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REVIEW

PIN-Dependent Auxin Transport: Action, Regulation, and Evolution

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Auxin participates in a multitude of developmental processes, as well as responses to environmental cues. Compared with other plant hormones, auxin exhibits a unique property, as it undergoes directional, cell-to-cell transport facilitated by plasma membrane-localized transport proteins. Among them, a prominent role has been ascribed to the PIN family of auxin efflux facilitators. PIN proteins direct polar auxin transport on account of their asymmetric subcellular localizations. In this review, we provide an overview of the multiple developmental roles of PIN proteins, including the atypical endoplasmic reticulum-localized members of the family, and look at the family from an evolutionary perspective. Next, we cover the cell biological and molecular aspects of PIN function, in particular the establishment of their polar subcellular localization. Hormonal and environmental inputs into the regulation of PIN action are summarized as well.

POLAR AUXIN TRANSPORT

Early experiments on plant tropisms suggested that a mobile signal exists in the plant, which has the ability to relay information about an environmental cue from the site of its perception to the tissues that react by altering their growth rates. Not only does this signal move, but it must be transported through plant tissues in a highly controlled manner, for example, to create concentration gradients between the shaded and illuminated sides of a phototropically responding coleoptile (Went, 1974). This signal is the plant hormone auxin, and further research, aided by identification of transport inhibitors, strengthened the notion of the physiological importance of auxin relocation in the plant. Auxin is distributed in the plant body by two distinct, but interconnected, transport systems: first, a fast, nondirectional stream in the phloem along with photosynthetic assimilates, and second, slow and directional cell-to-cell polar auxin transport (PAT). While phloem transport provides a general way to deliver auxin from the sites of its synthesis (mostly in young leaves) to recipient organs, PAT distributes auxin in a precise manner that is critically important for the formation of local auxin maxima, mainly in developing tissues.

At the level of single cells, PAT is explained by the chemiosmotic hypothesis (reviewed in Goldsmith, 1977). This model is based on the chemical nature of the principal auxin form, indole-3-acetic acid (IAA). In the slightly acidic pH of the apoplast, a fraction of IAA exists in a protonated state (IAAH), which allows it to pass into the plasma membrane and enter the cell freely by diffusion. While in the cytosol, at a higher pH of around 7, virtually all auxin molecules are dissociated into the ionic form and thus cannot exit the cell passively. For auxin transport out of the cell to occur, the existence of plasma membrane-localized auxin efflux carriers was postulated, and the strict directionality of PAT required these transporters to be localized only on one side of the cell; thus, their polar localization being decisive for the direction of auxin movement.

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DISCOVERY OF PINs: THE POLARIZED AUXIN EFFLUX COMPONENTS PREDICTED BY CHEMIOSMOTIC HYPOTHESIS

The founding member of *PIN* gene family, *PIN1*, was identified following isolation of the *pin-formed1* (*pin1*) mutant in *Arabidopsis thaliana*, which is characterized by a stem nearly devoid of organs such as leaves or flowers (Okada et al., 1991). The absence of organs on the stems of *pin1* mutants traces back to the shoot apical meristem, where primordia fail to form. The morphological phenotype of *pin1* could be phenocopied by a treatment with PAT inhibitors, and *pin1* mutants showed reduced PAT. Molecular cloning of the *PIN1* gene revealed that PIN1 encodes a transmembrane protein with similarity to bacterial and eukaryotic carrier proteins (Gälweiler et al., 1998). The protein was found to be localized on the basal side of cells in the vascular tissue of the stem, just as one would predict for the auxin efflux carrier postulated by the chemiosmotic hypothesis. Around the same time, a mutant with agravitropic root was isolated independently by four labs (Chen et al., 1998; Luschnig et al., 1998; Müller at al., 1998; Utsuno et al., 1998) and the underlying gene turned out to encode a protein highly homologous to PIN1, with the same predicted membrane topology, thus named PIN2. Root gravitropism has been long associated with PAT. The agravitropic *pin2* phenotype along with the polarized localization of PIN2 in the root (Müller at al., 1998) identified PIN2 as the PAT component of root gravitropic response. The auxin efflux capacity of PIN proteins has since been shown in a number of transport assays in different systems (Petrásek et al., 2006; Yang and Murphy, 2009; Barbez et al., 2013; Zourelidou et al., 2014). An important question concerned the relation between PIN polarity and directionality of auxin transport. When PIN1 is expressed under the control of the PIN2

promoter in the agravitropic *pin2* mutant, it is, in contrast to PIN2, localized predominantly basally (toward root tip) in epidermis cells. Therefore, the auxin transport capacity in basipetal (shootward) direction, necessary for correct gravitropism, is not achieved. However, a mutant version of PIN1, containing a green fluorescent protein (GFP) insertion that presumably interferes with a polarity-determining signal, localizes more apically (shootward) and was able to rescue the agravitropism of *pin2* by mediating the auxin flux away from the root tip, similarly to PIN2 function in the wild type. Thus, by switching the polarity of ectopically expressed PIN1 in otherwise identical conditions, and observing resultant auxin redistribution and tropic response, the causal link between PIN polar localization and auxin flow direction was proven (Wiśniewska et al., 2006).

DEVELOPMENTAL ROLES OF PIN-DRIVEN AUXIN TRANSPORT

The phenotypes of mutants in the first two *PIN* genes indicated that PIN-driven PAT is crucial for processes as diverse as aboveground organogenesis and the root gravitropic response. *Arabidopsis* contains a total of eight *PIN* sequences, and isolation of other *pin* mutants extended the repertoire of processes mediated by this auxin efflux carrier family. Auxin transport mediated by PINs is necessary from the very beginning of multicellular plant body development, during the laying down of the main apical-basal body axis in early embryogenesis (Friml et al., 2003). Embryos express four *PIN* genes, namely, *PIN1*, *3*, *4*, and *7*, and developmental defects in early embryogenesis can be found in single *pin4* (Friml et al., 2002a) and *pin7* mutants (Friml et al., 2003), with increasingly severe aberrations in multiple mutant combinations (Friml et al., 2003; Blilou et al., 2005; Vieten et al., 2005). Preferential accumulation of auxin is first seen in the apical cell originating after zygote division, and auxin remains present in the apical part of the embryo until a switch occurs around the 32-cell stage, following which auxin maximum is instead detected in the basal parts of the embryo, as well as the uppermost suspensor cell (Friml et al., 2003; Figure 1C). Polar localizations of PINs correspond well with these auxin fluxes, as initially PIN7 is expressed in basal domains of the embryo and polarized toward apical cells, while later both PIN7 in the suspensor and PIN1 in provascular cells of the embryo proper exhibit basal polarity (Friml et al., 2003; Figure 1D). Recent modeling studies combined with experimental approaches revealed that the PIN polarity switches during embryogenesis occur as a result of feedback regulation by auxin sources. In this scenario, local auxin production, first in the suspensor and presumably in the maternal tissues, and later at the apical end of the embryo, polarize auxin fluxes to define the apico-basal embryonic axis (Robert et al., 2013; Wabnik et al., 2013). As embryo development progresses, additional peaks of auxin response appear at the sites of cotyledon formation. Accordingly, PIN1 protein can be detected in the epidermal layer with polarities facing toward these auxin maxima, while a canal expressing basally localized PIN1 forms in the inner embryo body, driving auxin away from the primordium and defining future vascular strands (Benková et al., 2003). Indeed, cotyledon development defects, such as single, triple, fused, or improperly shaped cotyledons are observed in *pin* mutants (Benková et al., 2003; Friml et al., 2003).

