which mediates sorting back to the ER (Donohoe et al., 2007). Finally, the recruitment processes occurring throughout the formation of such vesicles require ADP-ribosylation factor (ARF) GTPases and the ARF GTPase guanine-nucleotide exchange factor (ARF-GEF) *Arabidopsis* GNOM-LIKE 1 (GNL1), which are essential for ER/Golgi trafficking processes as well as for protein recycling between endosomes and the plasma membrane (Richter et al., 2007; Teh and Moore, 2007; Du et al., 2013a).

Despite the conservation, little is known about the early steps that are involved specifically in the processing and sorting of *de novo* synthesized PIN proteins. There is also a lack of knowledge concerning PIN post-translational modifications such as glycosylation, which is involved in the control of protein maturation. In addition, there is currently little information about PIN proteolytic processing events, including signal peptide recognition and cleavage, although a report from Mravec et al. (2009) represents a remarkable exception; this report describes the identification of a *cis*-acting motif that causes PIN1 retention in the ER. This motif, which is found within the central domain shared by all *Arabidopsis* PINs, contains an NPxxYxxΦ signature that combines NPxxY and YxxΦ motifs (with x representing any amino acid and Φ representing a residue with a bulky hydrophobic side chain). Upon site-directed mutagenesis of the entire motif, a significant proportion of PIN1 accumulates in the ER instead of being sorted to the plasma membrane (Mravec et al., 2009), which is suggestive of aberrations in protein domains essential for PIN1 ER exit. However, as both affected motifs are implicated in the regulation of endocytic protein sorting by interaction with clathrin-associated proteins (Ohno et al., 1995; Bouley et al., 2003), the ER retention of the NPxxY- and YxxΦ-defective PIN1 might also result from improper folding rather than from mutation in a specific ER exit motif.

Accessory proteins, which are necessary for the ER exit of plasma membrane proteins, have also been reported in plants. For example, PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR 1 (PHF1) has been shown to facilitate ER exit of *Arabidopsis* PHOSPHATE TRANSPORTER 1 (PHT1), while AUXIN RESPONSE 4 (AXR4) specifically releases the auxin influx transporter AUX1 from the ER (Gonzalez et al., 2005; Dharmasiri et al., 2006). Consistent with a crucial function in early sorting events, loss of PHF1 or AXR4 leads to severe defects in phosphate homeostasis or auxin responses, respectively.

From the Golgi to the plasma membrane

The post-Golgi exocytic sorting of *de novo* synthesized plasma membrane proteins (including PINs) seemingly proceeds along trafficking routes in the context of TGN/EE-mediated cargo sorting. Similar to ER-Golgi trafficking, vesicle formation and movement as well as vesicle tethering and fusion are influenced by GTP-binding proteins of the ARF and Ras genes from rat brain (Rab) types. The expression of dominant-negative alleles encoding proteins that exhibit impaired GTP binding has shown that the activity of members of different *Arabidopsis* Rab subclasses is linked to distinct sorting events (Batoko et al., 2000; Pinheiro et al., 2009). Members of the Rab-E subclass in particular are implicated in post-Golgi trafficking to the plasma membrane, as indicated by Golgi retention of otherwise secreted cargo upon expression of RAB-E1^{d} (Zheng et al., 2005). Another dominant-negative *rab* allele was discovered in a mutant screen that aimed to identify specific effectors of PIN1 exocytic sorting (Feraru et al., 2012). This approach was based on the analysis of intracellular plasma membrane cargo retention upon treatment with the fungal toxin Brefeldin A (BFA), which interferes with exocytic protein sorting by reversible inhibition of ARF-GEFs (Donaldson et al., 1992; Helms and Rothman, 1992). Upon washout of the drug, protein sorting to the plasma membrane resumes, as reflected in a gradual disappearance of intracellular BFA-induced compartments (Steinmann et al., 1999; Geldner et al., 2001). Using this approach, the mutant *BFA-visualized exocytic trafficking defective* (*bex5-1*) was identified and was shown to carry a mutation in *RABA1B* that results in elevated intracellular retention of PIN1 after BFA washout. This indicates a requirement for BEX5/RABA1B in correct post-Golgi PIN sorting to the plasma membrane (Fig. 3), underlined further by its localization to the TGN/EE (Feraru et al., 2012).

Potential regulators of Rab- and ARF-type GTPases implicated in post-Golgi sorting have also been identified. Recently, *Arabidopsis VASCULAR NETWORK DEFECTIVE 4* (*VAN4*) was found to encode a putative TRS120 ortholog that acts as a GEF for Rab GTPases (Naramoto et al., 2014). The colocalization of a VAN4 reporter protein with RABA1B at the TGN/EE, together with the intracellular accumulation of PIN reporter proteins caused by loss of *VAN4*, points to a role for VAN4 as a positive regulator of PIN exocytic sorting to the plasma membrane (Naramoto et al., 2014). However, a GEF that acts on ARF-type GTPases, known as GNOM, currently represents the best-characterized effector of PIN sorting (Richter et al., 2010). *gnom* loss-of-function alleles were shown to cause severe embryo patterning defects (Liu et al., 1993; Mayer et al., 1993), and extensive analysis in the following years demonstrated a prominent role for GNOM in the control of PIN sorting (Shevell et al., 1994; Busch et al., 1996; Steinmann et al., 1999; Geldner et al., 2004). *GNOM* encodes a BFA-sensitive ARF-GEF that is responsible for the retention of PIN1 and other membrane proteins in intracellular compartments upon BFA treatment (Steinmann et al., 1999; Geldner et al., 2003). In agreement with a central function in protein sorting, PIN1 distribution is severely disrupted following a complete loss of *GNOM*, whereas the expression of a BFA-resistant *gnom* allele results in plants that are no longer responsive to the toxin (Steinmann et al., 1999; Geldner et al., 2003). These and several additional reports established GNOM as key regulator of auxin-dependent developmental processes via its control of directional

Box 1. How do PIN proteins function?

A range of transport assays in plant-derived setups as well as in heterologous systems have demonstrated a role for PIN proteins in the translocation of auxin across plasma membrane borders (Petrasek et al., 2006; Yang and Murphy, 2009). However, whether PINs per se are sufficient to mediate such translocation remains to be demonstrated. PINs could also form part of a heteromeric protein complex, constituting a plasma membrane-localized auxin efflux module (Blakeslee et al., 2007; Titapiwatanakun et al., 2009). In addition, PINs were suggested to mediate the release of auxin into the extracellular apoplastic space by modulating vesicular transport of the growth regulator to the plasma membrane (Schlicht et al., 2006). Regardless of their mode of action, the regulation of PIN function appears to rely to a large extent on controlled variations in PIN intracellular location and abundance, whereas only a few reports have pointed to a role for PIN activity that is uncoupled from their intracellular sorting (Zourelidou et al., 2009; Willige et al., 2013).

