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REVIEW

Auxin-Signaling: Short and Long

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Long-standing major questions in auxin biology are now being answered through the latest discoveries and characterizations of auxin receptors and transporters. An F-box protein TIR1 and its close homologs are emerging as potent auxin receptors, which directly modulate the degradation of transcriptional repressors for auxin-responsive genes. The membrane proteins for polar auxin transport, intuited by Darwin almost 130 years ago, have been characterized over the past decade and implicated in diverse aspects of auxin-mediated plant development. This growth regulator is now considered to be a plant equivalent of morphogen because of how crucial the formation of its transporter-associated concentration gradient is to the patterning processes of plants. Such long-distance auxin-signaling from the source to the target cell via transporters has helped advance our understanding of plant development as a holistic system. Here, we summarize recent achievements in the study of molecular and long-distance signaling mechanisms for auxin, and discuss their biological meaning.

Keywords: auxin, auxin-signaling, morphogen, polar auxin transport, root hair

PINPOINTING DARWIN'S MOBILE STIMULUS

In 1880, Charles Darwin investigated the phototropic movement of canary grass coleoptiles and speculated on the existence of a signal that could transmit from one part of the plant to another (Darwin, 1880). Since a signaling molecule was first isolated as auxin in 1926 by Fritz Went (Went, 1926), an exponential expansion of knowledge has occurred in auxin biology. Both Darwin's insight into a movable signal and Went's discovery of that auxin molecule were derived from the same phenomenon of 'polar auxin transport (PAT)', namely, the downward movement of auxin from the shoot apex. Compared with the pace of earlier auxin research, the gains made in defining PAT and auxin-signaling at the molecular level have quickly accelerated our understanding because of the major achievements made just in the past decade, as described in many reviews (Blakeslee et al., 2005; Badescu and Napier, 2006; Geisler and Murphy, 2006; Leyser, 2006; Parry and Estelle, 2006; Quint and Gray, 2006; Scheres and Xu, 2006; Kerr and Bennett, 2007). Here, we focus primarily on the biological aspects of PAT as part of long-distance auxin-signaling during plant development.

The cell-to-cell movement of auxins and their concentration-dependent manner of signaling have led researchers to make analogies with morphogen in animal models (Baluska et al., 2003; Bhalerao and Bennett, 2003; Leyser, 2005; Esmon et al., 2006). Two criteria are required when defining a morphogen: concentration-dependent action through diffusive movement from the source, and direct transfer to the target cells/tissues (Turing, 1952). However, the now-expanded concept of morphogens in animals accommodates diverse means for the formation of a concentration gradient (Bhalerao and Bennett, 2003). Auxin satisfies the current concept in that it forms a gradient via PAT, which in turn is linked to downstream gene expression and developmental processes.

The formation of such a gradient by vectorial auxin transport has long been implicated in various aspects of development, including primary axis formation, tropic movements in the shoots and roots, lateral organ formation, wood-patterning, and other organogenesis. Studies of mutants have led to discoveries of the key auxin-transporting components. Auxin carriers are transmembrane proteins that mediate the influx or efflux of auxin at the plasma membrane. Bacterial amino acid permease-like AUX1 and its homolog LAXs (LIKE AUX) are known as auxin influx carriers, while PINs (PIN-FORMED) and p-glycoproteins (PGP1 and PGP19) act as efflux carriers. Both AUX1 and PINs show clear, asymmetric sub-cellular localization in certain tissue types, thereby providing a mechanism for polar auxin movement from the source to the target cells. Protein phosphorylation is involved in the regulation of this localization and the activity of PINs (Friml et al., 2004; Lee and Cho, 2006).

Our understanding of how auxin-signaling relays into the target cells has been greatly deepened by the recent discovery of auxin receptors (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Auxin directly adheres to a small group of F-box proteins (TIR1 and its homologs) to mediate the interaction between those F-box proteins and the transcriptional repressors whose degradation is required for auxin responses.

In this review, we briefly introduce the molecular mechanism for a cellular auxin-signaling process that is perceived and mediated by the F-box proteins. We also provide more detail about the biological roles of auxin transporters and their regulators.

CELLULAR SIGNALING OF AUXIN

Molecular signaling of auxin in a cell, namely from auxin to gene expression, is much shorter than previously thought.

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The auxin receptor, discovered independently in laboratories led by Ottoline Leyser and Mark Estelle, directly interacts with a family of transcriptional repressors.

AUX/IAAs and ARFs

Most prominently responsive to auxin are three gene families: *SAUR*s (Small Auxin-Up RNAs), *GH3*s, and *Aux/IAAs* (Hagen and Guilfoyle, 2002). Their promoter regions contain one or more auxin-responsive elements (AREs; consensus='TGTCTC') (Hagen and Guilfoyle, 2002). Identification of AREs has led to finding the Auxin Response Factor 1 (ARF1), a transcription factor that binds to ARE (Ulmasov et al., 1997). In all, 23 *ARF* genes have now been identified in *Arabidopsis* (Liscum and Reed, 2002). ARFs are characterized by a conserved N-terminal ARE binding domain (DBD), a non-conserved regulatory middle region (MR), and a prevalently conserved C-terminal domain (CTD) that probably functions by forming a dimer in response to auxin concentrations. The MR region acts either as an activation domain (Q-rich) or as a repressor domain (S, SG, or SP-rich), depending upon its amino acid composition (Tiwari et al., 2003). *Aux/IAA* genes are early auxin-response genes that encode short-lived nuclear proteins with four conserved domains, referred to as I, II, III, and IV. *Arabidopsis* Aux/IAA proteins function as transcription repressors, as seen in protoplast transfection assays of auxin-responsive reporter genes (Tiwari et al., 2001). These ARFs and Aux/IAAs form homo- and hetero-dimers both within and between families (Kim et al., 1997; Fig. 1). The *Arabidopsis* Aux/IAA protein family contains 29 members (Liscum and Reed, 2002). Domain II and Domains III and IV play roles in protein stability and dimerization, respectively. Domain I, which possesses a conserved LxLxL motif, is believed to be an active repressor domain that is transferable and dominant over the activation domains (Tiwari et al., 2004).