When seeds germinate underground, or in vitro in darkness, the elongated hypocotyl forms at its apical end a curved structure called the apical hook, designed by nature to protect the all-important shoot apical meristem from physical damage in the soil. During the development of this structure, an auxin response gradient is formed with a maximum in the inner side of the hook (Friml et al., 2002b; Vandenbussche et al., 2010; Žádníková et al., 2010). The importance of PAT for generating this local auxin maximum and for the resulting differential cell elongation is evident by a complete loss of apical hook formation following treatment with PAT inhibitors (Žádníková et al., 2010). Time-lapse imaging revealed disrupted dynamics of apical hook development in *pin1*, *pin3*, *pin4*, and *pin7*, as well as certain double mutant combinations. Interestingly, PIN3 and PIN4 are preferentially expressed in the outer side of the apical hook, and the increased drainage of auxin from this region may result in its predominant accumulation on the opposite side (Žádníková et al., 2010).

From the seedling stage on, PINs function in maintaining the activity of the root apical meristem. The local auxin maximum in the root tip has been recognized as the pattern- and organ polarity-organizing signal (Sabatini et al., 1999) established by directional auxin transport driven by PINs (Friml et al., 2002a; Blilou et al., 2005). Joint action of PIN1, PIN2, PIN3, PIN4, and PIN7 establishes a local "reflux loop" of auxin (Blilou et al., 2005). Studies on PIN function in the root apical meristem revealed that PIN proteins exhibit partial functional redundancy in this developmental context, aided by ectopic expression of some *PIN* genes to complement lack of others in *pin* mutants (Blilou et al., 2005; Vieten et al., 2005).

The elaboration of plant body architecture at later stages of development involves auxin- and PIN-dependent postembryonic formation of new organs. The root system is extended into a branched network by the process of lateral root development. New roots arise from the pericycle layer of the primary root, at sites of elevated auxin response (Benková et al., 2003; Dubrovsky et al., 2008). The progression into a newly emerged organ involves dynamic changes in PIN-driven auxin distribution, leading to gradual concentration of auxin at the apical end of the growing lateral organ (Figures 1E and 1F). In *pin* mutants, lateral roots are generated at lower frequency, progress more slowly through development, or cannot be formed at all, instead resulting in a disorganized array of cells with diffuse auxin distribution (Benková et al., 2003).

While the development of underground tissues depends on the collaborative action of multiple PINs, the phenotypes of *pin1* clearly imply a less redundant role of PIN1 in aerial organogenesis. As stated above, *pin1* is characterized by the presence of nearly naked stems, which only rarely form defective cauline leaves or flowers (Okada et al., 1991). In the shoot apical meristem, where these organs originate, PIN1 is expressed in the outermost tissue layer, with polarities converging at an auxin maximum in the primordium tip (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Figures 1A and 1B). Files of cells with basally localized PIN1 drain auxin from this maximum, into inner parts of the tissue. Later during development of the flower, local auxin response maxima mark the apical end of each developing floral organ. When ovules develop inside the gynoecium, their tips also show auxin accumulation, and an auxin peak is seen at the tip of each developing integument (Benková et al., 2003).

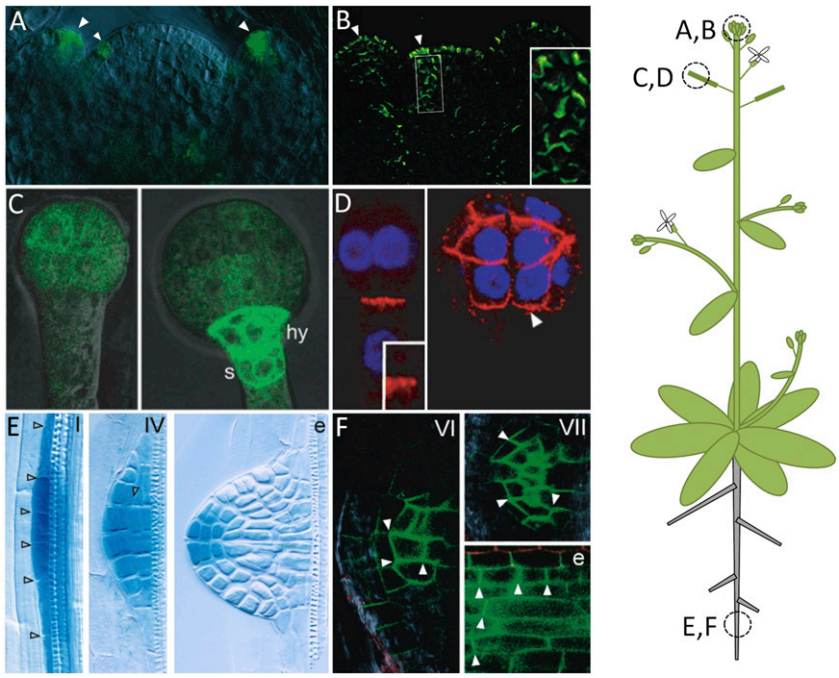

Figure 1. Examples of Auxin-Mediated Developmental Processes.

Auxin response maxima (visualized by transcriptional auxin response reporters; green in **[A]** and **[C]**; blue in **[E]**) are established by the action of polarly localized PIN proteins (green in **[B]** and **[F]**; red in **[D]**) during the development of shoot apical meristem-derived primordia (**[A]** and **[B]**), embryo (**[C]** and **[D]**), and lateral root (**[E]** and **[F]**). s, upper suspensor cell; hy, hypophysis I, IV, VI, and VII; e, developmental stages of lateral roots. Reproduced with modifications from Benková et al. (2003) (**[A]**, **[B]**, **[E]**, and **[F]**) and Friml et al. (2003) (**[C]** and **[D]**).

(A) In the shoot apical meristem, auxin accumulates at the position of incipient primordia and in primordium tips (indicated by arrowheads in **[A]** and **[B]**).

(B) In epidermis, PIN1 is polarized toward these auxin maxima, while in the inner tissues, basally localized PIN1 presumably drives auxin away from the primordium (inset).

(C) During embryogenesis, auxin response is first observed in apical parts of the embryo (left; eight-cell stage) and later in the basal parts (right; globular stage).