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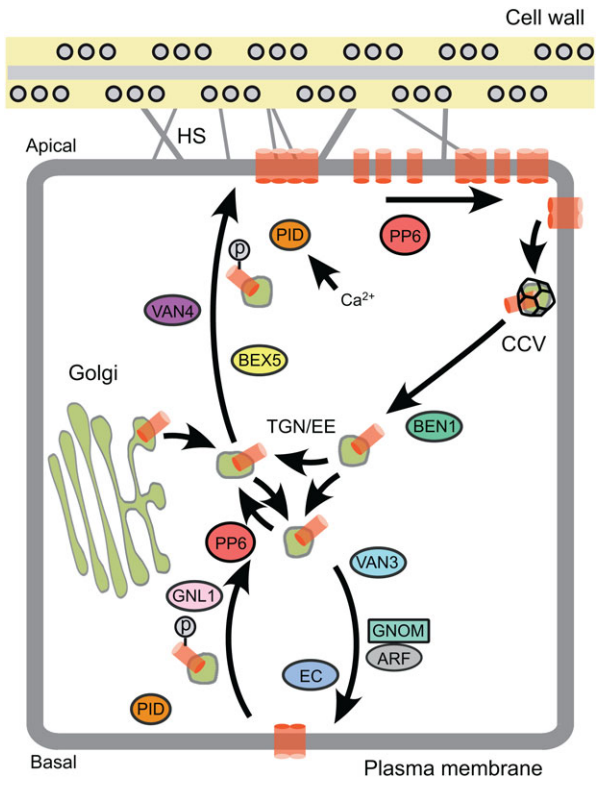

Fig. 3. The regulation of plasma membrane protein recycling and polar localization. The ADP-ribosylation factor (ARF) GTPase guanine-nucleotide exchange factor (GEF) GNOM plays a key role in the recycling of PIN proteins (orange cylinders) between TGN/EE compartments and basal plasma membrane domains. In addition, regulators such as the Rab-type GTPase BEX5 and the ARF-GEF proteins BEN1 and GNL1 are implicated in PIN trafficking. This process also depends on the activity of the exocyst complex (EC), which is suggested to modulate the exocytic sorting of PINs. PIN targeting to the apical plasma membrane domain has been linked to PIN phosphorylation (p), presumably involving the activity of the AGC3-type protein kinase PINOID (PID), which in turn appears to be under the control of Ca^{2+} and phosphoinositide signaling. The activity of the PP6 phosphatase antagonizes PID, promoting dephosphorylation and PIN sorting to basal domains. PIN targeting to the plasma membrane is suggested to involve 'super-polar' exocytosis, as reflected in the accumulation of PIN protein clusters at polar plasma membrane domains. Lateral diffusion of such protein clusters appears to be slow and depends on as-yet-unidentified crosstalk with cell wall components. For example, Hechtian strands (HS) that bridge the space between the cell and plasma membrane have been suggested to act in this process. Adjacent to polar plasma membrane domains, clathrin-mediated endocytosis enforces internalization of PINs in clathrin-coated vesicles (CCVs), preventing their further diffusion within the plasma membrane.

auxin transport and of hormone gradient formation (Geldner et al., 2004; Moriwaki et al., 2011; Wolters et al., 2011; Okumura et al., 2013). However, even in very severe *gnom* alleles, a substantial fraction of PIN proteins is still sorted to the plasma membrane, contrasting with the intracellular protein retention observed upon BFA treatment (Steinmann et al., 1999) and highlighting redundant BFA-sensitive sorting activities acting in addition to GNOM. In line with this, the ARF-GAP (GTPase-activating protein) VASCULAR NETWORK 3 [VAN3; also known as SCARFACE (SFC)] has been shown to be involved in the regulation of BFA-responsive PIN sorting. VAN3 localizes to the TGN/EE and appears to modulate intracellular PIN distribution in response to BFA (Koizumi et al., 2005; Sieburth et al., 2006). Furthermore, VAN3 and GNOM have been implicated in the regulation of cargo endocytic sorting and transcytosis (Naramoto et al., 2010) (see below), highlighting the versatility of the small GTP-binding protein molecular toolbox found in higher plants (Fig. 3). Unraveling the identity of the ARF proteins that are recognized by GNOM and VAN3 will clearly advance our understanding of the timing and specificity of these diverse activities in the regulation of PIN protein sorting (Xu and Scheres, 2005).

In recent years, a role for the exocyst complex in PIN sorting has also emerged. Exocyst represents an evolutionarily conserved component of the eukaryotic sorting machinery that functions as a tethering complex for exocytic vesicles upon fusion with the plasma membrane (Fendrych et al., 2013). In yeast and mammals, exocyst was described as a cytosolic complex composed of eight proteins, orthologs of which have been identified in plant genomes (Zarsky et al., 2013). Loss of the exocyst subunits EXO70A1 or SECRETORY 8 (SEC8) in *Arabidopsis* was demonstrated to drastically slow down the release of PIN from BFA compartments after drug removal, indicating that these mutants are impaired in PIN targeting to the plasma membrane. Consistent with this observation, the analysis of fluorescently tagged exocyst subunits revealed that they colocalize at the cell surface with the endocytic tracer FM4-64, as would be predicted for a plasma membrane tethering complex (Fendrych et al., 2013). Notably, *Arabidopsis* exocyst mutants exhibit a fairly regular distribution of membrane proteins and are, as such, not deficient in plasma membrane targeting of PINs unless treated with BFA (Fendrych et al., 2013). However, variations in PIN2 localization have been described for *exo70a1* mutants, in which PIN2 was found to colocalize with RABA5D, a marker for protein recycling vesicles (Fendrych et al., 2013). This implies a role for exocyst in PIN recycling between endocytic compartments and plasma membrane domains (Fig. 3).

Regulated internalization: *cis*- and *trans*-acting determinants of protein endocytosis

Several pathways exist for the internalization of plasma membrane proteins and extracellular cargo molecules in eukaryotic cells. These endocytic pathways can be divided into clathrin-dependent and clathrin-independent pathways, which exert distinct and overlapping activities. Plasma membrane proteins undergo internalization for different purposes. For example, endocytosis can allow the removal of ligand-bound receptors from the site of perception leading to cell desensitization to a given stimulus, as proposed for FLS2 and BRI1 (Robatzek et al., 2006; Geldner et al., 2007; Smith et al., 2014), or can sequester transporters such as IRT1 away from their extracellular substrates as part of homeostatic control (Barberon et al., 2011, 2014). In the case of the ETHYLENE-INDUCING XYLANASE 2 (LeEIX2) receptor, internalization is also associated with endosomal signaling (Sharfman et al., 2011), similar to what has been reported for several receptors in mammals (Murphy et al., 2009). The mechanisms driving the constitutive and regulated internalization, and the vacuolar targeting, of plasma membrane proteins are slowly emerging in plants.

