Signaling and Communication in Plants

Rujin Chen
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# Polar Auxin Transport

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*Editors*
Rujin Chen
Plant Biology Division
The Samuel Roberts Noble Foundation
Ardmore, Oklahoma
USA

František Baluška
University of Bonn
IZMB
Bonn, Nordrhein-Westfalen
Germany

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# Preface

The plant hormone auxin plays essential roles in diverse plant developmental processes including morphogenesis, organogenesis, vascular tissue differentiation, and growth responses to biotic and abiotic signals. The involvement of auxin in the broad spectrum of biological processes is mainly mediated by its regulation of cell division, expansion, and differentiation. Experimental evidence indicates that local auxin gradients generated by three families of auxin transporter proteins are essential for auxin actions. The molecular cloning of auxin transporter proteins in the past two decades marks a milestone in auxin research. Importantly, the biological activity of auxin transport proteins is associated with their characteristic plasma membrane localization. The PIN-FORMED (PIN) family of auxin efflux proteins has been shown to exhibit asymmetric plasma membrane localization and determine the direction of auxin flow. The maintenance of PIN protein plasma membrane localization involves clathrin-coated vesicle endocytosis, a process that is evolutionarily conserved in plant, yeast, and animal cells, and intracellular trafficking targeting proteins to distinct plasma membrane domains or lytic vacuoles for turnover. Auxin reinforces its own transport by regulating transcription and endocytosis and recycling of its transport proteins. Recently, a group of new auxin transport proteins, the so-called "short" PINs, has been demonstrated to localize to the endoplasmic reticulum membrane and play a role in intracellular transport and homeostasis of auxin.

Experimental evidence supports that membrane microdomains regulated by the composition of membrane lipids and sterols play a role in endocytosis of auxin transport proteins. Intriguingly, differences exist in the clathrin-mediated endocytosis of auxin influx and efflux proteins. Within plant cells, sequence-specific phosphorylation by plant AGC kinases counterbalanced by protein phosphatase-mediated dephosphorylation is necessary to channel PIN proteins to distinct intracellular trafficking pathways destined to specific membrane domains. Auxin transport proteins are also targeted to lytic vacuoles for protein turnover. Interestingly, gravity and light, two prevalent environmental signals, have been shown to modulate intracellular trafficking and targeting of auxin transport proteins during plant tropic responses.

Amongst numeric milestones in the field of auxin research are the identification and characterization of auxin signaling pathways and the TIR1/AFB family of auxin receptors. Development of different auxin sensors and quantification methods with increased sensitivities has greatly facilitated auxin research in the model species *Arabidopsis thaliana*. The advanced molecular and genetic tools have gradually become available in other plant species with interesting biological processes, providing new insights into our understanding of the role of polar auxin transport in developmental processes such as plant–microbe interactions.

As sessile organisms, higher plants evolved various mechanisms to cope with changing environmental conditions. Biotic and abiotic stresses have profound effects on plant development and reproduction in part through manipulation of the level, response, and distribution of plant hormones. As summarized in this book series, polar auxin transport is a prime target/mediator of abiotic and biotic stress responses of plants, as well as a practical target for agronomic trait improvements in crop plants. While tremendous progresses have been made in auxin research, new insights of the role of auxin transport in diverse developmental processes await to be gained.

Ardmore, OK, USA — Rujin Chen
Bonn, Germany — František Baluška

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# Signaling in Polar Auxin Transport

**Anindya Ganguly and Hyung-Taeg Cho**

**Abstract** Polar auxin transport, directionally delivering auxin and generating local auxin gradients, is pivotal to fundamental developmental processes in plants. The directional auxin flow is attributable to polar-localized auxin transporters. In particular, over the past several years, the cytological and molecular mechanisms of how auxin transporters distribute asymmetrically in the plasma membrane have become greatly understood. The cellular polarity players for auxin transporters include intracellular trafficking components, cargo phosphorylation cues and corresponding kinases, membrane lipid composition, ubiquitylation, and extracellular auxin receptor-mediated modulation of cytoskeleton. In addition to these internal polarity regulators, other major plant hormones, signaling molecules, environmental stimuli, and nutrients have been implicated in regulation of auxin transporters, reflecting the communication between diverse developmental/environmental signals and auxin-mediated plant development. In this chapter, we review the recent studies that have elucidated the regulatory mechanisms of auxin transport.

## 1 Introduction

He got up early as 6:40 A.M. in the summer morning, walked to a quite sizable dark box on the table, and carefully opened the top. He slowly looked over the stems and leaves of a verbena plant inside the dark box and then cautiously placed the fifth dot on the glass plate precisely above the tiny bead affixed through a glass filament to the verbena stem apex. Over the night, the bead had traveled from the east to the southwest (Fig. 1).

A. Ganguly • H.-T. Cho (✉)
Department of Biological Sciences and Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-742, South Korea
e-mail: htcho@snu.ac.kr

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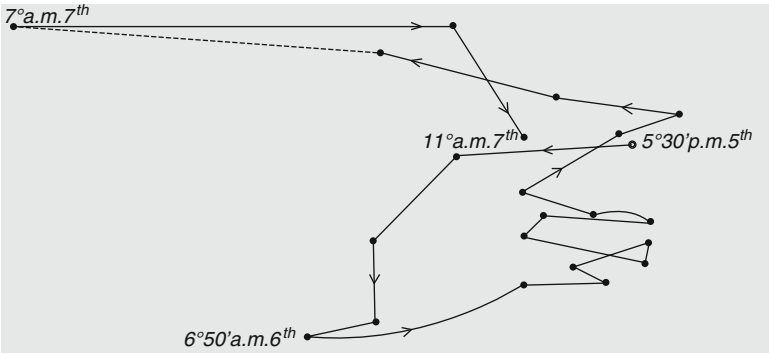


**Fig. 1** *Verbena melindres*: circumnutation of stem in darkness, traced on vertical glass, from 5.30 P.M. on June 5 to 11 A.M. June 7. The *broken line* represents a nocturnal course of the movement (Darwin 1880)

Just a few years before he passed away, Charles Darwin with his son Francis had left pioneering insights on the field of auxin physiology. The Darwins reported 157 tracings (like Fig. 1) of movements from more than 40 plant species in their book "The Power of Movement in Plants" (Darwin 1880). Darwin's great contribution to the phytohormone physiology in this volume is the prediction of auxin and its directional transport. The polar auxin transport concept was suggested by Darwin as a mobile "influence" which necessarily translocates from the tips of stem and root, which sense the direction of light and gravity sources, respectively, to the bending or differentially growing part of the organs. Forty-eight years later, the moving "influence" materialized into "auxin" (Went 1928). Since Darwin's prediction, it took more than 100 years to identify the auxin-transporting proteins (Bennett et al. 1996; Chen et al. 1998; Gälweiler et al. 1998; Luschnig et al. 1998). However, over the past decade since the discovery of auxin transporters, ~350 research papers on the major auxin transporters have been published, and now we have a much more detailed and enriched picture of plant developmental mechanism, illustrating at the cellular and molecular level how the embryonic apical/basal axis forms, how the leaf regularly emerges from the plane shoot apical dome, how fate determination and emergence of a lateral root occur in the pericycle cell, how organs under tropism acquire auxin gradients across them, and many other auxin-mediated developmental processes.