Aux/IAA degradation and auxin receptors

Aux/IAA proteins are degraded via the ubiquitin-mediated protein degradation pathway (Fig. 1). Aux/IAA proteins are ubiquitinylated by ubiquitin protein ligases (E3s) that further target them to the 26S proteasome-mediated pathway for degradation. One type of E3 ligase, SCF, comprises an SKP1, a Cullin, and an F-box protein (Deshaies, 1999; Petroski and Deshaies, 2005). The fourth element of SCF is the RBX1 protein, which acts as an ubiquitinylating protein-forming dimer with the Cullin proteins. This RBX1/Cullin complex is linked with the F-Box protein by SKP1. Nearly 700 F-box genes have been found in the *Arabidopsis* genome, compared with 24 in *Drosophila*, 14 in yeast, and 337 in *Caenorhabditis elegans* (Ruegger et al., 1998). F-box proteins interact with the SKP1 protein through a ~40 amino acid N-terminal F-box motif. The C-terminal end of the F-box proteins is responsible for recruiting the substrates that are subsequently ubiquitinylated by the SCF complex for degradation. One F-Box protein in *Arabidopsis*, identified as TIR1 (Transport Inhibitor Response 1), forms a SCF^{TIR1} complex and is required for auxin responses (Ruegger et al., 1998).

All the results from these studies have led researchers to

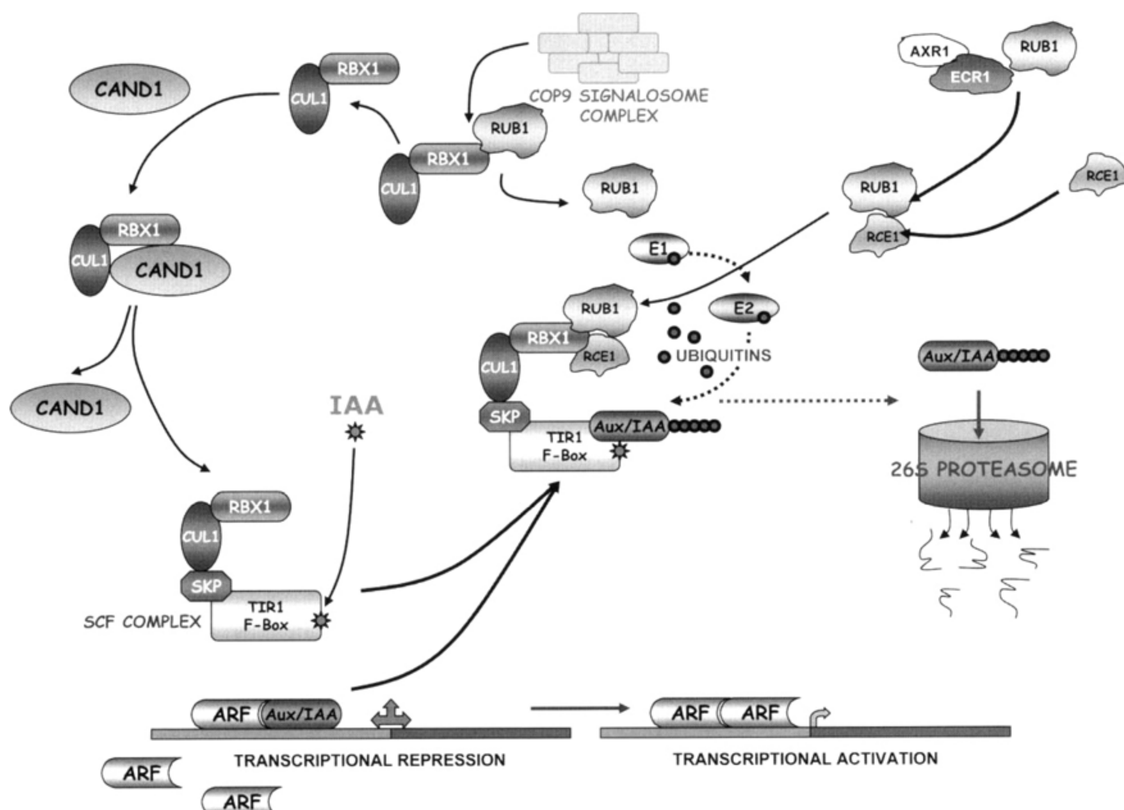

Figure 1. Auxin molecular signaling, from auxin perception by TIR1 F-box to degradation of Aux/IAAs by 26S proteasome. Details and abbreviations are included in the text.

propose that SCF^{TIR1} guides the repressors of auxin-signaling via ubiquitin-mediated degradation, and that this method is auxin-dependent. The interaction between Aux/IAA and TIR1 has been further proven in biochemical studies conducted by M. Estelle's group. They have shown that SCF^{TIR1} is needed for Aux/IAA degradation, that SCF^{TIR1} interacts with AXR2/IAA7 and AXR3/IAA17, and that Domain II of these proteins is necessary and sufficient for this interaction (Gray et al., 2001).