Clathrin and adaptor proteins: key mediators of protein endocytosis in plants

In yeast and mammals, cargo internalization predominantly involves the formation of clathrin-coated vesicles (CCVs) (Motley et al., 2003). Plasma membrane clathrin coats are composed of the structural protein clathrin, which comprises heavy and light chains (CHC and CLC, respectively), the heterotetrameric Adaptor protein 2 (AP2) complex, and various accessory factors such as AP180, epsin 1, eps15 and eps15R (Traub, 2009). Additional key players include the large GTPase dynamin, which is thought to be directly involved in pinching off endocytic vesicles from the plasma

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membrane (Bashkirov et al., 2008; Pucadyil and Schmid, 2008; Roux and Antonny, 2008). The genome of higher plants contains genes encoding homologs of CHC and CLC, adaptins (which are subunits of AP complexes), as well as additional adaptors and dynamins, although it lacks genes encoding the cargo-specific adaptors arrestins (Becuwe et al., 2012). However, the role of clathrin and the machinery associated with clathrin-mediated endocytosis (CME) has emerged only recently in plants (Fig. 4A).

Pharmacological and dominant-negative approaches using the C-terminal HUB domain of CHC, which competes for binding with CLCs, interfere with CME in plant cells (Dhonukshe et al., 2007), and the analysis of *Arabidopsis* clathrin loss-of-function mutants confirmed these results and revealed a crucial role for CME in bulk endocytosis, with a particular focus on PIN proteins (Dhonukshe et al., 2007; Kitakura et al., 2011; Di Rubbo et al., 2013). Moreover, and in accordance with a requirement for endocytosis in the polarization of *de novo* synthesized PIN1 and PIN2, clathrin appears necessary for the proper establishment of PIN polarity (Kleine-Vehn et al., 2011). Besides PINs, several other plasma membrane proteins, such as the iron transporter IRT1, the boron transporter BOR1, BRI1, and the aquaporin water channel PIP2, are also dependent on clathrin for their internalization (Dhonukshe et al., 2007; Takano et al., 2010; Barberon et al., 2011; Irani et al., 2012; Di Rubbo et al., 2013). In line with such a general role in endocytic sorting, genetic interference with clathrin function in plants results in severe developmental defects, including alterations in root elongation, root and hypocotyl gravitropism, and lateral root primordia initiation (Kitakura et al., 2011; Wang et al., 2013). *Arabidopsis* clathrin chains associate with AP2 subunits and form punctate foci at the plasma membrane (Fan et al., 2013; Kim et al., 2013; Yamaoka et al., 2013). As such, and similar to clathrin mutants, plants impaired in AP2 subunits show alterations in general endocytosis and PIN internalization and/or polarity, as well as defects in the endocytosis of BRI1, which correlates with severe developmental defects (Di Rubbo et al., 2013; Fan et al., 2013; Kim et al., 2013; Yamaoka et al., 2013). More recently, the activity of another, apparently plant-specific complex – the TPLATE adaptor complex – has been linked to early events in CME. TPLATE consists of eight core subunits and has been demonstrated to accumulate at distinct sites at the plasma membrane, preceding the recruitment of further components required for CCV formation (Gadeyne et al., 2014), thereby establishing TPLATE as a major adaptor complex that acts during early stages of CCV formation in plant cells (Fig. 4A).

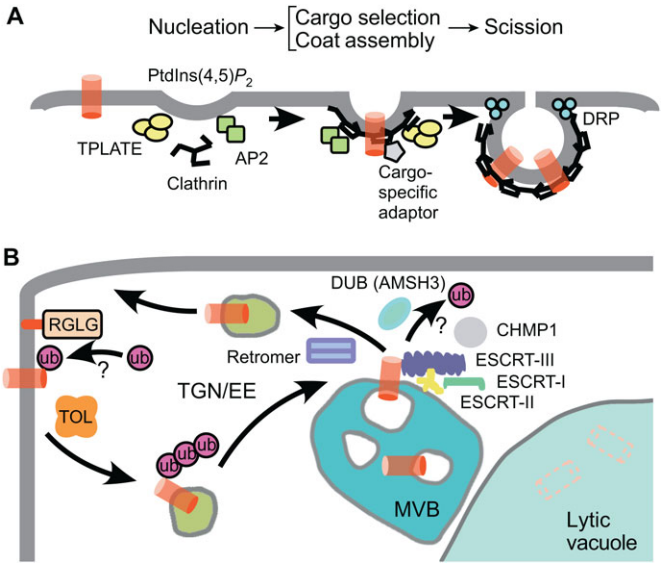

Fig. 4. Elements controlling the endocytic sorting and degradation of *Arabidopsis* plasma membrane proteins. (A) Regulators of clathrin-coated vesicle (CCV) formation thus far characterized in plants are shown. During the early steps ('nucleation'), TPLATE and AP2 adaptor proteins accumulate at designated invagination sites. This event, which is analogous to the situation in mammalian cells, might be triggered by a local enrichment of PtdIns(4,5)P_2 at such membrane domains. Adaptor association is a prerequisite for the subsequent event of clathrin recruitment and coat assembly during the later stages of CCV formation. The selection of cargo (orange cylinders) might require association with thus far uncharacterized cargo-specific adaptor proteins. Mature CCVs are then cleaved from the plasma membrane ('scission') by means of dynamin-related proteins (DRPs), thereby initiating the endocytic sorting of cargo-loaded CCVs. (B) The endocytic sorting of plasma membrane proteins destined for degradation in the lytic vacuole appears to be promoted by ubiquitylation (ub). In the case of PIN2 (orange cylinders), membrane-associated RGLG E3 ubiquitin ligases might be involved in ubiquitylation at the plasma membrane. TOL proteins appear to function as adaptors for ubiquitylated cargo, promoting cargo internalization from the plasma membrane. Efficient endocytic sorting towards the vacuole appears to depend on the attachment of K63-linked poly-ubiquitin chains. MVB- or TGN/EE-associated retromer subunits function as a gatekeeper, promoting the retrieval of endocytosed plasma membrane proteins back into the recycling pathway. Prior to such recycling or sorting into intraluminal vesicles of the MVB, cargo needs to be deubiquitylated, and this may involve the activity of deubiquitinases (DUBs) such as AMSH3. ESCRT complexes appear to be essential for cargo sorting into intraluminal vesicles, with ESCRT-III function controlled by CHMP1-type proteins. Once sequestered into intraluminal vesicles, MVB fusion with the lytic vacuole results in vesicle release and the proteolytic degradation of cargo.

Following clathrin and cargo recruitment, dynamins regulate membrane scission. In plants, the dynamin-related proteins DRP1 and DRP2 have been implicated in clathrin-mediated membrane trafficking (Collings et al., 2008; Bednarek and Backues, 2010; Fujimoto et al., 2010). PIN1 and DRP1 proteins transiently associate at the cell plate of dividing cells, presumably to allow for clathrin-mediated PIN1 redistribution after cell division. In line with a role for DRP1 in PIN sorting, *drp1* loss-of-function mutants show auxin-related growth defects such as agravitropism, altered polarity establishment during embryogenesis as well as organ positioning defects (Mravec et al., 2011). DRP1A also interacts with the ARF-GAP VAN3, which, together with GNOM, localizes to clathrin foci at the plasma membrane, thus linking ARF activity to CME (Sawa et al., 2005). Consistently, mutants in components of the ARF machinery, including *GNOM*, *GNL1*, *VAN3* and *ARF1*, are impaired in endocytosis and internalization of plasma membrane proteins, including PINs (Xu and Scheres, 2005; Teh and Moore, 2007; Naramoto et al., 2010; Irani et al., 2012). The apparently widespread function of ARF GTPase-based signaling in both intracellular compartments and CME explains the strong developmental alterations associated with these mutants (Geldner et al., 2003, 2004; Xu and Scheres, 2005; Richter et al., 2007; Teh and Moore, 2007; Naramoto et al., 2010).