The Darwinian auxin transport is achieved by cell-to-cell movement of auxin, which requires both influx and efflux carriers in the plasma membrane. Three major families of auxin-transporting proteins have been so far studied: AUXIN-RESISTANT1 (AUX1)/AUX1-LIKEs (LAXs) for auxin influx and PIN-FORMEDs (PINs) and several ATP-binding-cassette B (ABCB)/P-glycoprotein (PGP) members for auxin efflux (for a recent review, see Grunewald and Friml 2010). A recent study has added a new group of membrane proteins, PIN-LIKES (PILS), as auxin transporters, which mediate intracellular auxin transport (Barbez et al. 2012). As the major natural auxin, indole-3-acetic acid ($pK_a = 4.75$) is

predominantly ionized in the cytosol, the activity of efflux carriers is essential for the cell-to-cell auxin movement. In particular, PIN proteins play the critical role for the directionality of auxin movement (so the formation of local auxin gradients) because they asymmetrically localize in the plasma membrane and dynamically change their intracellular distribution in response to developmental and environmental signals (Grunewald and Friml 2010). In this chapter, we introduce the molecular properties of different auxin-transporting proteins and their regulatory mechanisms.

## 2 AUX1/LAXs

Although a portion of apoplastic auxin is lipid permeable, the majority are in their ionized form, indicating that influx carriers can facilitate uptake of auxin into the cytoplasm. AUX1/LAXs, a small conserved group of amino acid/auxin permease-like proteins with >60 % amino acid identity and 11 transmembrane spans (Kerr and Bennett 2007), have been identified as auxin influx carriers in plants and heterologous systems (Yang et al. 2006; Péret et al. 2012). AUX1/LAXs have been implicated in auxin-related biological functions such as root gravitropism, lateral root formation, root hair growth, and vascular patterning (Bennett et al. 1996; Cho et al. 2007b; Swarup et al. 2008; Jones et al. 2009; Péret et al. 2012). While the localization of AUX1 is either polar or nonpolar depending on cell type, for example, apical in the protophloem cells and basal in the lateral root cap cells but no polarity in the columella cells (Bennett et al. 1996; Swarup et al. 2001), LAXs do not show particular polar localizations (Swarup et al. 2008; Péret et al. 2012). AUX1 is thought to function for long-distance auxin transport from the shoot to the root through the vasculature, and LAXs seem to primarily maintain local auxin gradients (Swarup et al. 2002, 2004, 2008).

AUX1 exhibits subcellular localization not only in the plasma membrane but also in internal compartments such as Golgi and endosomes (Kleine-Vehn et al. 2006). A fungal toxin brefeldin A (BFA) inhibits recycling of PINs, which requires the BFA-sensitive ADP-ribosylation factor-GDP/GTP exchange factor (ARF-GEF)-mediated vesicle trafficking (Donaldson and Jackson 2000; Geldner et al. 2001). However, in the case of AUX1, BFA is inhibitory for its internal trafficking but not for its trafficking to the plasma membrane, suggesting that BFA-insensitive ARF-GEFs are implicated in AUX1 trafficking to the plasma membrane (Kleine-Vehn et al. 2006). The plasma membrane targeting of AUX1 requires AUXIN-RESISTANT 4 (AXR4), an endoplasmic reticulum (ER) chaperone, whereas PINs' trafficking does not need the protein (Dharmasiri et al. 2006). These studies collectively demonstrate the existence of different trafficking tools and pathways between AUX1/LAXs and PINs, reflecting the need of diversified regulatory mechanisms between auxin influx and efflux.

Although they all show auxin influx activities, AUX1/LAXs seem to have different regulatory mechanisms and biological functions (Péret et al. 2012). Not only their expression patterns and mutant phenotypes are unique to each member, but also the regulatory mechanism for their intracellular trafficking differs between

the members. For example, while they are properly targeted to the plasma membrane in their own expression domains, LAX2 and -3 fail to be targeted to the plasma membrane in AUX1-expressing tissues (Péret et al. 2012). This indicates that there are molecular cues in each influx carrier and some cell type-specific regulators operate for intracellular trafficking of each AUX1/LAX species. Péret et al. (2012) proposed that each AUX1/LAX-specific ER chaperone may exist for the specific trafficking as shown for the case of AXR4-mediated AUX1 trafficking (Dharmasiri et al. 2006).

A couple of recent studies have demonstrated that organ-level signals are implicated in regulation of AUX1-mediated auxin transport activity. Shoot-supplied ammonium inhibits lateral root initiation. Because AUX1 is required for lateral root formation and shoot-supplied ammonium suppresses AUX1 expression in vascular tissues, ammonium in the shoot seems to modulate lateral root number by controlling AUX1-mediated shoot-to-root auxin transport (Li et al. 2011). Shoot-applied iron also facilitates lateral root formation by inducing AUX1 expression in the lateral root, which increases auxin supply in the lateral root for its initiation and elongation (Giehl et al. 2012). The promoter analysis of the AUX1 gene and following search for the upstream factors would reveal diverse regulatory pathways for AUX1-mediated auxin transport in response to various internal and external signals.