This SCF^{TIR1} protein complex also undergoes several post-translational modifications (Fig. 1). The CUL1 subunit of that complex is covalently altered by the RUB1 protein (related to the ubiquitin protein; also known as Nedd8 in some species), which gets attached to the CUL1 protein by a conserved lysine residue to become an active protein complex. In this case, RUB1 is activated by the E1 AXR1-ECR1 (AUXIN RESISTANT 1 -E1 C-TERMINAL RELATED 1) protein and E2 enzymes called RCE1 (RUB conjugating Enzyme 1) that form a thiolester linkage with RUB1. Here, RBX1 takes the role of E3 ligase. Each of these molecules is equally important for proper functioning of the SCF^{TIR1} complex, and defects in any of these genes leads to reduced activity (del Pozo and Estelle, 1999; Gray et al., 2001, 2002; Dharmasiri et al., 2003b).

The multi-protein complex of COP9 SIGNALOSOME (CSN) cleaves the RUB1 protein from the CUL1 subunit of the SKP complex (Fig. 1). In contrast, CAND1 (CULLIN-ASSOCIATED and NEDD8-DISSOCIATED 1) competes with SKP1 and only binds to the CUL1 that is not conjugated to NEDD8. This ensures negative control of the SCF ubiquitin ligases and serves as an important regulator for SCF complex assembly-disassembly (Schwechheimer et al., 2001; Petroski and Deshaies, 2005).

The discovery of auxin action in a cell-free system (Dharmasiri et al., 2003a) paved the way for SCF^{TIR1} to finally be identified as an auxin receptor (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Estelle's and Leyser's groups co-purified radio-labelled auxin with an association of Aux/IAA and SCF^{TIR1}, tagging both of them with epitopes for the pull-down assay.

Aux/IAA does not bind auxin directly, but the F-box is necessary to form the functional SCF complex, more specifically for TIR1-Aux/IAA interaction, which is stabilized by auxin-binding. The mechanism by which this auxin-TIR1-Aux/IAA complex is formed is still not understood and is under investigation (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). However, it is already quite clear that auxin directly binds to TIR1, forming a stable complex with the Aux/IAA proteins to mediate their degradation via ubiquitinylation. Therefore, this action is responsible for the consequent changes in expression of auxin-regulated genes (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005).

The related F-box proteins -- AFB1, AFB2, and AFB3 (Auxin Signalling F-box proteins) -- also contribute to auxin responses by regulating the degradation of Aux/IAA proteins. Further, genetic studies have indicated that AFBs and TIR1 have overlapping and redundant functions in embryogenesis and other developmental phases (Dharmasiri et al., 2005b). Analysis of the auxin response in TIR1- and AFB-deficient seedlings has revealed that these proteins have an essential role in auxin-signaling. As with TIR1, they interact with Aux/IAA proteins in an auxin-dependent manner. AFBs also bind auxin and act as auxin receptors. Thus, TIR1, along with the AFBs, serve as auxin receptors and collectively mediate auxin responses throughout diverse plant processes (Dharmasiri et al., 2005b).

AUXIN-BINDING PROTEIN 1 (ABP1), first detected in the binding of auxin in crude membrane preparations of etiolated coleoptiles, is named for its auxin-binding affinity (Hertel et al., 1972). In response to auxin, ABP1 acts as an activator of proton pump ATPase (Rück et al., 1993), protoplast swelling (Steffens et al., 2001), potassium channels (Thiel et al., 1993) and voltage-dependent anion channels (Zimmermann et al., 1994; Barbier-Brygoo et al., 1996), as well as cell elongation and division during embryogenesis (Chen et al., 2001). Biochemical studies have revealed ABP1 as an auxin-binding protein (Napier et al., 2002). However, its binding affinity (*Kd*) for the natural auxin IAA is much lower than that of TIR1, with values of 5 to 10 mM and 25 to 84 nM, respectively (Venis and Napier, 1995; Napier et al., 2002; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Even the synthetic auxin 1-naphthylacetic acid (1-NAA) seems to be a much better ligand for ABP1, with an affinity of 50 to 200 nM. The difference in auxin affinities between ABP1 and TIR1 presents the possibility that these two auxin receptors might take part in separate auxin-responsive pathways with different auxin sensitivities.

In terms of their biochemical properties and biological functions, TIR1 and its homologs seem to be representative auxin receptors in plants. Nevertheless, because distinctive receptors for the same ligand molecule have been reported in animal science, we may expect plants to also have multiple auxin-receptor systems. To confirm this, we must investigate further biological roles for ABP1 and TIR1-related receptors.

BIOLOGICAL ROLES OF LONG-DISTANCE AUXIN CARRIERS

AUX1 and its homologs

The natural auxin IAA is a weak acid (pKa 4.8) and, thus, it partly exists as a protonated form (IAAH) in apoplastic spaces where the pH is ~5.5. This non-polar form can enter the cell by diffusion, and is de-protonated in the cytosol, where the pH is higher (~7.0). Although auxin can simply diffuse into cells, carrier-mediated uptake seems to play a major role in auxin influx. A family of putative auxin influx carriers, identified in *Arabidopsis* as AUX1 and LAXs, has been suggested as being involved in active uptake (Bennett et al., 1996; Swarup et al., 2000). The first clue that AUX1 serves in auxin uptake was obtained from studies of the *aux1* mutant phenotypes, which are highly resistant to exogenous IAA and show agravitropism in their roots (Bennett et al., 1996). AUX1 expression in the root cap and elongating epidermal cells is consistent with IAA resistance and agravitropic phenotypes in *aux1* (Swarup et al., 2001).