Besides CME, a number of endocytic pathways originating from membrane microdomains have been linked to cargo internalization. A well-characterized pathway in mammals uses caveolae (Kurzchalia and Parton, 1999), but the structural constituents of caveolae are absent from plants. Flotillins have also been implicated in a myriad of processes, including endocytosis, in mammals (Otto and Nichols, 2011). Notably, plant membranes contain flotillins in microdomains that are distinct from CCVs, and plant flotillin function has been associated with endocytic events during symbiotic bacterial infection, although no cargo has been clearly identified to date in plants (Haney and Long, 2010; Salome et al., 2012). Only the PIP2 aquaporin has

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been reported to colocalize and co-migrate with the FLOT1 flotillin, which is in accordance with PIP2 internalization associated with membrane rafts (Li et al., 2011).

cis-acting sorting signals in the control of endocytosis

The sorting of plasma membrane proteins to endosomes is mediated by signals present within their cytosolic domains. In mammalian cells, the interaction between AP2 and cargo proteins regularly relies on recognition of either YxxΦ or dileucine endocytic motifs ([DE]xxxL[LI]) (Bonifacino and Traub, 2003). Both motifs have been identified in plant plasma membrane proteins (Geldner and Robatzek, 2008), but only YxxΦ motifs have so far been linked to endocytosis. This was experimentally demonstrated in the case of PIN2, BOR1 and the tomato receptor LeEIX2 (Bar and Avni, 2009; Takano et al., 2010; Kleine-Vehn et al., 2011). The role of dileucine-based motifs in plasma membrane proteins is far less documented. Mutations in such motifs, for example in that of the cell plate-localized endo-1,4-β-glucanase KORRIGAN 1 (KOR1) or the vacuolar potassium channel TWO PORE CALCIUM CHANNEL PROTEIN 1 (TPC1), lead to protein relocation to the plasma membrane, but the underlying mechanisms have not been explored (Zuo et al., 2000; Larisch et al., 2012).

In yeast and mammals, post-translational modification by ubiquitin signals cargo internalization from the cell surface after recognition by epsin/Eps15-like adaptor proteins (Traub, 2009), and different types of ubiquitylation are associated with the endocytosis of plasma membrane proteins (Lauwers et al., 2010). Whereas mono-ubiquitylation of a single lysine is sufficient for cargo internalization, multi-ubiquitylation of several lysines or the formation of K63-linked poly-ubiquitin chains appears to accelerate cargo internalization by mediating higher avidity with endocytic ubiquitin-binding receptors (Galan and Haguenauer-Tsapis, 1997; Blondel et al., 2004; Hawryluk et al., 2006; Paiva et al., 2009). In plants, evidence for a role of cargo ubiquitylation in the early stages of endocytosis came from analyses of artificially ubiquitylated plasma membrane cargos, such as PIN2, plasma membrane H^+-ATPase (PMA) and a synthetic plasma membrane-localized TMD23-RFP reporter protein, which all exhibited enhanced internalization and vacuolar degradation (Herberth et al., 2012; Leitner et al., 2012b; Scheuring et al., 2012). The ubiquitylation of several endogenous plasma membrane proteins has also been demonstrated in *Arabidopsis* (Abas et al., 2006; Göhre et al., 2008; Barberon et al., 2011; Kasai et al., 2011; Leitner et al., 2012b), although unambiguous demonstration of a role for this modification in internalization from the cell surface is still lacking. By contrast, strong evidence supports a crucial function of ubiquitylation in endocytic sorting of plant plasma proteins (Kasai et al., 2011; Leitner et al., 2012b).

Developmental and environmental control of cargo internalization

Internalization may be constitutive or controlled by endogenous or external cues to serve developmental programs and adaptation to environmental conditions (Table 1). CME is regulated by several plant hormones as well as environmental factors, and this is likely to reflect extensive crosstalk between different pathways involved in sorting decisions. For example, strigolactones affect shoot branching by modulating PIN1 CME (Shinohara et al., 2013), while salicylic acid was found to repress the endocytosis of different

Table 1. Plant plasma membrane proteins and associated endocytic sorting mechanisms

Protein	Signal	Regulated step	Trafficking mechanism	References
PIN1	Phototropism	Not characterized	?	(Blakeslee et al., 2004)
	Blue light	Recycling	GNOM, PID/PP2A	(Blakeslee et al., 2004; Zhang et al., 2014)
	Cytokinin	Vacuolar targeting	BEN1 and BEN2	(Marhavy et al., 2011)
	Strigolactones	Internalization	CME	(Shinohara et al., 2013)
	SA	Internalization	CME	(Du et al., 2013b)
	JA	Internalization	?	(Sun et al., 2009)
	Auxin	Internalization	BIG, ABP1-TMK, ROP-RIC	(Paciorek et al., 2005; Robert et al., 2010; Xu et al., 2010, 2014)
PIN2	Gravity	Vacuolar targeting	Ub, TOL, SNX1	(Kleine-Vehn et al., 2008a; Leitner et al., 2012a,b; Korbei et al., 2013)
	Dark/light	Not characterized	?	(Laxmi et al., 2008)
	JA	Internalization	?	(Sun et al., 2011)
	Auxin	Internalization	BIG, TIR1/AFBs?	(Paciorek et al., 2005; Pan et al., 2009)
		Vacuolar targeting	Ub, TIR1/AFBs	(Leitner et al., 2012b; Baster et al., 2013)
	GA	Vacuolar targeting	?	(Willige et al., 2011; Löfke et al., 2013b)
	Salt	Internalization	PLD	(Galvan-Ampudia et al., 2013)
	Heat	Recycling	SNX1	(Hanzawa et al., 2013)
PIN3	Gravity	Internalization and transcytosis	GNOM	(Kleine-Vehn et al., 2010; Rakusova et al., 2011)
	Light/phototropism	Transcytosis	GNOM, PID	(Ding et al., 2011)
	Shade avoidance	Recycling	?	(Keuskamp et al., 2010)
	Fruit development	Transcytosis	PID and WAG2	(Sorefan et al., 2009)
FLS2	Flagellin	Vacuolar targeting?	Ub?	(Göhre et al., 2008)
BOR1	Boron	Vacuolar targeting	Ub	(Kasai et al., 2011)
IRT1	Non-Fe metals	Vacuolar targeting?	Ub, IDF1-dependent	(Barberon et al., 2011, 2014; Shin et al., 2013)
PIP2	Salt	Internalization	CME, raft-mediated endocytosis	(Li et al., 2011)
	Auxin	Internalization	BIG	(Paciorek et al., 2005)

GA, gibberellic acid; JA, jasmonate; SA, salicylic acid; CME, clathrin-mediated endocytosis; Ub, ubiquitin.