## 3 ABCBs

Among 21 ABCB members, ABCB1, ABCB4, and ABCB19 have been implicated in auxin transport (Noh et al. 2001; Multani et al. 2003; Geisler et al. 2005; Terasaka et al. 2005; Cho et al. 2007b). In plant cells, these three ABCBs play mainly as auxin efflux transporters (Geisler et al. 2005; Cho et al. 2007b; Kubes et al. 2012). Auxin-transporting (AT) ABCBs consistently localize to the plasma membrane mainly with no polarity but with polarity in certain cells (Geisler et al. 2005; Terasaka et al. 2005; Blakeslee et al. 2007; Wu et al. 2007; Cho et al. 2007b, 2012; Mravec et al. 2008). Being predominantly nonpolar, the AT-ABCBs may control the auxin amount available for PIN-mediated polar auxin transport (Mravec et al. 2008). Several lines of evidence have pointed toward a direct interaction between AT-ABCBs and PINs in coordinating polar auxin transport (Bandyopadhyay et al. 2007; Blakeslee et al. 2007). Genetic interactions between AT-ABCBs and PINs also have been shown to be important for both embryogenesis and organogenesis (Mravec et al. 2008). These studies suggest that AT-ABCBs together with PINs may also play the major role for cellular auxin efflux or play alone for local auxin loading for PIN-mediated long-distance auxin transport (Bandyopadhyay et al. 2007; Blakeslee et al. 2007; Bailly et al. 2008).

## 4 ABCB Endocytosis

AT-ABCBs generally more stably reside in the plasma membrane than PINs. Among three AT-ABCBs, ABCB19 and ABCB4 show more stable plasma membrane localization compared with ABCB1, which shows some endocytic movement (Blakeslee et al. 2007; Titapiwatanakun et al. 2008; Cho et al. 2012). Fluorescence recovery after photobleaching (FRAP) experiments have revealed that ABCB4 and ABCB19, stably anchoring to the plasma membrane, seldom recycle between the endosome and the plasma membrane (Titapiwatanakun et al. 2008; Cho et al. 2012). In contrast to PINs whose polarity is maintained by dynamic recycling, the nonpolarity of AT-ABCBs may not require the active recycling. The difference in BFA sensitivity also shows the diverged intracellular trafficking pathways between different AT-ABCBs. ABCB19 was shown to be more resistant to BFA than did ABCB1 and ABCB4, consistently with the observation that ABCB19 takes GNOM-independent but GNL1-dependent trafficking mechanism (Titapiwatanakun et al. 2008; Cho et al. 2012; Wu et al. 2010). On the other hand, ABCB4 trafficking seems to be dependent on GNOM as well as other ARF-GEFs (Cho et al. 2012).

## 5 Phosphorylation-Mediated Regulation of ABCB Trafficking

Similarly to PIN proteins, AT-ABCBs also are regulated by AGC (protein kinase A, G, and C family) kinases (Christie et al. 2011; Henrichs et al. 2012). However, while the AGC kinases act as positive regulators for PIN activities, they either activate or deactivate the AT-ABCBs depending on the kinase-ABCB combinations and environmental conditions. For example, while PHOT1, the blue-light receptor AGC kinase, inhibits ABCB19 auxin efflux activity in the hypocotyl by directly phosphorylating ABCB19 in response to light (Christie et al. 2011), the PINOID (PID) AGC kinase directly phosphorylates ABCB1 to increase its efflux activity in protoplast and yeast assay systems (Henrichs et al. 2012). The inactivation of ABCB19 by PHOT1 increases auxin levels in and above the hypocotyl apex to halt vertical hypocotyl growth in response to blue light (Christie et al. 2011). PHOT1 also regulates the direction of auxin flow during the hypocotyl phototropism where PHOT1 in response to light decreases PID transcription and in turn may affect the lateral PIN3 polarization as will be mentioned in the later section for PINs (Ding et al. 2011). Altogether, it seems that PHOT1 regulates simultaneously the ABCB19 activity and the PIN3 polarization in order to channelize auxin to the elongation zone of the hypocotyl upon phototropic stimuli and promote the hypocotyl bending toward the light source.

TWISTED DWARF1 (TWD1), an immunophilin-like protein, was found to directly interact with the C-terminal end of ABCB1 and ABCB19, and the loss-of-function *twd1* mutant phenotypes are similar to those of the *abcb1*/*abcb19* mutant (Murphy et al. 2002; Geisler et al. 2003). Based on these results, TWD1 was

proposed to be an activator of the membrane-localized ABCB complexes by causing conformational changes in the ABCB proteins (Geisler et al. 2003; Bouchard et al. 2006; Bailly et al. 2008). In the *twd1* mutant, the plasma membrane localizations of ABCB1, ABCB4, and ABCB19 were significantly compromised, suggesting that TWD1 is also implicated in the trafficking of AT-ABCBs to the plasma membrane (Wu et al. 2010). As TWD1 has been demonstrated to be localized both in the plasma membrane and ER (Geisler et al. 2003; Wu et al. 2010), it is conceivable that ER-localized TWD1 functions for ABCB trafficking and plasma membrane-localized TWD1 modulates the ABCB activity.

It has been lately shown that TWD1 directly binds to PID, and this TWD1–PID interaction modulates the ABCB1-mediated auxin transport activity (Henrichs et al. 2012). In the absence of TWD1, PID (but not WAG1, a PID paralog) phosphorylates a single serine residue (634) in the ABCB1 linker domain and enhances ABCB1-mediated auxin efflux activity. Corresponding phospho-defective (S634A) and phospho-mimic (S634E) versions of ABCB1 also showed reduced and elevated auxin export activities, respectively. Conversely, the presence of TWD1 inhibits PID-induced enhancement of ABCB1-mediated auxin transport activity most likely by inhibiting PID-mediated phosphorylation of the S634 residue in the ABCB1 linker domain. PID phosphorylates the NSVSSPIMTR motif of ABCB1, and this motif bears no sequence similarity to the known phosphorylation motifs of PINs as will be mentioned later.

## 6 PINs

PIN genes have been found throughout the land plant lineage consistently with the presence of auxin signaling components and auxin responses in land plants (Krecek et al. 2009; Lau et al. 2009). The Arabidopsis PIN family consists of eight members, and their homologs in angiosperms can be clustered roughly into four subgroups: PIN1 and 2 subgroups; PIN3, 4, and 7 subgroups; PIN6 subgroup; and PIN5 and 8 subgroups. On the other hand, PINs from moss and Lycopodiopsida form outgroups in the molecular phylogeny (Krecek et al. 2009), indicating that the functional diversification among different PIN subgroups could have occurred during the angiosperm evolution.