(D) PIN7 localizes apically in the basal cell of a two-cell embryo (left; magnified in inset) and PIN1 basally in provascular initials at 16/32-cell stage (right; indicated by arrowhead).

(E) Auxin concentrates at the apical end of a developing lateral root (arrowheads indicate cell division planes).

(F) PIN1 gradually establishes polarized localizations (indicated by arrowheads) in the inner tissues of the developing lateral root.

The examples of auxin-mediated formation of organs, such as cotyledons, lateral roots, leaves, or flowers, point at a common mode of auxin action (Benková et al., 2003), in which PIN proteins, by their coordinated polarized localizations, direct auxin transport such that auxin accumulates locally and defines sites of organogenesis. It is interesting to notice that in each case, auxin is transported toward the tip of the root through the central part of the tissue, a flux that in root-derived organs supplies the local maximum with auxin, while in apical organs drains it; thus, complementary fluxes through the outer tissue layers also have opposite directions in apical and basal organs. These two patterns of auxin flow in organogenesis have been termed "fountain" and "reverse fountain" (Benková et al., 2003).

As already hinted in the previous paragraphs, the vascular tissue, which serves as a connecting tract for transport of photosynthetic assimilates, water, and minerals between the various plant organs, also forms in an auxin-dependent manner, and PIN1 appears to have a principal role in this process—at least when aboveground tissues are considered. *pin1* mutant plants show vascular abnormalities in stems and leaves (Gälweiler et al., 1998; Mattsson et al., 1999), which can be mimicked by chemical inhibition of PAT with 1-*N*-naphthylphthalamic acid (NPA; Mattsson et al., 1999). Accordingly, the pattern of vascular development in leaves is preceded by expression of PIN1 and an elevated auxin response (Scarpella et al., 2006). During leaf vasculature formation, the "reverse fountain" auxin flow pattern can be observed, as PIN1 polarities converge in epidermis and form a stream reaching inside the leaf. PIN1 also controls the establishment of leaf shape, in particular the outgrowth of serrations at the leaf margin (Hay et al., 2006; Bilsborough et al., 2011). The epidermal auxin maxima that correspond to future veins in subepidermal tissues also mark sites for serration development. Consistently, *pin1* mutants as well as NPA-treated plants exhibit smooth leaf margins (Hay et al., 2006).

In contrast to the developmental processes outlined above, the opening of *Arabidopsis* fruit appears to depend on a conceptually novel local auxin minimum, rather than formation of an

auxin maximum (Sorefan et al., 2009). *Arabidopsis* silique opens along thin margins between the replum and the two valves in a process dependent on the transcription factor INDEHISCENT (IND) expressed specifically at the valve margin. The proposed model suggests that fruit opening requires a localized auxin depletion resulting from IND- and phosphorylation-dependent modulation of PIN3 polarity and redirection of PAT.

AUXIN TRANSPORT ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE

The canonical, plasma membrane-localized PINs, which consist of two transmembrane regions separated by a long hydrophilic loop, are represented in *Arabidopsis* by five members: PIN1-4 and PIN7. By contrast, PIN5, PIN6, and PIN8 are characterized by a reduction in the middle hydrophilic loop, partially in PIN6 and more pronounced in PIN5 and PIN8. Surprisingly, localization studies revealed that PIN5, PIN6, and PIN8 predominantly localize to the endoplasmic reticulum (ER; Mravec et al., 2009; Dal Bosco et al., 2012; Ding et al., 2012; Bender et al., 2013; Sawchuk et al., 2013), although instances of plasma membrane localization of PIN5 and PIN8 have been reported as well (Ganguly et al., 2014). Auxin transport and auxin content measurements indicate that PIN5 likely mediates auxin transport into the ER lumen (Mravec et al., 2009), while PIN8 appears to counteract this activity, which is further supported by antagonistic genetic interactions observed between mutants and overexpressors of these two transporters (Ding et al., 2012; Sawchuk et al., 2013). The proposed role of PINs at the ER membrane is the regulation of auxin homeostasis by subcellular auxin compartmentalization, as auxin inserted into ER lumen is likely unavailable for participation in PAT and nuclear signaling and becomes a potential substrate for inactivation by ER-localized auxin conjugating enzymes (Mravec et al., 2009). PIN6- and PIN8-mediated translocation of auxin from the ER lumen into the nucleus has also been hypothesized (Sawchuk et al., 2013).

ER-localized PINs play both distinct and overlapping functions in plant development. PIN8, which is highly expressed in the male gametophyte, has an important role in pollen development and functionality (Dal Bosco et al., 2012; Ding et al., 2012), while PIN6 has been ascribed a function in the production of nectar and proper development of short stamens (Bender et al., 2013) as well as root growth and lateral root development (Cazzonelli et al., 2013). PIN5 appears to be necessary for fine-tuning of auxin function, as a *pin5* mutant revealed only minor developmental phenotypes (Mravec et al., 2009). Moreover, all three ER-localized PINs are necessary in leaf vascular patterning (Sawchuk et al., 2013) where their intracellular auxin transport activity interacts with the intercellular PAT driven by PIN1.

Recently, a novel family of putative auxin transporters, designated PIN-likes (PILS), has been discovered, based on in silico search for proteins with predicted topology similar to that of the PINs (Barbez et al., 2012). Reduction or upregulation of PILS activity leads to alterations in auxin-mediated developmental processes, such as root and hypocotyl growth and lateral root formation. Analysis of transcriptional auxin responses suggested that PILS action leads to decreased auxin signaling, while auxin accumulation assays showed their ability to increase intracellular auxin retention, with a shift toward conjugated forms of the molecule. This mode of action is reminiscent of ER-localized PIN5; indeed, PILS transporters were found to localize to ER in all systems analyzed. Thus, PILS emerged as a novel family of cellular auxin homeostasis regulators.

EVOLUTION OF PIN PROTEINS

In the flowering plant species *A. thaliana*, PIN proteins have become highly specialized, as evidenced by their differential expression patterns, subcellular localizations, and developmental roles. An interesting question, then, arises about the evolutionary origin of this diversity in PIN function and the roles of PIN-mediated auxin transport in taxonomic groups ancestral to the flowering plants. Recently, experimental insights into the developmental functions of PIN transporters in the moss model species *Physcomitrella patens* have been reported. The *P. patens* genome encodes four *PIN* sequences, denoted Pp-*PINA*-*PIND*, of which Pp-*PINA*-*PINC* represent long, PIN1-type transporters, while Pp-*PIND* encodes a short, PIN5-type protein. Accordingly, Pp-PINA shows polar, plasma membrane localization at the apical ends of moss filaments and in leaves, while Pp-PIND does not colocalize with plasma membrane markers and has localization pattern reminiscent of ER (Bennett et al., 2014; Viaene et al., 2014). The auxin transport activity of Pp-PINs was verified by various means, including measurements of IAA export into the media from *P. patens* tissues.