Therefore, what is the role of AUX1 in the patterning of auxin accumulation that modulates plant development and

growth? Active auxin uptake against a diffusion gradient might retard the diffusive loss of auxin from the cell – this makes AUX1 a more efficient tool for amassing high levels of auxin in the target tissue. Yang et al. (2006) have demonstrated, using a *Xenopus oocyte* expression system, that AUX1 facilitates high-affinity auxin transport with substrate specificity. However, from a practical perspective, the IAAH diffusion process is too slow to have any developmental meaning. The defect of 'auxin maximum' in the *aux1* root apex is most likely linked to the phloem-unloading of auxin by AUX1 (Swarup et al., 2001). In addition, AUX1 expression and its asymmetric sub-cellular distribution, together with PIN2, in the elongating root epidermis suggest that AUX1 is responsible for uptake in the epidermis to mediate gravitropic root-bending (Swarup et al., 2005; Fig. 2B). Although its molecular properties are better understood, the developmental influence of AUX1-mediated auxin flow remains to be further characterized.

PINs

Most representative of long-distance polar auxin movement are the properties of PINs. In the whole-plant system, auxin flows from the young leaves and shoot apices all the way through the stem downward to the root apices before turning upward to the zone of root epidermal elongation (Fig. 2A, B). For asymmetric tropic-bending, auxin also moves laterally across the stem and root tissues. The expression patterns and sub-cellular localizations of PINs provide reasonable explanations for these holistic auxin-streaming networks. The most detailed model for the relationship between auxin flow and PINs has been established with the root meristem region. In the root tip, auxin, which has traveled from the shoot through the vascular parenchymal cells, accumulates at the quiescent center and columella root cap initials to form an 'auxin maximum'. Auxin leaves this maximum down through the columella and is transported up to the epidermis via the lateral root cap. This bidirectional transport and simultaneously formed auxin gradient contribute not only to the continuity of root meristematic activity but also to epidermal cell elongation and root hair differentiation. Auxin in the elongation zone then turns back to the quiescent center through the central tissues, completing its flow circuit in the roots. This circuit is thought to be important to the maintenance of root meristematic activity; this theory is supported by coordinated polar localizations and the expression patterns of individual PIN members within corresponding tissues (Blilou et al., 2005).

PIN proteins, distantly related to some bacterial transporters, are the major auxin efflux carriers. They have been studied primarily in *Arabidopsis*, which has eight PIN homologs, PIN1 to PIN8. All except PIN5 and PIN8, which lack the large hydrophilic middle domain, share a similar primary structure and putative topology in their membranes (Paponov et al., 2005). The name of PIN is derived from the knitting needle-like inflorescence phenotype of the *pin-formed1* (*pin1*) mutant (Okada et al., 1991). Each member of the PIN family shows a tissue-specific expression pattern, and their mutant phenotypes are consistent with developmental defects that can be caused by disruption of the corresponding auxin flow.

PIN1, localized on the lower cell face of xylem parenchyma cells in both stems and roots, is responsible for transporting auxin to the root tip (Fig. 2A, B). The PIN2 protein is expressed in the root epidermis and cortex, and localizes basally (toward the shoot/ root junction) in the epidermis, and apically (toward the root tip) in cortical cells around the meristematic and elongation zones (Luschnig et al., 1998;

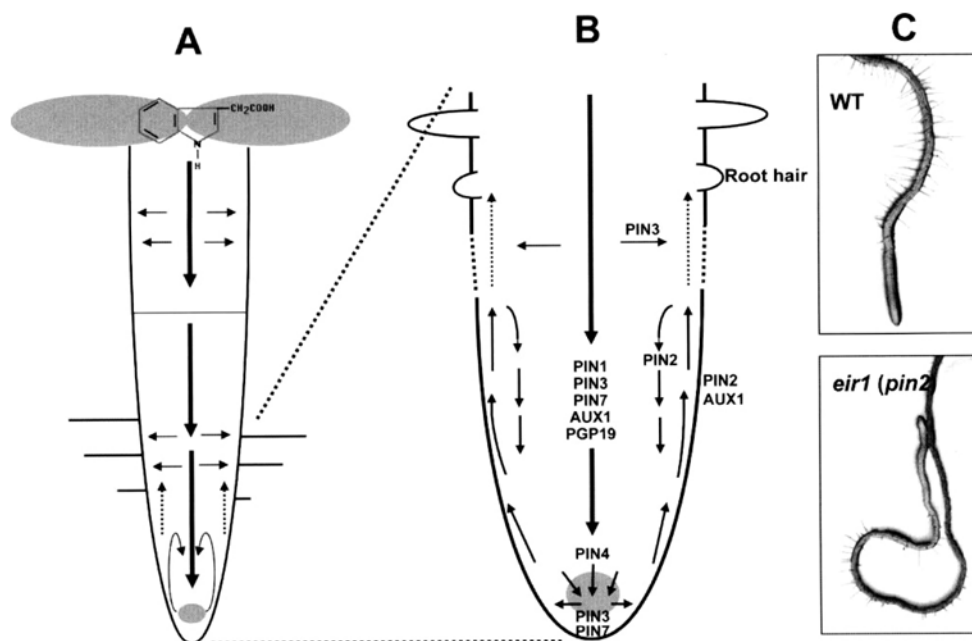

Figure 2. Long-distance auxin polar movement and auxin transporters. **(A)** Whole-plant-level auxin movement. Gray circle at root tip indicates 'auxin maximum' that is needed for maintaining root meristematic activity. **(B)** Auxin flows in root, and auxin transporters corresponding to flows. **(C)** Short root hair phenotype in loss-of-PIN2 (EIR1) mutant that is most likely due to insufficient auxin supply from root tip to hair-initiating epidermal cells in mutant root.