All PINs have two sets of five transmembrane helices, each in the N-terminal and the C-terminal regions, which are connected by a varying length of central hydrophilic loop depending on PIN species (Krecek et al. 2009; Ganguly et al. 2010, 2012a, b). PIN1–4, 6, and 7 and their orthologs have a long hydrophilic loop (>300 residues, so-called long PINs), and PIN5 and 8 and their orthologs have a short hydrophilic loop (<50 residues, so-called short PINs). Long PINs generally localize in the plasma membrane in diverse cell types, whereas short PINs have been shown to localize predominantly in internal compartments such as ER (Krecek et al. 2009; Mravec et al. 2009; Ganguly et al. 2010; Dal Bosco et al. 2012; Ding et al. 2012). However, PIN8, when ectopically expressed in Arabidopsis root hair

cells and tobacco Bright Yellow (BY)-2 cells, showed a dual localization pattern in both the plasma membrane and ER-like compartments, which is contrasted with the observation that PIN5 consistently localized only to the ER-like compartment in both cell types (Ganguly et al. 2010). These observations suggest that the hydrophilic loop of PINs includes certain molecular cues for intracellular PIN trafficking and those cues could be operational in a cell type-dependent manner. Because the short PINs show the partial plasma membrane targeting depending on cell type, the short hydrophilic loop of short PINs might carry limited molecular code for plasma membrane trafficking. On the other hand, the hydrophilic loop of long PINs includes diverse molecular cues for clathrin-mediated endocytosis, ubiquitylation, and phosphorylation which collectively modulate trafficking, stability, and subcellular polarity of long PINs (Grunewald and Friml 2010; Kleine-Vehn et al. 2011; Ganguly et al. 2012b; Leitner et al. 2012).

Most long PINs localize asymmetrically to certain plasma membrane side of the cell, which necessarily generates the directional flow of auxin and the formation of auxin gradients. Most long PINs such as PIN1, 3, 4, and 7, localizing at the basal (toward the root tip) side, contribute for the downward auxin flow in the root (Friml et al. 2002a, b; Blilou et al. 2005). Apically localized PIN2 in root epidermal cells transports auxin from the tip to the upper part of the root (Luschnig et al. 1998; Müller et al. 1998). Laterally localized PIN3 in the pericycle and basally localized PIN2 in the cortex contribute for redirecting auxin back to the root meristem (Blilou et al. 2005). Redistribution of PIN3 and PIN7 in the columella upon gravity vector changes plays a role for gravitropic bending of the root (Friml et al. 2002b; Kleine-Vehn et al. 2010). During phototropism of the hypocotyl, the abundance of PIN3 in the outer lateral membrane of the endodermal cell in the illuminated hypocotyl side is decreased so as to transport auxin to the shaded side of the hypocotyl (Ding et al. 2011). PIN1 in the shoot apical cells localizes at the membrane toward the incipient leaf primordium, establishing auxin maxima for the initiation of leaf development (Reinhardt et al. 2003). Mutant phenotypes of PINs reflect the biological role of each PIN's subcellular polarity in plant development. The Arabidopsis *pin1* mutant, as the name of PIN-FORMED originated, lacks lateral organs in the inflorescence stem (Okada et al. 1991). Loss of PIN2 causes defects in root gravitropism and root hair growth (Chen et al. 1998; Luschnig et al. 1998; Müller et al. 1998; Utsuno et al. 1998; Cho et al. 2007a). Consistently with their expression and dynamics in polarity changes responding to gravity, PIN3 and PIN7 are required for root gravitropism (Friml et al. 2002b; Kleine-Vehn et al. 2010), and phototropism is partially defective in the *pin3* mutant (Friml et al. 2002b).

Internally localized short PINs seem to be implicated in internal relocation of auxin such as sequestration, nuclear transport, and compartmentalization of active auxin or auxin derivatives (Mravec et al. 2009; Ganguly et al. 2010; Dal Bosco et al. 2012). In contrast to long PINs, the mutant phenotypes of short PINs are difficult to be inferred from their internal localization and probable function. *pin5* mutants are defective in hypocotyl and root growth as well as lateral root formation (Mravec et al. 2009), and loss of PIN8 causes defects in pollen germination but with normal pollen tube growth (Ding et al. 2012). Ectopic overexpressions of PIN8 (PIN8ox)

and PIN5 (PIN5ox) show opposite effects: auxin export, longer hypocotyl and root, and inhibition of root hair growth in PIN8ox versus auxin import, shorter hypocotyl and root, and stimulation of root hair growth in PIN5ox (Mravec et al. 2009; Ganguly et al. 2010; Ding et al. 2012). These results are difficult to be explained because both PIN5 and PIN8 seem to be localized mainly in the ER, but the auxin responses mediated by these two short PINs are opposite. In the root hair cell, because PIN8 is also localized in the plasma membrane, PIN8ox could decrease the auxin response of the root hair by lowering cellular auxin levels (Ganguly et al. 2010). In contrast, higher auxin responses by PIN8ox in the root meristem and hypocotyl (Ding et al. 2012) may take place via more complicated processes.

## 7 Modulators for the Regulation of PIN Polarity

The subcellular polarity of PINs is not determined by polar trafficking of newly synthesized proteins but by polar recycling of preexisting proteins in the plasma membrane (Dhonukse et al.2008). There are three major intracellular PIN trafficking pathways (1) ER → Golgi → trans-Golgi network (TGN)/early endosome (EE) → plasma membrane, for the exocytic pathway of newly synthesized PINs, (2) plasma membrane → TGN/EE → recycling endosome (RE) → plasma membrane, for recycling of preexisting PINs, and (3) plasma membrane → TGN/EE → pre-vacuolar compartment (PVC)/multivesicular body (MVB) → vacuole, for the vacuolar lytic pathway (Grunewald and Friml 2010). Here, we will focus on the several regulatory players for the polar recycling pathway of PINs.