The first stage of gametophyte development in moss is a structure consisting of one-cell-wide filaments called protonemata, which grow by apical cell divisions, gradually changing their character from chlorophyll-rich chloronema to chlorophyll-poor caulonema. This developmental switch in cell identity seems to be regulated by auxin (Prigge et al., 2010; Jang and Dolan, 2011). Presumably, Pp-PINs mediate auxin transport from the base of protonema filaments to their tips, influencing auxin content in each cell and thus their developmental fates (Viaene et al., 2014).

During later development, mosses generate gametophores with leaves consisting of two-dimensional sheets of cells. Auxin transport inhibition, external auxin application, and long-type *pin* knockouts suggest that PIN-driven auxin transport is required for multiple aspects of gametophore development, such as apical meristem activity, leaf initiation and growth, and tropisms (Bennett et al., 2014; Viaene et al., 2014). Furthermore, development of the *P. patens* sporophyte depends on Pp-PIN function (Bennett et al., 2014), consistent with the presence of long-range PAT in moss sporophytes (Fujita et al., 2008)

The first experimental insights into PIN protein function in *P. patens*, a representative of most basal land plants, indicate that auxin transport mediated by polarly localized PINs has been recruited in the dominant gametophyte stage of moss, controlling a suite of processes including the development of relatively simple morphologies, such as one-dimensional protonemal filaments or two-dimensional cell sheets that are the moss leaves.

SUBCELLULAR TRAFFICKING FOR PIN POLAR LOCALIZATION

Every plasma membrane-localized PIN protein in *Arabidopsis* exhibits polarized localization in some instances. How is that achieved? Today, there is mounting evidence supporting subcellular

trafficking of PINs as the primary factor in the establishment of their polarities. Although one may presume that PIN proteins are statically deposited at the plasma membrane, in fact, PINs turned out to be continuously, dynamically cycling between their polar domain at the plasma membrane and endosomal compartments (Figure 2). This conclusion was first drawn from observations that the fungal toxin brefeldin A (BFA) triggers intracellular accumulation of PIN1 in so-called "BFA compartments," which is fully reversible after removal of BFA (Geldner et al., 2001). BFA inhibits ADP-ribosylation factor guanine-nucleotide exchange factors (ARF-GEFs), which activate ARFs, molecular players necessary for the formation of coated vesicles mediating various trafficking events in the endomembrane system. Putting these facts together, a BFA-sensitive ARF-GEF action mediates constitutive recycling of PIN1 from an endosomal compartment to the polar domain at the plasma membrane, and when this recycling step is blocked, the constitutive internalization of PIN1 is revealed by its intracellular accumulation. A candidate for the protein that may mediate this BFA-sensitive polar delivery of PIN1 is the ARF-GEF GNOM. *gnom* mutants demonstrate severe defects in development, characterized, in extreme cases, by a complete loss of apical-basal body plan (Mayer et al., 1991; Shevell et al., 1994). These defects originate at embryogenesis, where coordinated cell polarities are not correctly established, as manifested by the loss of correct polarization of PIN1 (Steinmann et al., 1999). BFA-sensitive PIN1 recycling was conclusively linked with GNOM function with the use of an engineered, BFA-resistant, yet fully functional variant of GNOM (Geldner et al., 2003). While originally a model was proposed in which GNOM mediates the recycling step from a hypothetical "recycling endosome," recent in-depth analyses using high-end microscopy revealed that GNOM in fact acts at the Golgi apparatus (Naramoto et al., 2014). Thus, GNOM may be indirectly influencing the function of *trans*-Golgi network/early endosome compartment, from where polar cargoes might be recycled. The GNOM-mediated pathway is to a large extent responsible specifically for the basal targeting of PIN proteins, while the apical pathway may be mediated by other, BFA-resistant ARF-GEFs (Kleine-Vehn et al., 2008a). Additional components of PIN delivery to the plasma membrane, namely, the ARF family member ARF1A1C (Tanaka et al., 2014) and a small GTPase RabA1b (Feraru et al., 2012), were identified in a forward genetic screen based on PIN1-GFP fluorescence imaging (reviewed in Zwiewka and Friml, 2012).

PIN proteins are internalized from the plasma membrane by clathrin-mediated endocytosis (Dhonukshe et al., 2007). Interference with clathrin-coated vesicle formation by expression

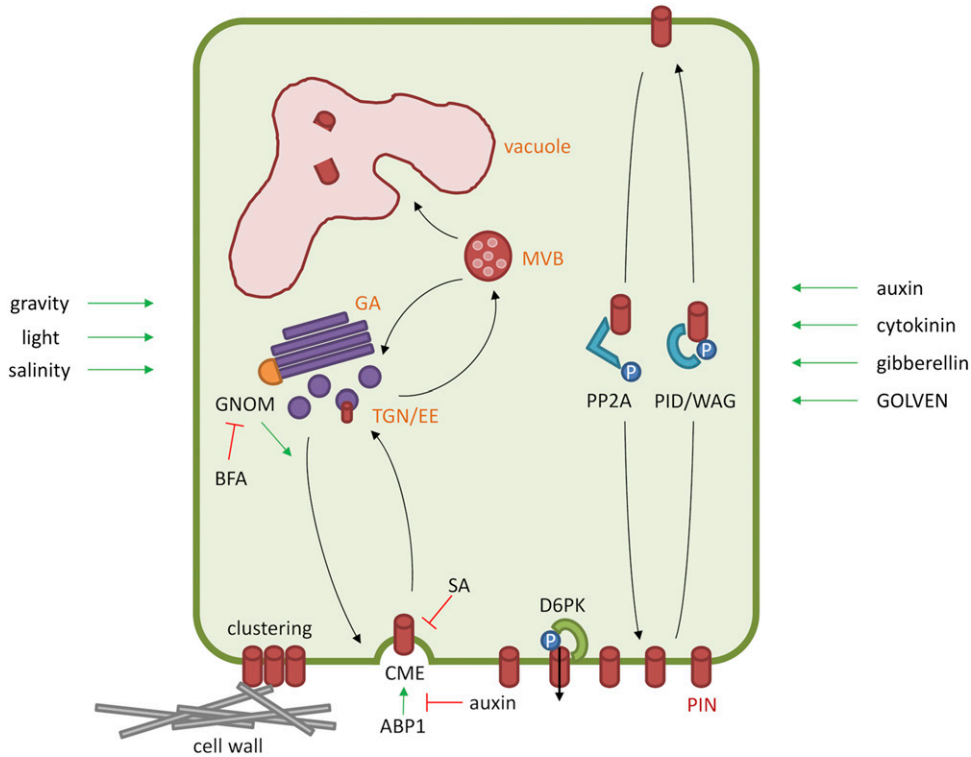

Figure 2. Subcellular Trafficking and Polarity Maintenance of PIN Proteins.