Müller et al., 1998). These localization patterns suggest a role for PIN2 in transporting auxin from the root tip to the elongation zone and returning to the root tip (Müller et al., 1998). PIN3 proteins are expressed in the root provascular cells, columella cells, pericycle, and stem starch sheath cells (Friml et al., 2002b). Their sub-cellular localization implicates them in tropic movements by the stem and root because these proteins are laterally localized in the pericycle and stem starch sheath cells. However, they do change their localization in columella cells upon gravitational stimulus. PIN4 is found in the quiescent center and its surrounding cells, and is apically localized to the provascular cells (Friml et al., 2002a). This protein assists in forming a high auxin concentration in the root meristematic cells. PIN7 is localized at the basal and lateral faces of provascular cells of the root meristem and elongation zone, and symmetrically in columella cells (Blilou et al., 2005). PIN6 is expressed in the lateral root primordia, and is involved in the formation of those roots (Benkova et al., 2003).

Dynamic sub-cellular localization of PIN proteins depends on the particular developmental stage. During early embryogenesis, these proteins determine auxin flow, thereby affecting the tissue pattern formation. At the four-cell stage, PIN1 locates laterally in the apical cells whereas PIN7 moves apically in the basal cells, resulting in auxin accumulation in the former. However, at the globular stage, auxin begins to be synthesized in the embryo and is transported reversely. A new 'auxin maximum' is then formed in the hypophyseal region (i.e., future quiescent cells), this action being mediated by PIN1 and PIN4. Interestingly, at this stage, PIN7 localization changes to the basal side, possibly mediating auxin transport from the embryo to the maternal tissue (Friml et al., 2003).

Studies of mutants have provided us with insight into the relationship between PINs and auxin distribution. Single mutants for PIN1, PIN2, and PIN3 show the respective phenotypes of pin-formed stems (Okada et al., 1991), waves and defects in both root gravitropism (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998) and root hair growth (Fig. 2C), and defects in stem and root gravitropism (Friml et al., 2002b). Paradoxically, however, these single PIN mutants show few phenotypic effects in their patterns of auxin distribution, most likely because the remaining PIN proteins are able to change their expression patterns to compensate for those losses (Leyser, 2006). In the *pin3pin4pin7* triple mutant, PIN1 expression is extended to the lateral-basal membrane of the endodermis, and PIN2 is also found at the basal end of the provascular cells where, normally, PIN3 and PIN7 would be expressed. The expression pattern of PIN4 takes over for that of PIN3 in the *pin3* single mutant and in the *pin2pin3* double mutant, which is observed in the three tiers of columella cells. In *pin3pin7*, PIN4 expression also expands to the lateral root cap (Blilou et al., 2005). Leyser (2006) has hypothesized that these dynamic alterations in auxin accumulation patterns trigger changes in PIN expression patterns to correct for auxin distribution patterns.

Environmental factors also can modulate the sub-cellular polarity of PINs to modify auxin accumulation patterns and, thus, the developmental responses to them. Gravitropism is a well-studied example. In the stationary state, PIN3 proteins spread symmetrically throughout the entire membrane of columella cells, which sense this stimulus. However, when the gravity vector changes, these proteins quickly re-localize asymmetrically to the membrane facing the new vector, resulting in a greater supply of auxin to the lower part of the root and an inhibition of cell elongation. The differential auxin accumulation between the lower and upper sides of the root is preceded by increased PIN2 degradation on the upper side of the lateral root cap and epidermis, which occurs in a proteasome-dependent manner (Abas et al., 2006). This degradation depends on intracellular molecular-trafficking, indicating the importance of vesicular-trafficking in the regulation of PIN-functioning and auxin accumulation patterns.

How do PIN proteins change their sub-cellular localization patterns? As described earlier, different PINs are expressed with cell-type specificity. When expressed ectopically in the root hair cells, they loose their polarity, showing symmetrical localization (Lee and Cho, 2006). This suggests that the cell type itself emanates unique determinants for the specific localization of PIN proteins. However, this polar localization can also be determined in a molecule-specific manner. When PIN1-HA (hemaglutinin) fusion proteins are expressed in the root epidermis under the *PIN2* promoter, these proteins are localized at the bottom of the cell while PIN2 is localized to the upper side (Wiśniewska et al., 2006). It is intriguing, however, that PIN1 tagged with GFP in a certain position within its cytosolic domain is localized in an identical pattern as PIN2 in the epidermis. This strongly supports the idea that individual PIN molecules also carry the cue for sub-cellular localization.

The plasma membrane localization of PINs can be explained by membrane-trafficking pathways. The reversible cycling of auxin carrier proteins between plasma membrane and endosomes appears to be a regulatory point for the abundance and polarity of auxin carrier proteins in the plasma membrane. Developmental and environmental cues affect this process to modulate auxin distribution patterns, as discussed earlier. The vesicular membrane-trafficking process for auxin carrier proteins is actin-dependent, appears to be protein-specific, and is possibly mediated by different endosomal compartments (Geldner et al., 2001; Dharmasiri et al., 2006; Jaillais et al., 2006). The trafficking pathway of PIN1 is regulated by GNOM, an ADP-ribosylation factor (ARF)-GDP/GTP exchange factor (ARF-GEF), whereas AUX1 is controlled by a novel trafficking pathway that depends upon AUXIN-RESISTANT 4 (AXR4) (Geldner et al., 2001; Dharmasiri et al., 2006). Additionally, PIN2-trafficking is mediated by novel endosomal compartments that include SORTING NEXIN 1 (SNX1) (Jaillais et al., 2006). Such a mechanism that incorporates these trafficking machineries would explain the different polar localizations among auxin transporters and their rapid sub-cellular relocation in response to environmental or developmental cues. Moreover, these mechanisms might prompt researchers to reform the classical chemiosmotic model (Rubery and Sheldrake, 1974; Raven, 1975), which has been widely accepted for explaining the mechanism for cellular auxin transport.