### *7.1 ARF-GEFs and Their Modulators*

The first characterized regulators for polar PIN trafficking are ARF-GEFs. The fungal toxin BFA, an ARF-GEF inhibitor, blocks RE → plasma membrane exocytic and TGN/EE → PVC/MVB lytic PIN trafficking resulting in PIN accumulation in "BFA compartments" inside the cell (Geldner et al. 2001; Kleine-Vehn et al. 2008a). Conversely, the plasma membrane → TGN/EE endocytic process of PIN is not affected by BFA (Geldner et al. 2003). The basal PIN targeting is regulated by GNOM, a BFA-sensitive ARF-GEF (Steinmann et al. 1999; Geldner et al. 2001, 2003). The basal PIN1 localization was compromised in the *gnom* loss-of-function mutant, while apically localized PIN2 and AUX1 remained mostly unaffected (Steinmann et al. 1999; Kleine-Vehn et al. 2006, 2008a). Moreover, the response to BFA was comparatively much stronger in PIN1 than PIN2 and AUX1, suggesting that the apical localization of certain membrane proteins is mainly controlled by BFA-resistant ARF-GEFs (Kleine-Vehn et al. 2006, 2008a). GNOM-like (GNL) ARF-GEFs also are implicated in PIN trafficking. GNL1, a BFA-insensitive ARF-GEF, is important for ER → Golgi trafficking (Richter et al. 2007) and

interestingly mediates PIN2 endocytosis rather than exocytosis (Teh and Moore 2007). GNL2 is required for germination and tip growth of the pollen (Jia et al. 2009; Richter et al. 2011) and functionally compatible with GNOM in PIN1 recycling from endosome to (basal) plasma membrane (Richter et al. 2011).

A small GTPase ARA7 (a Rab5-GTPase) and its regulator VPS9A play for endocytosis-mediated PIN polarization (Dhonukshe et al. 2008). In contrast to GNOM, which is predominantly implicated in basal PIN recycling, ARA7 and VPS9A seem to regulate the endocytosis of both apical and basal PINs. ARA7 has been demonstrated to localize in several intracellular compartments such as early/late endosomes, multivesicular bodies, and tonoplast but not in the plasma membrane (Ueda et al. 2004; Haas et al. 2007; Ebine et al. 2011). Hence, it may not be directly involved in PIN endocytosis from the plasma membrane. However, because the PIN polarity, but not its PM localization, is defective in the dominant negative ARA7-expressing plant, ARA7 may indirectly regulate PIN endocytosis (Dhonukshe et al. 2008).

## *7.2 PIN Phosphorylation*

Protein phosphorylation has long been implicated in regulation of auxin transport. In tobacco suspension cells, protein kinase inhibitors inhibited cellular auxin efflux and phosphatase inhibitors inhibited both efflux and influx of auxin (Delbarre et al. 1998). Arabidopsis protein phosphatase 2A (PP2A) was shown to have a negative effect on auxin transport (Rashotte et al. 2011), and the mutations of PID have been related with defects in auxin transport (Bennett et al. 1995; Benjamins et al. 2001). Consistently with these previous studies, PID was shown to play as a positive effect or for auxin efflux transport in Arabidopsis root hair cells and tobacco BY-2 cells (Lee and Cho 2006).

### 7.2.1 A Subset of AGC Kinases Phosphorylate the Hydrophilic Loop of PINs

A series of recent studies on phosphorylation of PINs have provided cellular and molecular mechanisms to understand auxin-mediated plant growth and development (for a recent review, see Ganguly et al. 2012a). The hydrophilic loop of PINs has recently been shown to be the direct phosphorylation target by a subgroup of AGC kinases (AGC1 or AGCVIIIa subgroup, Bögre et al. 2003). In vitro and transient protoplast phosphorylation assay systems, four members of AGC1 kinases, PID, WAG1, WAG2, and D6PK, were able to phosphorylate the hydrophilic loop of PINs (Michniewicz et al. 2007; Zourelidou et al. 2009; Huang et al. 2010; Dhonukshe et al. 2010; Ding et al. 2011; Ganguly et al. 2012b). PID-mediated phosphorylation of PIN1 was antagonized by PP2A (Michniewicz et al. 2007).

These studies have identified multiple phosphorylation motifs in the PIN hydrophilic loop. The serine residue in the "TPRXS" (where X is any amino acid residue)

motif of the PIN1 and PIN2 hydrophilic loop was shown to be phosphorylated by PID, WAG1, and WAG2 in vitro (Huang et al. 2010; Dhonukshe et al. 2010). The hydrophilic loop of long PINs carries three repeats of TPRXS motif, and phosphorylation of any one of these motifs seems to be enough for the biological function of PIN, indicating the functional redundancy among three TPRXS motifs (Huang et al. 2010). Recently, another phosphorylation motif, RKSNASRRSF(/L) (called "3m1" motif), has been identified from the PIN3 hydrophilic loop by in vitro and *in planta* phosphorylation assays (Ganguly et al. 2012b). The 3m1 motif is located a few residues ahead of the first TPRXS motif (the "M3" motif together with 3m1), and the capacity for phosphorylation and biological function of the 3m1 motif was augmented when it operated together with the neighboring TPRXS motif. The 3m1 or M3 motif was shown to be the target of PID but not of WAG1. This study also showed that PID phosphorylates other residues in the PIN3 hydrophilic loop in addition to the M3 motif. The third phosphorylation motif, Ser337/Thr340, was biologically identified which is necessary for proper polarity and biological function of PIN1 (Zhang et al. 2010). However, this Ser337/Thr340 motif was not phosphorylated by PID, suggesting that other kinases are implicated in the phosphorylation of this motif.

Multiple phosphorylation motifs in the PIN hydrophilic loop and the target specificity of their kinases suggest the possibility that diverse combinatorial phosphorylation codes are operational to determine subcellular polarity of different PINs under different cellular conditions. These functionally characterized phosphorylation motifs in the hydrophilic loop have been largely conserved among long PINs even in moss and Lycopodiopsida (Huang et al. 2010; Ganguly et al. 2012b; our unpublished multiple-alignment analysis), implying that the phosphorylation-mediated regulatory tools for PIN polarity and directional auxin transport had emerged from the beginning of land plant evolution.

### 7.2.2 Phosphorylation of the PIN Hydrophilic Loop Modulates the Subcellular Polarity of PINs

Overexpression of PID led to the basal-to-apical polarity switch of PIN1, PIN2, and PIN4 in the Arabidopsis root (Friml et al. 2004). Consistently, PINs are preferentially targeted to the basal membrane in *pid* loss-of-function mutants (Friml et al. 2004), and the loss of PP2A leads to preferential apical PIN targeting in Arabidopsis embryos and roots (Michniewicz et al. 2007). These lines of evidence suggest that phosphorylated PINs are primarily recruited to the apical targeting pathway, whereas the dephosphorylated PINs are recruited to the basal targeting pathway (Michniewicz et al. 2007). PID, WAG1, or WAG2 is localized in the membrane with no particular polarity. These kinases phosphorylate the TPRXS motifs of PINs to lead them to the apical membrane, and phospho-defective mutations of these motifs result in basal targeting of PINs (Huang et al. 2010; Dhonukshe et al. 2010).