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cell surface proteins, including PINs and PIP2, by blocking clathrin recruitment at the plasma membrane (Du et al., 2013b). Likewise, jasmonate, another plant hormone, has been demonstrated to regulate PIN2 endocytosis, although a connection with CME has not been established (Sun et al., 2011). Recently, common salt was identified as another trigger for PIN2 CME in root meristem cells, and it was suggested that it causes auxin redistribution resulting in root growth in a direction away from harmful salt concentrations (Galvan-Ampudia et al., 2013). Auxin, by contrast, inhibits the CME of several plasma membrane-resident proteins including PIN1, PIN2, PIN4, PIP2 and PMA (Paciorek et al., 2005). In particular, auxin modulates its own transport by inhibiting PIN CME via a signaling module composed of the putative auxin receptor ABP1 and its associated transmembrane kinase (TMK) receptor kinase that modulates the downstream effectors ROP GTPase and RIC (Rop-interactive CRIB motif-containing protein) (Robert et al., 2010; Xu et al., 2010, 2014; Chen et al., 2012; Lin et al., 2012). This feedback regulatory pathway appears crucial for a range of auxin-controlled growth responses, including root gravitropism and vascular patterning, as well for the establishment of interdigitated lobes and indentations found in leaf epidermal pavement cells. Environmental conditions also impact on clathrin-independent internalization pathways, as exemplified by enhanced internalization of PIP2 via a membrane raft-associated pathway upon salt stress (Li et al., 2011).

The fate of endocytosed plasma membrane proteins

The fate of endocytosed cargos is varied, and includes recycling to the cell surface or vacuolar targeting for degradation by vacuolar proteases. As we discuss below, these distinct fates, which lead to distinct developmental outcomes, are determined by different factors; whereas recycling appears to be dependent on the retromer complex, targeting for degradation is mediated by ubiquitylation.

Recycling and intracellular trafficking: the role of the retromer complex

The recycling and intracellular trafficking of PINs requires subunits of the retromer complex, which is a coat complex that localizes to the cytosolic face of endosomes and is involved in the intracellular sorting of specific transmembrane proteins (Fig. 4B). In particular, the SORTING NEXIN 1 (SNX1) and VACUOLAR PROTEIN SORTING 29 (VPS29) subunits of the complex have been implicated in PIN recycling (Jaillais et al., 2006, 2007). In mechanistic terms, SNX1 endosomes appear to be recruited to microtubules by direct interaction between retromer and microtubule-associated CLASP, which has been suggested to control cargo recycling to the plasma membrane (Ambrose et al., 2013). SNX1, in particular, appears to control variations in PIN2 degradation in gravistimulated roots and mediates PIN2 retrieval from late endosomes in response to increased temperature, thus linking retromer activity to adaptive growth responses (Jaillais et al., 2006; Kleine-Vehn et al., 2008a; Hanzawa et al., 2013). SNX1 has also been implicated in the recycling of internalized IRT1 from late endosomes to prevent its premature degradation (Ivanov et al., 2014). The VPS35A retromer subunit, by contrast, appears to be required for the functionality of late endosomal compartments, reflecting the differing functions of distinct elements of the plant retromer machinery (Nodzynski et al., 2013).

Targeting to the vacuole: developmental roles

For some proteins, vacuolar targeting appears to be flexibly controlled to serve developmental programs or adaptation to varying conditions (Table 1). This is well illustrated by the BOR1 transporter, which exhibits high boron-induced ubiquitin-mediated vacuolar targeting to limit boron overload and toxic effects (Takano et al., 2005). A similar scenario can be envisaged for the ubiquitin-regulated IRT1 iron transporter when exposed to its highly reactive secondary substrates zinc, manganese and cobalt (Barberon et al., 2011, 2014). Vacuolar targeting may also contribute to the desensitization of plant cell-surface receptors, as described for the flagellin receptor FLS2 (Robatzek et al., 2006). In further studies, gravistimulation and dark incubation were shown to trigger vacuolar targeting of PIN proteins, thereby locally adjusting auxin flow (Abas et al., 2006; Kleine-Vehn et al., 2008a; Laxmi et al., 2008). Phytohormones, such as auxin and cytokinin, were also found to impact on PIN abundance by triggering vacuolar targeting, and this appears to be crucial for organogenesis and adaptive growth responses (Marhavy et al., 2011; Leitner et al., 2012b; Baster et al., 2013). Likewise, the hormone gibberellic acid affects PIN2 vacuolar targeting to modulate auxin transport in roots that respond to gravity (Willige et al., 2011; Löfke et al., 2013b). In most of these cases, the mechanisms driving vacuolar targeting are unresolved, although a crucial role for cargo ubiquitylation has been established (Leitner et al., 2012b).

The emerging role of ubiquitin in vacuolar targeting

Prior to targeting to the vacuole, cargo needs to be sorted into intraluminal vesicles of MVBs, which subsequently fuse with the vacuolar compartment to release cargo for its degradation. This intracellular sorting and targeting to the lysosome/vacuole is highly dependent on K63-linked poly-ubiquitylation. In plants, the ubiquitin-mediated sorting of plasma membrane proteins, and the associated machinery, came to light only very recently and revealed some variety in signaling events (Reyes et al., 2011; Tomanov et al., 2014). For example, a tyrosine-based sorting motif mediates the internalization of BOR1 from the plasma membrane, but further sorting into late endosomes and vacuolar targeting require BOR1 mono- or di-ubiquitylation on a single lysine residue (Takano et al., 2010; Kasai et al., 2011). PIN2, by contrast, is modified by K63-linked poly-ubiquitin chains (Fig. 4B), which depend on a class of E3 ubiquitin ligases known as ring-domain ligases (RGLGs), although a direct role for RGLGs in PIN ubiquitylation remains to be determined (Yin et al., 2007; Leitner et al., 2012b). Notably, only the collective mutagenesis of several potential ubiquitylation sites interfered with efficient PIN2 poly-ubiquitylation and vacuolar targeting, suggesting highly redundant modes of PIN2 ubiquitylation. These mutant alleles also failed to rescue a *pin2* loss-of-function mutant, underling the role of ubiquitylation in the regulation of the PIN auxin transport machinery (Leitner et al., 2012b). Variations in plasma membrane protein ubiquitylation in response to defined stimuli further underline the biological significance of this post-translational modification. PIN2, for example, has been demonstrated to undergo increased ubiquitylation in response to gravity stimulation, which is likely to reflect reversible adjustments in protein turnover in gravity-responding roots (Leitner et al., 2012a). Related observations have been made for FLS2 upon treatment with the bacterial elicitor flagellin and the bacterial effector AvrPtoB, and for BOR1 in response to an increase in boron concentration (Göhre et al., 2008; Kasai et al., 2011), pointing to a key role for reversible cargo ubiquitylation in transmitting environmental signals to developmental and metabolic pathways.