Polar localization of PIN proteins is established by GNOM-mediated recycling and clathrin-mediated endocytosis (CME) and maintained by clustering in the plasma membrane as well as cell wall-plasma membrane connections. Apical-basal polarity is determined by reversible phosphorylation by PID/WAG kinases and PP2A phosphatase. Auxin transport activity of PIN is mediated by D6PK. PINs undergo trafficking through multivesicular body (MVB) for degradation in the lytic vacuole. Endogenous (hormones and signal peptides) and environmental (light, gravity, and salinity) signals influence various aspects of PIN trafficking. GA, Golgi apparatus; TGN/EE, *trans*-Golgi network/early endosome.

of a dominant-negative truncated variant of clathrin heavy chain (CHC), referred to as HUB, abolished the BFA-visualized internalization of PIN1 and PIN2 (Dhonukshe et al., 2007; Kitakura et al., 2011), while a similar, but weaker effect on PIN2 was observed in *clathrin light chain2 clathrin light chain3* (*clc2 clc3*) double mutants (Wang et al., 2013). The relevance of clathrin-mediated PIN endocytosis for correct auxin transport and auxin-mediated development is illustrated by improper auxin distribution in the above-mentioned HUB line, concomitant with root gravitropism and lateral root formation defects, embryo patterning defects in the *chc2* mutant, which leads to development of seedlings with improperly formed cotyledons and root apical meristems (Kitakura et al., 2011), as well as deficiencies in auxin transport and distribution in *clc2 clc3* mutant (Wang et al., 2013). Other than clathrin, ARF machinery has also been implicated in endocytosis. The ARF-GEF GNOM, apart from regulating recycling, functions in endocytosis in concert with ARF-GTPase-activating protein VASCULAR NETWORK DEFECTIVE3 (Naramoto et al., 2010), while the related ARF-GEF GNOM-LIKE1 participates more specifically in endocytosis of PIN2 (Teh and Moore, 2007). Downstream of endocytosis, the early endosomal trafficking of PINs is controlled by another ARF-GEF, BFA-visualized endocytic trafficking defective1, and the Sec1/Munc18 family protein BEN2 (Tanaka et al., 2009, 2013).

The proposed role of constitutive internalization of PIN proteins is to maintain, in concert with polar recycling, the polarized localization of PINs at the plasma membrane. Presumably, this is necessary to counteract lateral diffusion in the absence of diffusion barriers, such as junctions that separate polar domains in polarized epithelial cells (for a comparison of polarity determination mechanisms in animal and plant cells, see Kania et al. [2014]). Indeed, PIN proteins are depolarized in the dominant negative clathrin HUB line as well as *chc2* embryos (Kitakura et al., 2011). Experimental and modeling approaches suggest that endocytosis of PINs, necessary for maintenance of its polarity, may take place preferentially at lateral cell sides (Kleine-Vehn et al., 2011). The dynamic nature of PIN trafficking could be also highly relevant for rapid changes in PIN polar localization, occurring during embryo development (Friml et al., 2003), lateral root formation (Benková et al., 2003), root gravitropic response (Friml et al., 2002b; Kleine-Vehn et al., 2010), and hypocotyl tropic responses (Ding et al., 2011; Rakusová et al., 2011). In support of the notion of PINs being dynamically redistributed to new polar domains, a transcytosis-like process, in which the same PIN protein molecules undergo trafficking from one polar domain to another, was documented (Kleine-Vehn et al., 2008a) and implicated in gravity-induced PIN3 relocation in the root tip columella (Kleine-Vehn et al., 2010).

Endocytosis and polar recycling jointly establish the polar localization of PINs at the plasma membrane. An additional layer of regulation comes from ubiquitination-dependent PIN degradation in the vacuole (Kleine-Vehn et al., 2008b; Leitner et al., 2012), a process that requires proteasome function (Abas et al., 2006). Multiple trafficking components participate in vacuolar PIN sorting, including the adaptor protein complex 3 (Feraru et al., 2010; Zwiewka et al., 2011), the retromer (Nodzyński et al., 2013), and the endosomal sorting complexes required for transport (Spitzer et al., 2009; Gao et al., 2014).

PIN POLARITY MAINTENANCE BY CLUSTERING AND THE CELL WALL

Apart from the subcellular trafficking events describe above, an additional factor that might be important for polarity maintenance during the residence of PIN in the plasma membrane has been uncovered (Kleine-Vehn et al., 2011). PIN proteins have been observed to exhibit only limited lateral diffusion in the plasma membrane, when compared with non-polar plasma membrane markers. Following a detailed confocal and high-resolution microscopic analyses, PIN1 and PIN2 were found to be unevenly distributed in the plasma membrane, with a large fraction of the protein residing in so-called clusters, defined as agglomerations of PIN signals 100 to 200 nm in diameter. These clusters were immobile in the membrane, and since less clustering of non-polar membrane markers was observed, clustering was proposed as a factor limiting the lateral diffusion of PIN proteins out of their polar domains (Kleine-Vehn et al., 2011).

Another line of investigation aimed at identification of novel components of PIN polarity was the *regulator of pin polarity* (*repp*) forward genetic screen, designed to identify mutants with a basal-to-apical switch in localization of PIN1 ectopically expressed in root tip epidermis. Because the screen was performed in the agravitropic *pin2* mutant background (PIN2:PIN1-HA, *pin2* line), such apicalization of PIN1 in the epidermis could be identified easily, since it would lead, by mimicking native function of apically localized PIN2, to restoration of a correct auxin flow and, hence, root gravitropism (Feraru et al., 2011). The first mutant identified in this screen, *repp3*, was mapped to the *CELLULOSE SYNTHASE3* locus, and independent pharmacological or genetic interference with cellulose biosynthesis phenocopied *repp3.* This unexpected function of the cell wall for PIN polarity was substantiated by observations that PIN proteins are associated with domains that are physically connected to the cell wall and by the rapid loss of PIN polarization after cell wall digestion. Furthermore, the lateral diffusion of PIN2 was much faster when cell wall connections were lost following pharmacological inhibition of cellulose synthesis or cell plasmolysis (Feraru et al., 2011). The role of the plant cell wall in limiting lateral diffusion of plasma membrane proteins has been since further confirmed (Martinière et al., 2012). In summary, clustering and cellulose-based cell wall-to-polar domain connections presumably contribute to the maintenance of PIN proteins at their polar domains (Figure 2). The possible relationship between these two processes remains an open question for future research.