Prolonged BFA treatment causes apical-to-basal transcytosis of PIN1 (in the root epidermis) and PIN2 (in the root meristematic cortex) (Kleine-Vehn et al. 2008a, 2010; Rahman et al. 2010). These results suggest that distinctive ARF-GEFs are

implicated in apical-basal transcytosis of PINs: BFA-sensitive for basal and BFA-resistant for apical transcytosis (Kleine-Vehn and Friml 2008). Therefore, phosphorylation-mediated apical targeting and BFA-sensitive GNOM-mediated basal targeting are seemingly antagonistic to each other. A supporting observation for this notion is that loss of the phosphatase PP2A enhances the BFA-mediated apical targeting of cortical PIN2 (Rahman et al. 2010), indicating that both the defect of PIN dephosphorylation (thus more phosphorylated PINs) and the blocking of BFA-sensitive basal trafficking cause apicalization of PINs. Although the PIN localization had not been directly shown, AGC1 kinases are likely to redundantly operate for antagonizing BFA-sensitive ARF-GEF-mediated basal trafficking because the quadruple mutation of AGC1 kinases (PID, PID2, WAG1, and WAG2) was able to reduce the BFA sensitivity for root growth and gravitropism (Rahman et al. 2010). AGC1 kinases phosphorylate PIN proteins at the plasma membrane to lead them to the BFA-resistant apical targeting pathway (Kleine-Vehn et al. 2009; Dhonukshe et al. 2010), and compromised AGC1 activities lead to basal PIN targeting (Dhonukshe et al. 2010).

Three recent studies have suggested that PID also modulates lateral PIN polarity (Ding et al. 2011; Rakusová et al. 2011; Ganguly et al. 2012b). During phototropism of the Arabidopsis hypocotyl, PIN3 gradually disappears in the outer lateral membrane of the endodermal cell of the illuminated hypocotyls side, whereas PIN3 in the endodermal cell of the shaded side maintains apolar localization (Ding et al. 2011). PID is inhibitory to this light-mediated polarization of PIN3. Overexpression of PID strongly inhibits light-mediated inner lateral polarization of PIN3 in the endodermal cell of the illuminated side thereby making the hypocotyl nonresponsive to the light (Ding et al. 2011). Similarly, during gravitropism of the Arabidopsis hypocotyl, PIN3 polarizes to the lower side of the hypocotyl endodermal cell, and PID overexpression inhibits this PIN3 polarization (Rakusová et al. 2011).

In the Arabidopsis root pericycle cell, PIN3 localizes to the inner (toward the vasculature) and basal (toward the root tip) membranes (Blilou et al. 2005). Phospho-defective mutations of the 3m1 (RKSNASRRSF(/L)) or M3 (RKSNASRRSF(/L) + TPRPSNL) motif of the PIN3 hydrophilic loop, which are the targets of PID, made PIN3 apolar in the pericycle cell and failed to rescue the *pin3* mutant phenotypes such as shorter roots and defects in root gravitropism (Ganguly et al. 2012b). These observations demonstrate that PID is inhibitory in polar localization of PIN3 in the hypocotyl endodermis, whereas it is promotive for polarization of PIN3 in the root pericycle. It would be interesting to know whether the phosphorylation motif like 3m1 or M3 is also implicated in lateral PIN3 localization in the hypocotyl endodermis.

Phosphorylation-mediated intracellular trafficking and polarity determination of PINs seem to depend on certain cell type-specific factors. Phospho-defective mutations in the M3 motif of the PIN3 hydrophilic loop led PIN3 localization to the tonoplast in the root hair cell, whereas the same mutations led PIN3 localization normally to the plasma membrane but with no polarity in the root pericycle cell (Ganguly et al. 2012b). Interestingly, the basal PIN3 localization in the root vasculature remained unaffected by the M3 mutations (Ganguly et al. 2012b). These results

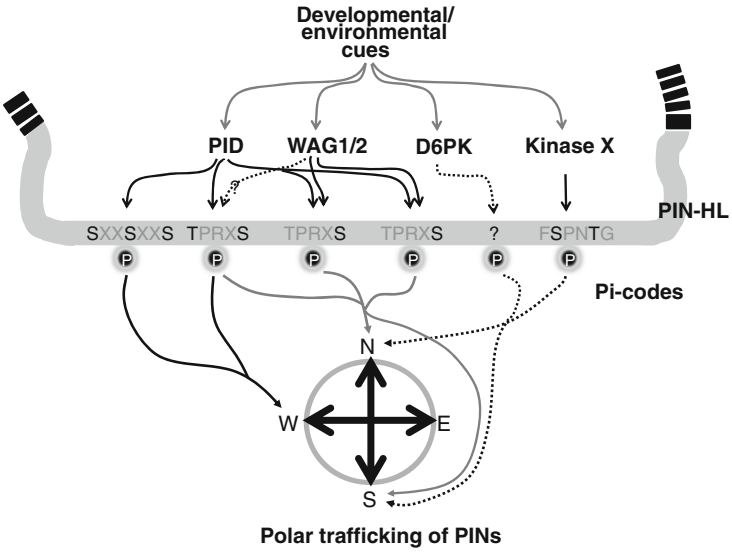


**Fig. 2** Phosphorylation codes in the PIN hydrophilic loop (HL) modulate PIN polarity. Developmental and environmental cues may regulate different protein kinases which in turn generate different phosphorylation codes on the PIN-HL for the specific PIN polarity depending on cell type and PIN molecule

suggest that the M3 phosphorylation code of PIN3 is interpreted as a polarity determinant in the root pericycle cells but is recognized primarily as a plasma membrane-targeting cue in the root hair cell.

As mentioned previously, multiple phosphorylation sites have been functionally identified in the PIN hydrophilic loop, and more sites would possibly exist. Some phosphorylation sites have been conserved among different PIN species, but those phosphorylation sites also show some diversity in their sequence structures. Different PINs show different subcellular polarity. Furthermore, cell-type specificity, stimuli responsiveness, and the existence of multiple protein kinases are added to this complexity. Those multiple phosphorylation motifs with some contextual diversity and multiple kinases may play combinatorially to generate a specific phosphorylation code for cell type-, stimuli responsiveness-, or molecule-specific trafficking and polarization of PINs (Fig. 2). So far, the downstream trafficking or polarity factors of phosphorylated (or dephosphorylated) PINs have remained to be identified. These downstream factors, together with the upstream components, would additionally contribute for the diversity of PIN polarization signaling.