K63-linked poly-ubiquitin chains are recognized by the ESCRT (endosomal sorting complex required for transport) protein-sorting machinery (Mukhopadhyay and Riezman, 2007; Lauwers et al., 2010). In yeast and mammals, ESCRT is composed of four subcomplexes:

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ESCRT-0 to ESCRT-III. Elements of the evolutionarily conserved ESCRT machinery are also found in plants and have been described as potential mediators of ubiquitylated cargo sorting (Winter and Hauser, 2006; Reyes et al., 2011) (Fig. 4B). Consistently, plants defective for ESCRT-I subunits show compromised endosomal sorting of the flagellin receptor FLS2, revealing a requirement for ESCRT-I in plant pathogen responses (Spallek et al., 2013). Interference with ESCRT-III activity, by contrast, was found to efficiently block the vacuolar sorting of constitutively ubiquitylated cargo protein (Herberth et al., 2012; Scheuring et al., 2012). Moreover, CHMP1 proteins, which are predicted to function as regulators of ESCRT-III, and the *Arabidopsis* AMSH3-type deubiquitinase (DUB) that acts in conjunction with ESCRT-III, were found to be required for the vacuolar sorting of plasma membrane protein cargo including PINs (Spitzer et al., 2009; Isono et al., 2010; Katsiarimpa et al., 2011) (Fig. 4B). However, homologs of ESCRT-0 components that are required for the initial recognition of ubiquitylated cargo have not been identified in plants, although potential homologs of mammalian Target of Myb (TOM) have been suggested to functionally substitute for ESCRT-0 in *Arabidopsis* (Korbei et al., 2013). Such TOM-like (TOL) proteins are required for the efficient vacuolar targeting and subsequent degradation of PIN2 and KNOLLE syntaxin, as well as artificially ubiquitylated PIN2 (Korbei et al., 2013) (Fig. 4B).

Establishing polarity: determinants of polar protein distribution at the plasma membrane

The intracellular distribution of plasma membrane proteins is under tight control. This appears of particular importance for proteins involved in directional transport or signaling processes at the plasma membrane, as such proteins frequently adopt an asymmetric, polar membrane distribution. Plasma membrane-localized PINs represent the prototype for polarly distributed membrane proteins in plants, and most of the research addressing asymmetric protein sorting has been performed on PINs. However, several other membrane proteins, including the nutrient transporters BOR1, NODULIN 26-LIKE INTRINSIC MEMBRANE PROTEIN 5;1 (NIP5;1), Low silicon rice gene 1 (Lsi1), IRT1, AtPGP1 (P-glycoprotein), as well as an exporter of the plant hormone precursor indole-3-butyric acid ABCG37 (ATP-binding cassette), the pathogen defense-related transporter PENETRATION RESISTANCE 3 (PEN3) and CASPs (Casparian strip membrane domain proteins), among others, are also targeted to polar membrane domains, and characterization of their sorting has contributed substantially to our knowledge about protein polarization in plants (Geisler et al., 2005; Ma et al., 2006; Ruzicka et al., 2010; Takano et al., 2010; Roppolo et al., 2011). Polarized protein distribution could, in principle, be achieved in different ways, involving the polarized secretion of *de novo* synthesized protein, targeted protein (re)cycling from intercellular sorting compartments to plasma membrane domains, as well as spatially restricted protein endocytosis operating only at specific plasma membrane domains. In fact, a combination of these processes seems to impact on the distribution of polarly localized membrane proteins in plants, highlighting the complexity of the mechanisms involved.

Exocytic sorting and variations in protein retention at the plasma membrane as a means of establishing polarity

The post-Golgi exocytic sorting of PINs has received a great deal of attention because directional sorting could represent a rate-limiting determinant for establishing and maintaining protein distribution in polar domains. Polarized exocytic sorting has been characterized in non-plant organisms (Altschuler et al., 2008), and related evidence has been presented particularly for polarized PIN exocytic sorting in *Arabidopsis* (Geldner et al., 2001). A combination of high-resolution imaging and modeling of protein sorting has revealed the existence of 'super-polar' exocytosis together with tightly controlled lateral diffusion at the plasma membrane (Kleine-Vehn et al., 2011) (Fig. 3). In this scenario, after sorting to polar plasma membrane domains, PINs, like other membrane proteins, accumulate in membrane clusters that exhibit a low tendency to diffuse laterally, thereby contributing to the maintenance of an already established polar protein distribution (Kleine-Vehn et al., 2011; Martiniere et al., 2012). Moreover, there is evidence for enhanced clathrin-dependent endocytic sorting of PIN proteins, specifically at sites proximal to PIN-enriched plasma membrane domains (Kleine-Vehn et al., 2011). Support for this comes from analyses of a *pin2* allele that is affected in a predicted PIN2 clathrin-sorting motif and exhibits ectopic protein accumulation at the lateral plasma membrane domains of root epidermal cells (Kleine-Vehn et al., 2011). A similar role for CME has been associated with the establishment of lateral polarity of the boron transporter BOR1 to the inner plasma membrane domain of root epidermal and endodermal cells (Takano et al., 2010). Targeted internalization of cargos by CME is not, however, involved in the enrichment of IRT1 at the outer plasma membrane domain of root epidermal cells (Barberon et al., 2014).

This scenario, which relies on variations in lateral diffusion of membrane proteins, only remotely resembles the situation in animal cells, in which tight junctions restrict diffusion from apical to basolateral plasma membrane domains (Wells et al., 2006), suggesting the evolution of analogous mechanisms for polarity determination in animal and plant kingdoms. In addition, several polarity determinants that are conserved in animals are not found in plant genomes, raising questions about the machinery involved in polar protein deposition and maintenance in plants (Geldner, 2009). As for lateral diffusion within the plasma membrane of plant cells, it appears that neither components of the cytoskeleton nor protein-protein interactions or variations in lipid composition exert any considerable effects on the kinetics of membrane protein diffusion (Martiniere et al., 2012). However, disruption of cell wall integrity and/or biogenesis does cause significant alterations in plasma membrane protein mobility (Martiniere et al., 2012). This was shown to be significant for the control of PIN localization when the *regulator of pin polarity 3* (*repp3*) mutant was identified as a novel allele of the *CESA3* cellulose synthase subunit, which is one of the key constituents for cellulose biosynthesis at the plant cell wall (Feraru et al., 2011). Intriguingly, *repp3*/*cesa3* mutant alleles, or interference with cellulose biosynthesis, cause a shift in the distribution of ectopically expressed PIN1 from basal to apical domains of *Arabidopsis* root cells (Feraru et al., 2011). Furthermore, circumstantial evidence for a role of cellulose in the control of PIN sorting came from approaches in which treatment with cellulose-degrading enzymes was shown to cause pronounced depolarization of PIN proteins at the plasma membrane (Feraru et al., 2011). Nevertheless, it remains to be determined how the cell wall, or components therein, could restrict lateral diffusion and loss of polar PIN distribution in mechanistic terms.