PGPs

The mechanism of PAT in plants has been mostly attributed to PIN proteins because their regulatory manners, tis-

sue-specific expression patterns, and polarized sub-cellular localizations are highly consistent with diverse developmental processes. Nevertheless, in addition to PINs, great progress has been made in the past few years toward understanding the role of PGPs in auxin transport.

PGPs, which are homologs of the mammalian ABCB/multidrug resistant/p-glycoprotein, are integral membrane ATP-binding cassette (ABC) transporters that consist of two similar halves, each containing a nucleotide binding fold (NBF) and six transmembrane domains (TMD). The two halves are joined by a highly divergent linker domain of ~60 amino acid (Ambudkar et al., 2005). Compared with the 6 isoforms found in humans, the plant PGPs are more expansive, with 21 expressed in *Arabidopsis* and 17 in rice (Martinoia et al., 2002; Blakeslee et al., 2005; Geisler and Murphy, 2006).

PGPs have been implicated in the movement of auxin, based on defects in PGP1 and PGP19 that result in decreased transport, reduced elongation growth, and altered tropic responses in *Arabidopsis* (*pgp1* and *pgp19*), maize (*brachytic2/zmpgp1*), and sorghum (*dwarf3/sbpgp13*) (Noh et al., 2001; Multani et al., 2003; Geisler et al., 2005). Biochemical studies have further demonstrated that PGPs function in auxin transport. For example, PGP1, 2, 4, 10, and 19 have been purified as high-affinity NPA (naphthylphthalamic acid, an auxin efflux inhibitor)-binding complexes (Murphy et al., 2002; Blakeslee et al., 2005; Geisler et al., 2005; Terasaka et al., 2005). The loss of PGP-functioning causes a decrease in cellular IAA export in *Arabidopsis* protoplasts, with this decline being even more severe in the double mutant (Geisler et al., 2005). Expression of PGP1 in heterologous yeast and HeLa cell systems enhances the efflux of IAA and 1-NAA, whereas such activity is normally blocked by auxin-transport and ABC transporter inhibitors. PGP19 also mediates auxin export when expressed in HeLa cells (Bouchard et al., 2006). Interestingly PGP4, which share 60% amino acid sequence identity with PGP1 but contains a unique coiled-coil protein interaction domain at its N-terminus, seems to be involved in ATP-dependent auxin influx in yeast and mammalian cells (Santelia et al., 2005; Terasaka et al., 2005). Furthermore, the CjMDR PGP-type transporter in *Coptis japonica* is capable of mediating the influx of the alkaloid berberine (Yazaki et al., 2001). Its closest homologue in *Arabidopsis* is PGP4 (with a 71% amino acid identity). However, the biochemical activity of PGP4 is not consistent with the mutant phenotypic effects seen in *Arabidopsis* (Santelia et al., 2005; Terasaka et al., 2005). Therefore, additional biochemical studies using plant systems are required to clarify the directionality of PGP activity.

Not only PINs but also PGP proteins exhibit tissue-specific expression and polar/apolar sub-cellular localization patterns (Blakeslee et al., 2007). Their localization patterns may overlap in some tissues but not in others. For example, PGP1 and PGP19 co-localize with PIN1 in the shoot apex and with PIN1 and PIN2 in root tissues. PGP19 and PIN1-GFP signals overlap in the root stele, endodermis, and pericycle. In cotyledonary nodes and upper hypocotyls, the expression of PIN1 and PGP19 overlaps in the vascular and bundle sheath tissues of seedlings. PGP19-HA and PIN2 signals overlap in root cortical cells, and PGP1, PIN1, and PIN2 are also co-localized to the roots (Fig. 2B).

Together with these expression profiles, it has been demonstrated that a synergistic increase in auxin transport, substrate specificity, and NPA sensitivity occurs when either PGP1 or PGP19 is co-expressed with PINs in HeLa cells (Geisler et al., 2005; Blakeslee et al., 2007). This PGP-PIN interaction has been confirmed in genetic double/triple mutant analyses, biochemical co-immunoprecipitation assays, and yeast-two-hybrid analyses. When PGP4 is co-expressed with PIN1 in the HeLa cells, a net efflux of auxin is observed, indicating that an interaction between PGP4 and PIN1 reverses the influx activity of PGP4. Moreover, when PGP4 is co-expressed with PIN2, cells retain more IAA than with PGP4 alone, suggesting a synergistic interaction similar to that observed between PIN1 and PGP1 (or PGP19). Co-expression of PGP4 with AUX1 synergistically increases the net IAA content in cells. More detailed study of the molecular interactions among PGPs, PINs, and AUX1/LAXs in *Arabidopsis* will further enhance our understanding of the organization and operation of this auxin transport complex.

REGULATION OF AUXIN TRANSPORT

Although the molecular mechanisms that control the polarity and amount of auxin movement are not well-elucidated, several studies have provided insight into the regulatory strategies that modulate PAT. Pharmacological and genetic data have suggested that protein phosphorylation/dephosphorylation is involved in controlling this transport (Delbarre et al., 1998; Benjamins et al., 2001; Rashotte et al., 2001; DeLong et al., 2002).

PINOID regulates auxin transport

The loss of a Ser/Thr protein kinase, PINOID (PID), shows phenotypic effects, as seen with *pin1* (Bennett et al., 1995). PID belongs to the family of AGC (cAMP-dependent protein kinase A, cGMA-dependent protein kinase G, and phospholipid-dependent protein kinase C) protein kinases (Bögre et al., 2003). When PID is over-expressed, using the 35S cauliflower mosaic virus promoter, the transformants show both auxin-resistant and auxin transport-defective phenotypes (Christensen et al., 2000; Benjamins et al., 2001). These results implicate PID both in auxin-signaling as a suppressor and in auxin transport as a positive regulator. However, two recent studies have clearly demonstrated that PID is a regulator for auxin transport rather than for signaling.