### 7.2.3 Upstream Regulatory Pathways for PIN Phosphorylation

Two PID-interacting proteins have been identified: TCH3 (TOUCH3, a calmodulin-like protein) and PBP1 (PID-BINDING PROTEIN1, an EF-hand motif protein) (Benjamins et al. 2003). The interaction between these proteins and PID requires

calcium ions, and these proteins are likely to function upstream of PID. The autophosphorylation activity of PID was shown to be enhanced by PBP1 (Benjamins et al. 2003). However, the *in planta* functions of these PID-interacting proteins in PID-mediated regulation of auxin transport still remain to be elucidated. The PID kinase activity is also boosted by 3-phosphoinositide-dependent protein kinase-1 (PDK1) which directly phosphorylates PID in vitro (Zegzouti et al. 2006). In this context, a recent study has implicated the inositol triphosphate (InsP3)-mediated calcium signaling in regulation of PIN polarity (Zhang et al. 2011). Increases of InsP3-mediated calcium signaling disrupted basal polarity but did not affect apical polarity of PIN localization. High levels of InsP3 and calcium also reduced PID overexpression-induced root collapse and strengthened cotyledon-defective phenotypes of the *pid* loss-of-function mutant. Although the mechanism how InsP3-mediated calcium signaling regulates the PID activity remains to be elucidated, this observation adds another evidence for the connection between InsP3-mediated calcium signaling and PID-regulated PIN polarity.

## *7.3 Membrane Lipid Composition*

The lipid composition of the plasma membrane is important for movement and polar localization of membrane proteins (Brown and London 2000). Defects in sterol-biosynthetic enzymes such as sterol methyltransferases (SMTs) and cyclopropylsterol isomerase1 (CPI1) disrupt particularly the polar localization of PINs and cause defects in auxin-related development such as cell polarity and embryogenesis (Willemsen et al. 2003; Men et al. 2008; Carland et al. 2010). PIN proteins localize to the newly formed cell plate of the dividing cell (Geldner et al. 2001; Dhonukshe et al. 2006). After cell division, the polar PIN protein like PIN2 needs to be removed from one side of the membrane in order to retain its usual apical polarity. In the *cpi1-1* mutant, this removal of PIN2 from one side of the daughter membrane is inhibited, and PIN2 is retained on both sides of the newly formed daughter cell plasma membrane (Men et al. 2008). Moreover, sterols co-localize with PIN2-positive endosomes and undergo BFA-sensitive and actin-mediated endocytic trafficking thereby indicating a sterol-mediated process in PIN polarity regulation (Grebe et al. 2003). Likewise in animals, the plant plasma membrane also harbors certain microdomains which are rich in sterols, detergent resistant, and implicated in plasma membrane-related signaling for endocytosis (Mongrand et al. 2004; Borner et al. 2005; Sharma et al. 2002; Titapiwatanakun and Murphy 2009). Although sterols are obviously involved in regulation of PIN polarity, the mechanism remains to be elucidated.

In addition to sterols, the metabolism of membrane phospholipids also is implicated in PIN trafficking. Phospholipase A2 (PLA2) is a fatty acyl hydrolase of membrane phospholipids. Pharmacological or genetic suppression of PLA2 interrupts the plasma membrane localization of PINs, causing their internalization (Lee et al. 2010). Although the mechanism how PLA2 regulates PIN trafficking is

currently speculative, the phospholipase, localizing in the Golgi, might be involved in modification of membrane phospholipids for the cargo-vesicle trafficking from Golgi to the plasma membrane.

## 7.4 Ubiquitylation

In addition to synthesis, secretion, phosphorylation, endocytosis, and recycling/transcytosis, degradation has been another orchestrating member for regulation of polar PIN localization. So far only PIN2 degradation by ubiquitin-mediated proteolysis has been studied in detail. A proteasome inhibitor MG132 is capable of blocking both internalization and degradation of PIN2, indicating that the proteasome activity is involved in these processes (Abas et al. 2006). Ubiquitylated PIN proteins in the plasma membrane have been shown to destine to the lytic vacuole (Abas et al. 2006; Laxmi et al. 2008; Kleine-Vehn et al. 2008b). A recent study has directly demonstrated that lysine63-linked polyubiquitin chain formation is involved in the vacuolar targeting and proteolytic degradation of PIN2 (Leitner et al. 2012). This study tested 22 out of 28 lysine residues in the PIN2 hydrophilic loop and found that multiple mutations (Lys to Arg) of 12 or 17 residues together were required to generate ubiquitylation-defective PIN2. Mutations of these lysine residues stabilized PIN2 in the plasma membrane and interfered with its vacuolar targeting and degradation (Leitner et al. 2012).

## 7.5 ABP1 and ROPs

Auxin promotes its own efflux from the cell by inhibiting endocytosis of PINs and AT-ABCBs, thereby increasing the abundance of these efflux transporters in the plasma membrane, and this process is independent of the TRANSPORTER INHIBITOR RESISTANT1/AUXIN-BINDING F-BOX-mediated nuclear auxin signaling (Paciorek et al. 2005; Cho et al. 2007b). Recent studies have shown that this auxin effect on PIN endocytosis is mediated by another auxin receptor AUXIN BINDING PROTEIN1 (ABP1). ABP1 mainly localizes in the ER but also partly localizes in the apoplast (Jones and Herman 1993; Tian et al. 1995; Henderson et al. 1997). Recently, apoplastic ABP1 has been found to regulate clathrin-mediated PIN endocytosis. Auxin binding to ABP1 inhibits the clathrin-dependent PIN endocytosis thereby leading to a high PIN abundance in the plasma membrane (Robert et al. 2010).

Currently, it remains to be elucidated how apoplastic ABP1 can regulate the clathrin-mediated endocytosis which basically occurs in the cytosolic side. It has recently been speculated that ABP1 might act through a transmembrane protein to transmit the signal from the extracellular space to the intracellular cytosol (Shi and Yang 2011). ABP1 may regulate endocytosis by affecting the phosphorylation

status of cargo proteins such as PINs or core components for the clathrin-mediated endocytic process. Future studies to identify certain ABP1-interacting proteins in the apoplast should shed light on the process of auxin-mediated inhibition of endocytosis. On the other hand, the role of ER-localized ABP1 remains unknown. Given the fact that membrane proteins like PINs are synthesized in the ER and secreted to the plasma membrane, it is tempting to speculate that ER-ABP1 may also play a role for PIN trafficking from ER.