A correlation between variations in PIN1 localization and microtubule orientation has been described in cells of the shoot apex (Heisler et al., 2010). In light of the role of microtubules in guiding cellulose synthase complexes (Paredez et al., 2006), it is possible that cytoskeleton-dependent variations in cell wall characteristics indirectly feed back on the localization of PINs, as further supported by studies proposing cell wall integrity as a

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potential mediator of microtubule effects on PIN distribution (Boutte et al., 2006). Aside from microtubules, actin microfilament conformation has been associated with polarized PIN distribution; however, no apparent link to cell wall components has been established in this context (Geldner et al., 2001; Nagawa et al., 2012). In addition, physical connections between the cell wall and the plasma membrane, known as Hechtian strands (Fig. 3), were suggested to impact on plasma membrane protein mobility (Feraru et al., 2011), but this lacks any clear-cut experimental support, thus leaving crosstalk between polarly localized membrane proteins and cell wall-associated polarity determinants enigmatic.

Membrane composition: implications for plasma membrane protein distribution

Variations in sterol and lipid composition, as seen in some biosynthesis mutants, also can affect the polar distribution of plasma membrane proteins. For example, the *orc* allele of the *C24-STEROL METHYLTRANSFERASE 1* (*SMT1*) locus exhibits severe alterations in organ patterning and associated defects in PIN distribution (Diener et al., 2000; Willemsen et al., 2003). In addition, the analysis of *CYCLOPROPYLSTEROL ISOMERASE 1* (*CPI1*) mutants has provided mechanistic insights into the role of sterols in PIN polarity establishment (Men et al., 2008). Specifically, *cpi1-1* loss-of-function seedlings retain PIN2 both at the apical and – ectopically – at the basal plasma membrane domain of root epidermis cells as a result of deficiencies in polarized PIN2 endocytosis upon completion of cytokinesis. Together, these findings establish membrane sterol composition as a PIN protein-sorting determinant.

In addition to sterols, deficiencies in the biosynthesis of very long chain fatty acids (VLCFAs) have been linked to defects in the control of PIN sorting. Characterization of the *PASTICCINO* (*PAS*) loci, which are implicated in VLCFA biosynthesis, demonstrated pleiotropic roles for VLCFAs in cell proliferation and organogenesis (Roudier et al., 2010; Markham et al., 2011). Moreover, mutations in *PAS1/GURKE* were found to affect the sorting and correct polar plasma membrane localization of PIN1, and these aberrations could be partially rescued upon external application of VLCFAs (Roudier et al., 2010). A subsequent analysis of the *Arabidopsis* ceramide synthase family, an enzyme class that incorporates fatty acids into sphingolipids, suggested that VLCFA-containing sphingolipids impact the distribution of both PINs and the auxin uptake carrier AUX1 during the very early steps of exocytic protein sorting (Markham et al., 2011).

Sterols and sphingolipids are structural constituents of lipid rafts, which are involved in post-Golgi sorting in non-plant organisms (Klemm et al., 2009). By analogy, variations in the levels of VLCFA-containing sphingolipids in plants could modulate the structural properties of membrane domains, which in turn might affect aspects of vesicular sorting processes that are crucial for the correct targeting of polarly localized membrane proteins. Furthermore, sterol-enriched detergent-resistant membrane (DRM) microdomains have been demonstrated in membrane preparations from higher plants, and a role for such domains in stabilizing PINs at the plasma membrane has been proposed (Titapiwatanakun et al., 2009). How such mechanisms might confer specificity in sorting subsets of plant membrane proteins remains to be determined.

Protein recycling and phosphorylation as mediators of membrane protein localization

Experiments demonstrating continuous (re)cycling of plasma membrane proteins, specifically PINs, represented a key step forward in our understanding of polar protein sorting decisions in plants. In particular, the accumulation of plasma membrane proteins in intracellular BFA compartments was found to be reversible upon BFA washout, even in the presence of protein synthesis inhibitors, indicating that continuous protein cycling occurs between plasma membrane domains and recycling endosomes that might constitute part of the TGN/EE (Geldner et al., 2001). Studies in which the fate of a photoconvertible Eos-PIN reporter protein was followed upon BFA treatment further confirmed protein internalization and recycling (Dhonukshe et al., 2007). This recycling suggests that simply modulating the equilibrium of internal and plasma membrane-associated PIN protein pools can allow for reversible adjustments in auxin transport rates and directionality.

The BFA-sensitive ARF-GEF GNOM was shown to be key for such continuous membrane protein recycling (Geldner et al., 2001, 2003), and additional GEFs have also been implicated in this process. The ARF-GEF BFA-VISUALIZED ENDOCYTIC TRAFFICKING DEFECTIVE 1 (BEN1), for example, localizes to early endocytic vesicles (Tanaka et al., 2009), while GNL1 has a role in the selective endocytosis of PIN proteins, although it is otherwise suggested to function at the Golgi (Teh and Moore, 2007). Protein phosphorylation, acting interdependently with GNOM, has also been identified as a major determinant of PIN sorting. The activity of the AGC3-type serine/threonine protein kinase PINOID (PID) and that of its close relatives was shown to impact polar PIN distribution, promoting accumulation at apical/shootward cellular plasma membrane domains in the case of PIN1 (Friml et al., 2004), whereas PIN3 distribution appears to shift from non-polar to lateral plasma membrane domains in endodermal cells (Ding et al., 2011; Rakusova et al., 2011). These sorting decisions are dependent on the phosphorylation status of PINs, as PINs encoded by mutant *pin* alleles mimicking constitutive phosphorylation were found to accumulate at apical domains, whereas those encoded by loss-of-phosphorylation alleles accumulated at basal domains, regardless of *trans*-acting *PID* activity (Zhang et al., 2010; Huang et al., 2010). Together with *in vitro* evidence for PID-mediated phosphorylation of bacterially expressed PIN fragments and PID-dependent PIN phosphorylation in transient protoplast expression assays (Michniewicz et al., 2007), this led to models suggesting that PINs represent substrates for PID, although we are still lacking conclusive *in planta* evidence for a direct interaction. PID activity appears to be antagonized by a PP6-type phosphatase heterotrimeric protein complex, consisting of PHYTOCHROME-ASSOCIATED SERINE/THREONINE PROTEIN PHOSPHATASE (FyPP), SAPS DOMAIN-LIKE (SAL) and the PP2AA regulatory subunit, which was shown to interact with PIN proteins and to antagonize their phosphorylation (Michniewicz et al., 2007; Dai et al., 2012). Intriguingly, PP2AA phosphatase regulatory subunits were shown to colocalize with PIN proteins at plasma membrane domains (Michniewicz et al., 2007), consistent with a scenario in which modulation of phosphatase activity at the plasma membrane directly feeds back on PIN phosphorylation status, thereby shaping PIN sorting decisions (Fig. 3).