Friml et al. (2004) have reported that PID acts as a molecular switch for sub-cellular PIN polarity. Higher levels of PID, either caused by overexpression or existing naturally in the wild type (WT, values compared with the *pid* mutant), result in upper-side localization of PIN1 (in the shoot meristem epidermis and root vasculature cells), PIN2 (in root epidermal and cortical cells), and PIN4 (in quiescent cells). However, lower-side localization for PIN1 is observed in the shoot meristem vasculature cells. By contrast, lower concentrations of PID (either in *pid* or WT) are associated with the lower-side localization of PIN1, PIN2 (in root cortical cells), and PIN4 (in shoot meristem epidermal cells), while PIN2 remains on the upper side of the root epidermal cell. These

data suggest that PID acts as a determinant of polar-trafficking in PIN-containing vesicles.

Another line of experimentation with *Arabidopsis* root hair cells and tobacco BY2 suspension cell systems has revealed that PID directly facilitates auxin efflux activity (Lee and Cho, 2006). Auxin is a well-known strong stimulator of root hair growth (Okada and Shimura, 1994; Schiefelbein, 2000). Lee and Cho (2006) have hypothesized that, if PID facilitates auxin efflux, then the level of auxin in those hair cells should decrease and, accordingly, hair growth should be reduced. In those tests, a root hair cell-specific promoter was used to over-express PID in a hair cell-specific manner, thereby avoiding any pleiotropic effects that might arise through PID overexpression at the whole-plant level. In fact, hair cell-expressed PID significantly reduced root hair growth but without phenotypic effects in other tissues. PIN3 overexpression in the hair cells also greatly inhibited such growth. This was to be expected based on the theory that PINs would pump out auxin from the hair cells so as to deplete the cellular auxin needed for hair growth (Fig. 3). Auxin treatment restored root hairs from PID (and PIN3) overexpression lines, suggesting that PID is not a suppressor of auxin-signaling. Exogenous NPA or brefeldin A (BFA), which inhibit auxin efflux, also recovered the root hair growth of PID overexpression lines, almost to the level measured in wild type (Fig. 3). The results from this inhibitor study indicate that PID blocks root hair growth by facilitating auxin efflux. Conclusions based on these data from the root hair cell system are further supported by direct auxin efflux assays of dexamethasone (DEX)-inducible PID- and PIN3-overexpressing tobacco BY2 cells, which retain lower amounts of labeled auxin than do uninduced cells.

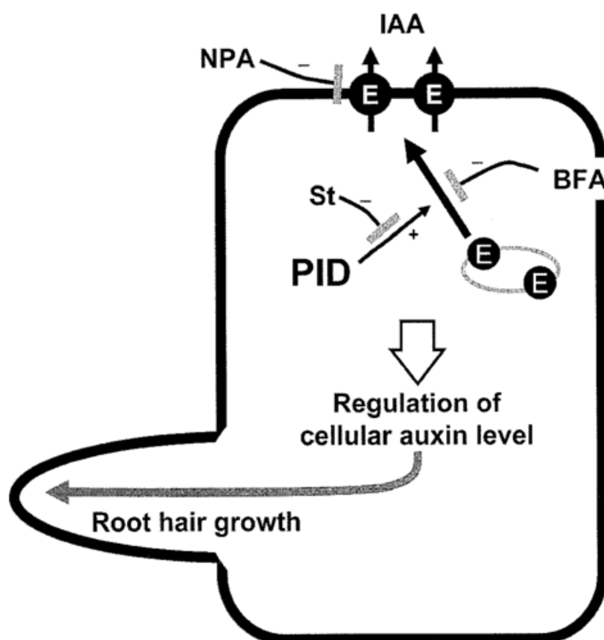

Figure 3. Root hair cell system used to study auxin transport and its regulation. High degrees of auxin efflux activity, either by greater abundance of auxin efflux carriers (E) in plasma membrane or by PINOID (PID) protein kinase-mediated activation, lower cellular auxin levels, subsequently resulting in inhibition of root hair growth. In contrast, blocking of auxin efflux activity by NPA and BFA, or protein kinase inhibition by staurosporine (St), restores root hair growth in plants with high auxin efflux activity.

An intriguing finding in this study is that PID is localized at the cell periphery, similar to the PIN proteins, and that this localization requires PID kinase activity. A point mutation in the ATP-binding domain of PID abrogates this peripheral localization. Moreover, a kinase inhibitor, staurosporine, disrupts the peripheral localization of PID and PIN3 and restores root hair growth in the roots of both types of transformants. From these data, Lee and Cho (2006) have proposed a model in which PID modulates the membrane-trafficking of PIN proteins by working in their proximity (Fig. 3).

Protein phosphatase 2A regulates auxin transport

A mutant *roots curl in NPA1* (*rcn1*) was isolated in a screen to identify genes involved in auxin transport or its regulation. These were determined through an assay for alterations in differential root elongation in the presence of NPA. The *RCN1* gene encodes a regulatory A subunit of protein phosphatase 2A (PP2A) (Garbers et al., 1996; Delbarre et al., 1998), and the *rcn1* mutant exhibits less PP2A activity and reduced organ elongation. PP2A is a highly conserved heterotrimeric enzyme in eukaryotes. It contains a catalytic (C) subunit that is bound by A and B regulatory subunits. The A subunit acts as a scaffold for the holoenzyme complex, and has direct regulatory effects on the activity of the catalytic subunit. B subunits are far more diverse than A and C subunits. Different B subunits alter the substrate specificity of PP2A activity.