Although the direct interacting partner of ABP1 remains elusive, several ABP1 downstream targets have been recently characterized. Xenbiao Yang's group has been studying the function of ROP (Rho of plant) small GTPases during interdigitated cell expansion of the Arabidopsis leaf epidermal pavement cells. Formation of lobes (tip) and indentations in the characteristic jigsaw puzzle-shaped pavement cells is coordinated by ROP2 and ROP6 (Fu et al. 2002, 2005). ROP2 is locally activated at the lobe-forming site and promotes cortical diffuse F-actin formation and lobe outgrowth by working with its effector ROP-interactive CRIB motif-containing protein 4 (RIC4) (Fu et al. 2005). In the lobe tip, ROP2 suppresses well-ordered cortical microtubule (MT) arrays by inactivating another effector RIC1, thus relieving MT-mediated outgrowth inhibition (Fu et al. 2002, 2005). In the opposing indenting zone, ROP6 activates RIC1 to promote MT organization and to suppress ROP2 (Fu et al. 2005, 2009).

Recently, ROP2 and ROP6 have been found to antagonistically control the formation of lobes and indentations in response to asymmetric auxin distributions (Xu et al. 2010). PIN1 proteins are polar-localized or more concentrated in the lobing plasma membrane than in the indenting membrane part of the pavement cell. This polar-localized PIN1 is thought to create local auxin accumulation in the lobe apoplast so as to cause binding of auxin to ABP1. Auxin-bound ABP1 is thereafter likely to activate the ROP2-RIC4 pathway in the lobe side to promote lobe formation. Conversely, auxin-free ABP1 activates the ROP6-RIC1 pathway in the indentation side of the adjacent cell, resulting in the development of interdigitated leaf pavement cells. ABP1-mediated activation of ROP2 seems to increase PIN1 abundance at the plasma membrane of the lobe side, which in turn leads to a positive feedback on ROP2-dependent formation of cortical diffuse F-actin.

Even though several lines of evidence have pointed toward an actin-dependent trafficking of PIN proteins (Geldner et al. 2001; Grebe et al. 2003), the question still remains how ROPs affect polar PIN1 localization in the pavements cells. It has been recently shown that the ROP2/RIC4-dependent auxin signaling pathway induces localized accumulation of cortical fine F-actin which inhibits clathrin-dependent PIN1-endocytosis and leads to PIN1 polarization (Nagawa et al. 2012), supporting the previous study where ROP2 was shown to regulate PIN2 polarization during gravitropic response (Li et al. 2005). However, the mechanism how local accumulation of actin can inhibit PIN endocytosis remains yet to be elucidated. In addition to the inhibition of PIN1 endocytosis, ROP2 signaling may also promote polar PIN1 recycling (Nagawa et al. 2012).

## 7.6 More Regulatory Signals for Auxin Transport

We have described diverse regulatory factors for auxin transport. In addition to these regulators, auxin transport is also modulated by many other factors such as plant hormones and other signaling molecules, nutrients, and even pathogens.

High cytokinin signaling in the root transition zone enhances *SHY2* (*SHORT HYPOCOTYL2*, encoding an AUXIN/INDOLE-ACETIC-ACID repressor) expression which in turn inhibits *PIN3* expression (DelloIoio et al. 2007, 2008). *PIN3* downregulation reduces auxin transport in the transition zone, ultimately leading to cell elongation and differentiation in the transition zone. Cytokinin regulates auxin transport not only through transcriptional regulation of *PIN* but also by promoting selective endocytosis and proteasome-mediated degradation of PIN1 in the Arabidopsis root and tobacco BY-2 cells (Marhavý et al. 2011). Cytokinin is also implicated in vascular development by promoting bisymmetric distribution of PIN proteins (Bishopp et al. 2011). Several studies have further demonstrated that cytokinin regulates the expression of *PIN* genes during root and other organ development (Laplaze et al. 2007; Ruzicka et al. 2009; Pernisová et al. 2009). Ethylene affects transcription as well as polar localization of PINs during Arabidopsis apical hook development (Zádníková et al. 2010). Brassinosteroid (BR) extends the *PIN2* expression domain from the root meristem to the elongation zone, resulting in enhanced root gravitropism (Li et al. 2005). This BR effect on root gravitropism might be achieved by way of ROP2 action because BR regulates the expression and subcellular localization of ROP2 and ROP2 modulates PIN2 polarity by affecting F-actin (Li et al. 2005; Lanza et al. 2012). Gibberellin has recently been shown to play as a positive factor to maintain PIN abundance most likely by modulating vacuolar degradation of PIN proteins (Willige et al. 2011). Jasmonic acid (JA) also has been known to regulate PIN trafficking. Low methyl JA (5 μM) inhibited PIN2 endocytosis through the TIR1/AFB-mediated auxin signaling, whereas higher (50 μM) methyl JA reduced PIN2 targeting to the plasma membrane in an auxin-independent manner (Sun et al. 2011).

Nitric oxide (NO), a regulator of plant development, decreases PIN1 protein levels in a proteasome-independent manner in the root meristem, which results in decrease of cell division and primary root growth (Fernández-Marcos et al. 2011). Furthermore, as previously mentioned, shoot-supplied ammonium and iron are involved in lateral root formation by affecting AUX1-mediated auxin transport (Li et al. 2011; Giehl et al. 2012). During the infection, plant-parasitic nematodes utilize the host PIN molecules, by modulating their gene expression and polarity, to direct auxin flow to the initial syncytial cell (Grunewald et al. 2009). A small group of transcription factors also regulate PIN expression for the proper leaf organogenesis in the shoot apex. Defects in three related PLETHORA-LIKE (PLT) AP2 transcription factors (PLT3, 5, and 7) cause alterations of phyllotaxis in Arabidopsis, and this is largely attributable to decreases in PIN1 levels in the shoot apical meristem (Prasad et al. 2011).

The diverse internal and external signaling pathways are operational in regulation of auxin transport, local auxin concentrations, and thus auxin-mediated developmental processes by affecting synthesis, trafficking, polarity, modification, and degradation of auxin-transporting proteins. Considering the broad and fundamental role of auxin in plant growth and development, it is natural to expect that such diverse signals interact to influence auxin transport and there could be more regulators to be identified. The regulatory process for the subcellular polarity determination of auxin transporters not only is important to understand how internal or environmental signals coordinate to affect plant development but also provides a model system to study how cellular polarity can be established in plants.

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