PHOSPHORYLATION-BASED REGULATION OF PIN-MEDIATED AUXIN TRANSPORT

In a genetic screen for mutants that have lost the ability to generate flower primordia, a mutant named *pinoid* (*pid*) was found, with phenotypic characteristics resembling those of *pin1* (Bennett et al., 1995). The PID protein encodes a member of AGCVIII family of protein kinases (Christensen et al., 2000). Although initially a role in auxin signaling was ascribed (Christensen et al., 2000), further analyses implicated PID in an auxin transport-related function (Benjamins et al., 2001). Overexpression of PID led to frequent collapse of the main root (Benjamins et al., 2001; Friml et al., 2004), which correlated with loss of local auxin maximum in the root apex. This phenotype was preceded by basal-to-apical switch of

polarities of PIN1, PIN2, and PIN4 (Friml et al., 2004), resulting in draining of auxin out of the root tip. Conversely, the *pid* mutant exhibits the opposite, apical-to-basal, polarity switch of PIN1 in the shoot apical meristem (Friml et al., 2004), confirming the function of PID in PIN apical versus basal polarity determination. PID and its homologs directly phosphorylate PIN protein hydrophilic loops at three highly conserved motifs, and the importance of these phosphorylation sites for PIN polarity determination was confirmed in planta by the analysis of phosphomutant versions of PINs (Michniewicz et al., 2007; Huang et al., 2010; Zhang et al., 2010).

The search for phosphatase activity that antagonizes PID function led to the identification of protein phosphatase 2A (PP2A) as an important factor in PIN polarity regulation. Initially, a mutant in one of the three *Arabidopsis* paralogs of the PP2A regulatory subunit A (PP2AA1) was isolated in a forward genetic screen for altered response to the PAT inhibitor NPA (Garbers et al., 1996). This and other phenotypes that indicated the involvement of PP2AA1 in auxin-mediated processes (Rashotte et al., 2001) prompted a more complete analysis that revealed severe auxin-related developmental defects during embryo and seedling development in multiple *pp2aa* mutants and artificial microRNA lines targeting PP2AA (Michniewicz et al., 2007). Genetic interactions supported the notion that PP2AA phosphatase function antagonizes that of PID kinase. Crucially, a basal-to-apical polarity shift of PIN1, PIN2, and PIN4 was observed in PP2AA-deficient plants (Michniewicz et al., 2007), indicating that dephosphorylation of PINs by PP2A promotes their basal localization.

Taking all these data together, a model emerged in which PID-mediated phosphorylation promotes apical PIN localization, whereas dephosphorylation by PP2A leads to basal PIN polarity (Figure 2). However, it remains unclear at which stage of the polar sorting processes and where in the cell these regulatory steps take place or whether they are relatively stable or more transient.

Recently, another group of proteins from the same AGCVIII family of protein kinases, consisting of D6 protein kinase (D6PK) and D6 protein kinase-likes (D6PKL), has been implicated in the regulation of PAT. Loss of D6PK activity led to typical auxin-related phenotypes that correlated with reduced PAT rates (Zourelidou et al., 2009, 2014; Willige et al., 2013). D6PKs phosphorylate plasma membrane-localized PINs (Zourelidou et al., 2014), but, unlike PID, do not affect their polar localizations (Willige et al., 2013). Instead, D6PKs act as activators of auxin efflux activity of PIN proteins: In *Xenopus laevis* oocytes, auxin was actively transported only when PINs were coexpressed with D6PK (Zourelidou et al., 2014). In planta, this transport activation presumably occurs at basal polar domains, where D6PK localizes (Barbosa et al., 2014; Figure 2). The auxin transport assays in *Xenopus* oocytes showed that PID and its close homolog WAG2 also activate PIN-driven auxin efflux (Zourelidou et al., 2014), showing that AGCVIII family kinases likely have both different and partially overlapping functions in regulating PINs.

AUXIN FEEDBACK REGULATION OF PIN-MEDIATED AUXIN TRANSPORT

The canalization hypothesis, proposed by Tsvi Sachs, suggests self-organizing properties of PAT on the level of organs and tissues (summarized in Sachs, 1991). In an undifferentiated group of cells exposed to a source and a sink of auxin, the cells gradually polarize their auxin transport activities toward neighbors that already happen to transport auxin most efficiently. This feedback mechanism, in which local flow of auxin affects the cells to modify this flow's direction and strength, results in emergence of a narrow, well-defined canal of cells efficiently transporting auxin to connect the source with the sink. The canalization hypothesis is intimately linked with several developmental roles of auxin, such as de novo vascular tissue formation or its regeneration. Cells forming the postulated canals of PAT will differentiate into vascular tissue connecting various parts of the plant body; indeed, it is by microscopic observation of vascular cell differentiation that Sachs assumed the PAT directions in his experiments.

According to the model described above, auxin itself should have the ability to influence the directionality and capacity of a given cell's auxin transport. Nowadays, we see mechanisms that could contribute to the feedback regulation of auxin on its own transport by affecting PIN polar localization and PIN protein abundance in the cell or, more specifically, at the plasma membrane. A series of experiments on wounded pea (*Pisum sativum*) epicotyls, inspired by Sachs' ideas, but extending them by direct observations of PIN proteins, was conducted by Sauer et al. (2006). The pea homolog of PIN1 coordinately polarized along the presumed path of auxin flow that forms around a wound, spatially corresponding to the vascular tissue that later differentiated in order to reconnect the severed vascular strand. When an auxin source was artificially provided, PIN1 became expressed and polarized so as to create a new canal, connecting that auxin source to the sink—the preexisting central vascular cylinder. The competition between different auxin sources has an impact on their ability to induce such canalization, providing a possible mechanism for regulation of apical dominance (Balla et al., 2011). Together, these observations show that PINs are suitable candidates to be targeted by the canalization mechanism, as both their expression and subcellular polarization are regulated by auxin itself. Auxin influence on PIN polarities can be also seen in the root apical meristem, where its external application leads to cell-type-specific lateral spreading of PIN1 and PIN2 via a mechanism involving the nuclear, Aux/IAA- and ARF-dependent auxin signaling pathway (Sauer et al., 2006).

As PINs are subject to dynamic subcellular trafficking, regulatory inputs of auxin into specific trafficking events could be envisioned as an efficient means for the hormone to regulate its own transport. Indeed, auxin was found to inhibit endocytosis, leading to stabilization of PIN proteins at the plasma membrane and enhancement of the cell's auxin efflux capacity (Paciorek et al., 2005). This rapid auxin effect is mediated by a nontranscriptional pathway involving the auxin receptor AUXIN BINDING PROTEIN1 (ABP1). These observations suggest a model (Figure 3) in which ABP1, when not bound to auxin, promotes clathrin-mediated endocytosis, while auxin binding would block this ABP1 activity, causing loss of clathrin from the plasma membrane and inhibition of endocytosis, including that of PIN cargoes (Robert et al., 2010). This extracellular ABP1 action on endocytosis, presumably coupled with PM-localized transmembrane kinase receptor-like kinases (Xu et al., 2014), can potentially be the central part of the mechanism by which auxin polarizes its own flux (Wabnik et al., 2010).