The phenotypic alterations in *rcn1* are consistent with lower PP2A activity because treatment with the phosphatase inhibitor cantharidin phenocopies *rcn1* (Delbarre et al., 1998). The *RCN1* gene is expressed in the root tips, lateral root primordia, pericycle, and vascular tissues. Auxin transport activity is altered in *rcn1* seedling roots. Basipetal auxin transport (from the root tip toward the root/shoot junction) is increased to over 150% of the wild-type value while the level of acropetal transport is normal. These distinct effects of *rcn1* -- basipetal vs. acropetal transport -- suggest differential regulation of the two transport streams by RCN1. PP2A might serve as a negative regulator of the basipetal transport stream. However, the RCN1-regulated target proteins are unlikely to be AGR1/EIR1/PIN2/WAV6 or AUX1 when one conjectures from a double mutant analysis in which *rcn1eir1-1* does not suppress the increased basipetal transport in *rcn1*. Phosphorylation status might affect the auxin-transport stream by controlling the biosynthesis of endogenous regulatory compounds such as flavonoids (Brown et al., 2001; Peer et al., 2004). Alternatively, conditions for RCN1-regulated protein phosphorylation might alter the degree of sub-cellular localization of auxin carriers by influencing the cytoskeleton organization (Baskin and Wilson, 1997).

ROOT HAIR CELLS FOR THE AUXIN TRANSPORT STUDY

The root hair is a tubular protrusion from the root epidermal cell. Its development follows three major stages: determination of hair/non-hair cell fate, hair initiation, and tip

growth (Schiefelbein, 2000). Auxin and ethylene are potent positive effectors for those latter two stages (Schiefelbein, 2000; Cho and Cosgrove, 2002). Mutant phenotypes also implicate an auxin transporter in root hair development. For example, the influx carrier *aux1* mutant is defective in root hair growth (Okada and Shimura, 1994), with likely causes being either a reduced auxin supply to the differentiation zone or a defect in auxin influx to the hair cell. The loss of an auxin efflux carrier, PIN2/EIR1, which is localized basally in the elongating root epidermal cells, also shows a short root hair phenotype (Fig. 2C). This defect in *pin2/eir1* is most likely due to a diminished auxin supply from the root tip to the root hair differentiation zone because PIN2 is the major basipetal auxin efflux transporter in this region (Fig. 2B). In contrast, the loss of PGP4 causes a longer root hair phenotype (Santelia et al., 2005; our observations). While PIN2 expression is confined to the elongation zone, that of PGP4 extends to the hair differentiation zone in the root (Terasaka et al., 2005). PGP4 could be one of the major auxin efflux carriers in the hair cell, as seen from its promoter-reporter gene assay (Terasaka et al., 2005). Thus, the defect of PGP4 may cause more auxin to be retained in the root hair cell, which, in turn, enhances hair growth. Although those auxin transport assays using heterologous systems demonstrate the influx activity of PGP4 (Santelia et al., 2005; Terasaka et al., 2005), its genuine *in planta* activity remains to be described.

As mentioned above, Lee and Cho (2006) have successfully adopted the root hair system to study auxin transport. They have established root hair growth, which is proportional to the level of intracellular auxin, as their *in planta* assay marker for measuring the activity of cellular auxin transport. The *Arabidopsis EXPANSIN A7* (*AtEXPA7*) promoter (Cho and Cosgrove, 2002; Kim et al., 2006) also has been utilized to specify the expression of auxin transport components and regulators only to the root hair cell in order to avoid any probable complications from the effects of non-cell-autonomous auxin transport. Overexpression of PID and PIN3 in *Arabidopsis* root hair cells follows well from what was expected from their model; namely, shorter root hairs that result from higher activities of auxin efflux, as described earlier (Fig. 3).

Another useful property of root hairs in studies of auxin transport is their robust growth under pharmacological treatments. The membrane-trafficking inhibitor brefeldin A (BFA) and the protein kinase inhibitor staurosporine are toxic to general cell metabolism (Satiat-Jeunemaitre et al., 1996; Yamaki et al., 2002). However, these inhibitors, within a certain range of concentrations, instead restores root hair growth in PID- and PIN3-overexpressing transformant roots (Fig. 3). This is surprising because wild-type hair growth continuously decreases in proportion to increases in chemical concentrations.

Auxin moves throughout the entire plant and, in a non-cell-autonomous manner, affects many developmental processes within diverse tissue types. Therefore, a single-cell system is keenly required for studying the regulators and components of the auxin-transport machinery. Heterologous culture systems, such as those used with yeast and mammalian cells, have certain limitations when implemented for examinations of plant-specific processes. In this regard, although it is an indirect marker system for auxin-transport activity, the root hair cell system enables either a single-cell or an *in planta* approach.

CONCLUDING REMARKS

The recent discovery of TIR1 and its homologs as auxin receptors has simplified the concept of auxin-signaling. Now, follow-up studies are necessary to confirm their biological implications in various auxin responses during plant development. Another task in the field of auxin-signaling will be to discover the role of the classical auxin binding protein ABP1 in auxin physiology. This past year has seen the presentation of the molecular activities of most auxin carrier proteins. However, the molecular and biological properties of LAXs (AUX1 homologs), PIN5 and PIN8 (non-canonical PINs), and PGP4 still must be characterized. The mechanism for molecular regulation of auxin transport is just now beginning to be revealed. Currently, a major focus is on membrane-trafficking so that we can elucidate the specific cellular and molecular properties of such pathways for different auxin transporters.

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