Having defined two interdependent sorting mechanisms, further studies addressed the interplay between GNOM and PID/PP6 activities in the sorting of PINs. Notably, a partial loss of *GNOM* results in apicalization of PIN proteins, similar to the phenotypes observed upon reduced PP6 activity or in *PID* overexpression lines (Kleine-Vehn et al., 2009). Genetic analysis indicated synergistic interactions of increased PID activity and reduced *GNOM* function, suggesting antagonistic roles for *GNOM* and *PID* in PIN polar sorting. In addition, BFA treatment of plants expressing a mutant

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pin allele mimicking constitutive phosphorylation revealed reduced BFA sensitivity of the PIN1 variant, indicating that phosphorylated PINs do not represent preferred targets for GNOM ARF-GEF-mediated protein sorting (Kleine-Vehn et al., 2009). In light of the various sorting decisions mediated by the GNOM- and PID-dependent pathways (Geldner et al., 2003; Kleine-Vehn et al., 2008b, 2010; Naramoto et al., 2010; Ding et al., 2011; Rakusova et al., 2011), variations in PIN phosphorylation could thus play a key role during distinct developmental responses by modulating the efficiency with which PINs enter either GNOM-dependent or -independent trafficking routes. This predicts the existence of mechanisms that sense and transmit variations in the phosphorylation status of PINs into variations in their intracellular sorting. Such factors, however, remain to be characterized.

GNOM-dependent effects on membrane protein sorting and polarity establishment appear common and are not restricted to PINs (Irani et al., 2012; Nielsen et al., 2012). Although dependent on ARF-GEF-mediated protein sorting, the establishment of laterally polarized ABCG37, PEN3 and BOR4 root transporters does not depend on any known factors mediating apical or basal protein distribution (Ruzicka et al., 2010), highlighting the existence of alternative pathways in polar sorting decisions in plants. More recently, the endosomal protein FYVE1 has been reported to interact with and to control the lateral polarity of IRT1 (Barberon et al., 2014). Overexpression of FYVE1 leads to the apolar localization of IRT1 at the cell surface and to defects in plant metal homeostasis. The precise mechanism by which FYVE1 participates in IRT1 polarity is still unclear, but it was suggested that FYVE1 overexpression is likely to cause misrouting of IRT1 beyond the outer polar plasma membrane domain on its way back to the cell surface (Barberon et al., 2014).

Conserved elements of the eukaryotic sorting machinery have also been implicated in PIN recycling (Kitakura et al., 2011; Fendrych et al., 2013). Furthermore, secondary messengers, namely calcium and inositol trisphosphate ($InsP_3$), exert major effects on polar sorting decisions in plants. In particular, *supo1*, a suppressor of agravitropic root phenotypes of seedlings ectopically expressing PIN1, was found to harbor mutations in a gene encoding inositol polyphosphate 1-phosphatase, an enzyme required for $InsP_3$ catabolism (Zhang et al., 2011). This mutant prevents ectopic PIN1 localization at basal plasma membrane domains of root epidermal cells, suggesting that increased $InsP_3$ levels in *supo1* interfere with basal targeting of PIN proteins. Indeed, manipulation of $InsP_3$ and calcium levels, the latter being controlled by $InsP_3$ signaling, revealed effects specifically on basally localized PIN proteins, such as PIN1 in the stele or PIN2 in young root cortical cells (Zhang et al., 2011). A similar effect on basally localized PIN was observed when calcium dissipation from the cytosol was perturbed via downregulation of Ca^{2+}-ATPase expression, demonstrating that cytosolic calcium levels function as a central signal in controlling polar protein sorting (Zhang et al., 2011).

It is not completely resolved how these secondary messengers could affect PIN polarity in mechanistic terms. Calcium, via binding to calcium-binding proteins that associate with PID, was found to activate PID (Benjamins et al., 2003). PID activation is predicted to promote apicalization of PINs, analogous to phenotypes observed in *supo1* and in Ca^{2+}-ATPase silencer lines (Friml et al., 2004; Zhang et al., 2011). In addition, an *Arabidopsis* Ca^{2+}/calmodulin-dependent kinase-related kinase 5 (*CRK5*) loss-of-function mutant demonstrated deficiencies in apical protein sorting, specifically of PIN2 in the root epidermis and in cortical root meristem cells (Rigo et al., 2013). This underscores the role of calcium in PIN polar targeting, suggesting that it could potentially act via modulating kinase activities in distinct signaling pathways (Fig. 3). Furthermore, PID activation has been observed upon its phosphorylation by a 3-phosphoinositide-dependent protein kinase (PDK1) *in vitro*, linking regulation of PID activity to phosphoinositide signaling, which in turn could impact on PIN polar sorting events (Zegzouti et al., 2006).

The endocytic sorting of PINs via clathrin-dependent pathways appears to represent another target for phosphoinositide signaling, as supported by evidence from reverse genetics approaches in which the function of two related phosphatidylinositol 4-phosphate 5-kinases (PIP5K1 and PIP5K2) has been analyzed (Ischebeck et al., 2013). A *pip5k1 pip5k2* double mutant characterized for diminished phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] content, exhibited auxin-related growth defects and reduced polarity in the distribution of PIN proteins at the plasma membrane. These aberrations coincided with alterations in clathrin dynamics and delayed PIN accumulation upon BFA treatment, altogether pointing to a function for PtdIns(4,5)P_2 in clathrin-mediated endocytic sorting of PIN proteins (Fig. 4A).

In summary, the analysis of polar protein sorting has revealed a role for exocytic and endocytic sorting as well as lateral membrane diffusion, but an integration of the various *cis*- and *trans*-acting determinants of this sorting into a coherent framework remains a challenge.

Conclusions

Early studies of plant plasma membrane dynamics were heavily influenced by data obtained in yeast and mammals and, although general traits and core components of the corresponding machineries appear conserved, originality in the plant secretory and endocytic pathways has only come to light in the past decade. The detailed analysis of the subcellular dynamics and polarity of PIN proteins as well as of a range of receptors and nutrient transporters has unraveled an amazingly complex network of pathways and factors that jointly modulate transport and ligand perception processes, translating into adaptive adjustments in plant growth and development. This is, in part, mediated by extensive crosstalk between different growth regulators and external stimuli. Although transcriptional networks are the basis of such signal integration, increasing evidence highlights the convergence of signaling pathways at the level of plasma membrane protein internalization, recycling and vacuolar targeting. Whereas the mechanisms driving such dynamics within cells are slowly emerging, the precise cascade of events involved in crosstalk with physiological readouts remains to be elucidated. Whether, for example, post-translational protein modifications involving phosphorylation and ubiquitylation are attributed to distinct sorting pathways that discriminate between different intracellular protein pools needs to be investigated in the future.

Overall, the analysis of plasma membrane protein trafficking has not only served the fascinating quest for understanding the cellular basis of transport and perception processes in plants, but has also shed light on commonalities and differences in membrane protein sorting strategies that have evolved in the different kingdoms.

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Competing interests

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