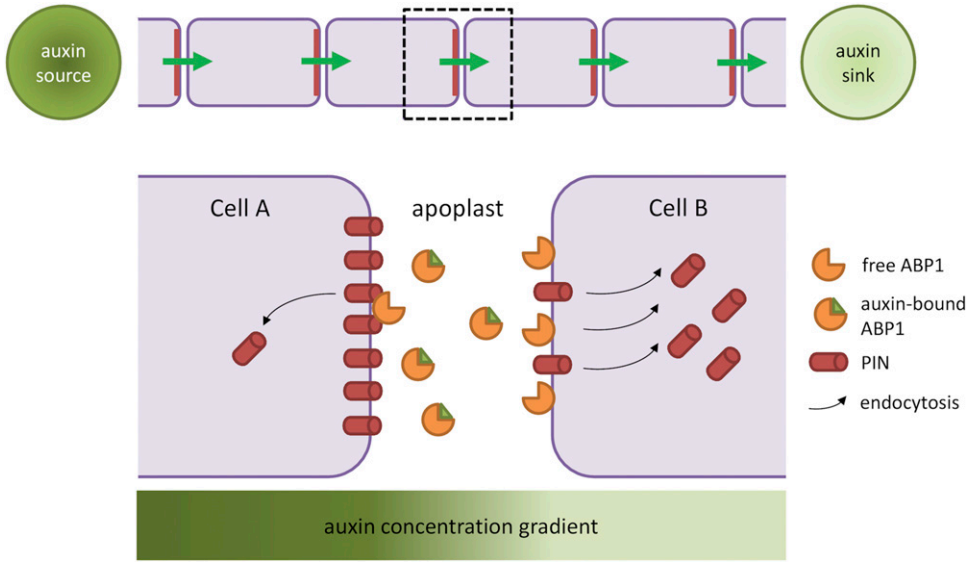

Figure 3. A Model of Auxin Transport Canalization by Extracellular Auxin Perception by ABP1.

This figure shows an update of the model presented by Wabnik et al. (2010). PIN proteins gradually polarize to form a canal of auxin flow connecting auxin source to the sink. Two neighboring cells share an apoplastic pool of ABP1 molecules. ABP1 exists in auxin-free and auxin-bound states, whereby it promotes endocytosis or is inactive, respectively. Due to an auxin concentration gradient across the apoplastic space, cell A (closer to the auxin source) experiences higher apoplastic auxin levels and fewer auxin-free ABP1 and thus has low PIN endocytosis rates, resulting in stabilization of PIN at the plasma membrane. The extracellular space near cell B has lower auxin concentration and more free ABP1 molecules that promote PIN removal from the plasma membrane.

Auxin also affects the abundance of PINs. Auxin influence on PIN degradation in the vacuole has been extensively studied on the model of gravistimulated root (Abas et al., 2006; Kleine-Vehn et al., 2008b; Baster et al., 2013). While auxin transiently stabilizes PIN2 by its inhibitory effect on endocytosis, both prolonged elevation and reduction of auxin levels leads to degradation of PIN2. Thus, only certain auxin optimum will not activate PIN2 degradation and so guarantee its stabilization at the plasma membrane (Baster et al., 2013). Furthermore, multiple *PIN* genes respond transcriptionally to auxin treatments, being upregulated in a tissue- and PIN- specific manner (Vieten et al., 2005).

The data outlined above lead to a multifaceted picture of the auxin feedback regulation of its transport directionality and capacity, and we have yet to fully understand the hierarchy of, and the interactions between, the various regulatory components involved.

ENDOGENOUS SIGNALS CONVERGING ON PINs

Apart from the feedback of auxin on its own transport, other endogenous signals, among them other phytohormones and secretory peptides, can modulate PIN protein activity (Figure 2). The plant hormone cytokinin (CK) exhibits antagonistic interaction with auxin, and the crosstalk between these two molecules mediates many aspects of root development (reviewed in Schaller et al., 2015). One of the mechanisms of such interaction is the CK effect on auxin transport in the root realized by modulation of *PIN* transcription (Dello Ioio et al., 2008; Růžička et al., 2009). Furthermore, CK has been shown to influence PIN function at the posttranslational level. CK affects PIN1 trafficking, promoting its delivery to the vacuole for degradation (Marhavý et al., 2011). Since this effect seems to be preferential for PINs at certain polar domains, it enables CK to regulate the auxin transport directionality (Marhavý et al., 2014). During lateral root development, PIN1 gradually reorients to drive auxin transport toward the tip of the newly developing root, in an axis perpendicular to the apical-basal axis of the main root. CK preferentially causes the removal of PIN1 from anticlinal membranes, while having little to no effect on periclinally localized PIN1, thus potentially contributing to the establishment of a new auxin stream driving lateral root organogenesis.

In contrast to CK, the plant hormone gibberellin (GA) exhibits a stabilizing effect on plasma membrane-localized PINs (Willige et al., 2011; Löfke et al., 2013). In GA biosynthesis-deficient conditions, PINs are preferentially targeted for vacuolar degradation, while exogenously applied GA reduces vacuolar PIN trafficking and promotes its residence at the plasma membrane. Although the specific molecular components recruited by GA remain unknown, it appears that a late trafficking step on the way to the vacuole is targeted. GA's input on PIN trafficking might be relevant for regulation of the root gravitropic response, and, remarkably, involves an asymmetric distribution of GA molecules between the upper and the lower root side after gravitropic stimulation.

While it seems that CK and GA effects on subcellular trafficking are relatively specific to PINs (Marhavý et al., 2011; Löfke et al., 2013), the plant hormone salicylic acid affects endocytosis of PIN proteins as a part of its general inhibitory effect on clathrin-mediated endocytosis (Du et al., 2013). This finding represents a novel and unexpected role of salicylic acid, which is molecularly distinct from

the well-established nuclear signaling pathway of this hormone, and its physiological functions remain unclear.

Strigolactone has been shown to cause depletion of PIN1 from the plasma membrane in xylem parenchyma cells of the stem (Shinohara et al., 2013). By regulating PIN1 levels at the plasma membrane, strigolactone can influence the capacity of bud-derived auxin to canalize toward the stem and thus modulate the bud activity and shoot architecture.

Apart from hormones, plants use small secretory peptides as short-range cell-to-cell signals for regulation of multiple developmental processes. Three peptides of the GOLVEN/ROOT GROWTH FACTOR (GLV/RGF) family have been implicated in gravitropism (Whitford et al., 2012). Overexpression of *GLV1-GLV3* genes, external application of corresponding GLV peptides, as well as loss-of-function mutants of the aforementioned genes lead to alterations in root and hypocotyl gravitropism. GLV3 peptide, within minutes of application, caused elevation of plasma membrane signal of PIN2, as well as an increase in intracellular, endosomal occurrence of PIN2. Internally synthesized GLV3 in a GLV3 overexpression line similarly promoted cellular PIN2 abundance, while a *glv3* RNA silenced line exhibited decreased PIN2 signals. Thus, while the detailed aspects of GLV/RGF peptide function await clarification, GLV3 likely mediates short-range signaling to contribute to the correct PAT streams in the gravistimulated root by modulating PIN2 subcellular trafficking dynamics.

Recent work describes two membrane phospholipid species, phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] as important components of PIN trafficking machinery and cell polarity in plants (Ischebeck et al., 2013; Tejos et al., 2014). Interference with these phosphoinositides in knockout mutants of PtdIns4P 5-kinases PIP5K1 and PIP5K2 led to multiple auxin-related phenotypes concomitant with defective polarization of PIN1 and PIN2. PIP5K1 and PIP5K2 participate in polarity determination presumably due to their preferential localization at apical and basal plasma membranes, where they regulate the balance between similarly locally enriched PtdIns4P and PtdIns(4,5)P_2 (Tejos et al., 2014). Such local regulation of phosphoinositides may influence the formation of clathrin-coated vesicles, thus being decisive for PIN endocytosis and so its polarity (Ischebeck et al., 2013). This provides another input avenue for signaling pathways, where PIN polarity and trafficking could be regulated via phosphoinositide metabolism and distribution.

Apart from these phosphoinositides, a role of inositol trisphosphate ($InsP_3$)-dependent Ca^{2+} signaling in PIN-driven auxin distribution has been recognized in a forward genetic screen for suppressors of PIN1 overexpression phenotypes (Zhang et al., 2011). While genetic and pharmacological upregulation of $InsP_3$ and cytosolic Ca^{2+} levels interfered with basal PIN polarity, decreasing the levels of these signaling molecules affected apical PIN targeting. The intermediate steps leading to these outcomes, and thus the exact mechanism by which Ca^{2+} influences PIN sorting, remain to be elucidated, but downstream regulation of PID activity is a likely component.

ENVIRONMENTAL INFLUENCES ON PIN-DRIVEN PAT

PAT is influenced by external signals in addition to endogenous regulation (Figure 2). Such regulatory inputs are necessary for auxin-mediated adaptive growth responses, clear examples of which are phototropism and gravitropism, the alignment of plant growth with light direction and gravity vector, respectively. The differential distribution of auxin between two sides of a responding organ, resulting in differences in growth rates, has been proposed by Cholodny and Went as a basis of tropic growth (summarized in Went, 1974). This auxin asymmetry has been documented during phototropism and gravitropism with auxin-inducible reporters (Luschnig et al., 1998; Rashotte et al., 2001; Friml et al., 2002b; Rakusová et al., 2011) and confirmed with a new generation of auxin sensors (Band et al., 2012; Brunoud et al., 2012). The role of PIN-driven PAT is evident from tropism deficiencies in *pin* mutants, such as the root agravitropic phenotype of *pin2* (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998), partial loss of root gravitropism in *pin3 pin7* (Kleine-Vehn et al., 2010), or reduced photo- and gravitropic bending of *pin3* hypocotyls (Friml et al., 2002b; Rakusová et al., 2011). Also, as seen in previous sections of this review, a number of molecular components regulating PIN trafficking and activities have been identified on the basis of tropism defects in corresponding mutants.

How can auxin transport be redirected by PINs in accordance to environmental cues? Within the PIN family, PIN3 exhibits a prominent ability to change its subcellular localization in response to gravity and light. In the columella cells of the root tip, where perception of gravity occurs (Chen et al., 1999), PIN3 is localized to the plasma membrane in an apolar manner. However, as early as several minutes after changing the gravity vector by rotating the plant, PIN3 begins to relocalize to the lateral, now lower side of the cells (Friml et al., 2002b). As such, it can redirect the auxin flow toward the lower side of the root for its further displacement by PIN2. In dark-grown hypocotyls, PIN3 shows prominent expression in the endodermis, without visible polarity. Both gravitropic stimulation (Rakusová et al., 2011) and unilateral light (Ding et al., 2011) cause polarization of the symmetrically distributed PIN3 to direct auxin flow laterally toward either the lower, or the shaded, side of the organ. Although the tissue or environmental signals concerned vary, the PIN3 polarization events described above share some similarities at the molecular level (Kleine-Vehn et al., 2010; Ding et al., 2011; Rakusová et al., 2011). In each instance, the ARF-GEF vesicle trafficking regulator GNOM was shown to be necessary for polarization to occur, and during hypocotyl tropisms, the prominent role of PID/WAG protein kinases has been shown.

Shade avoidance syndrome is a growth response triggered when plants are threatened to be out-competed for light by their neighbors. It is sensed as a lowered red to far-red (R:FR) light ratio, resulting from selective light absorption by chlorophyll in leaves of surrounding plants. Similarly to the phototropic response, a light signal is transduced by PIN3, which translocates to the outer endodermis cell side, redirecting part of the PAT to the outer tissue layers and thus promoting elongation (Keuskamp et al., 2010). Accordingly, seedlings of *pin3* mutant were unable to elongate hypocotyls upon sensing low R:FR ratio.

Not only gravity and light, but also salinity is an environmental variable to which plants react in an auxin- and PIN-dependent manner. Halotropism is a recently described tropic response, wherein roots grow away from high salt concentrations (Galvan-Ampudia et al., 2013). During halotropic

response, auxin accumulates differentially in the root tip, with more auxin at the side away from high salt concentration leading to asymmetric growth, analogous to the gravitropic response. This correlates with increased internalization of PIN2 at the side of salt perception, which likely leads to decreased auxin transport capacity. The proposed mechanism of this salt-induced promotion of PIN2 endocytosis involves recruitment of clathrin to the plasma membrane by increased activity of phospholipase D, presumably through synthesis of phosphatidic acid, a phospholipid that has been shown to bind components of clathrin machinery (McLoughlin et al., 2013).

In summary, a number of environmental responses involve downstream regulation of dynamic subcellular trafficking of PINs, typically leading to polarization to specific domains in order to redirect auxin flow according to external cues. However, the exact mechanisms by which perception of signals such as gravity, light, or salinity leads to regulation of PIN trafficking or polar sorting are still unclear.

SUMMARY

Auxin mediates an impressive variety of developmental processes. In virtually all its activities, the intercellular, and possibly also intracellular, transport mediated by PIN auxin transporters is of key importance. For their diverse roles to be fulfilled, PIN proteins are tightly controlled by an array of regulators at the levels of transcription as well as cellular polarity resulting from secretion, endocytosis, recycling, and vacuolar trafficking. Endogenous signals, including auxin itself and other hormones, influence these regulatory steps in order to fine-tune PIN localization and function. A number of external inputs are also decisive for regulating PIN activities, thus enabling the environmental conditions to be integrated into auxin-dependent developmental programs. In conclusion, from early on in their lineage, plants have gradually evolved a complex and versatile mechanism, in which integration of endogenous and exogenous signals, converging on the PINs and distribution of auxin, provides instructions for many aspects of growth and development.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the article.

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