AtPIN4 Mediates Sink-Driven Auxin Gradients and Root Patterning in Arabidopsis

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Summary

In contrast to animals, little is known about pattern formation in plants. Physiological and genetic data suggest the involvement of the phytohormone auxin in this process. Here, we characterize a novel member of the PIN family of putative auxin efflux carriers, Arabidopsis PIN4, that is localized in developing and mature root meristems. Atpin4 mutants are defective in establishment and maintenance of endogenous auxin gradients, fail to canalize externally applied auxin, and display various patterning defects in both embryonic and seedling roots. We propose a role for AtPIN4 in generating a sink for auxin below the quiescent center of the root meristem that is essential for auxin distribution and patterning.

Introduction

Developing organisms are growing populations of cells that exchange information about their relative positions and respond accordingly, resulting in spatial patterns of differentiated cell types. The concept of positional information proposes concentration gradients of morphogens, which instruct cells within a field about their position (Wolpert, 1996). Experiments in animals identified several molecules with morphogen properties, such as Wingless, Hedgehog, and DPP in Drosophila imaginal discs (reviewed in Teleman et al., 2001), Squint in the Zebra fish embryo (Chen and Schier, 2001), or the Sonic Hedgehog in the chicken neural plate (Briscoe et al., 2001). By contrast, morphogen gradients have not been demonstrated in plants, although pattern formation occurs also beyond embryogenesis in specialized regions, the meristems, where cells continuously divide and differentiate according to their position (Steeves and Sussex, 1989). The plant hormone auxin (indole-3-acetic acid, IAA) has long been implicated in pattern formation based on indirect evidence from physiological and genetic studies (Sachs, 1985, 2000). Patterning defects have been reported for mutants perturbed in auxin signaling, such as mp, ett, axr3, as well as in embryos and seedling roots upon manipulation of auxin levels (reviewed in Berleth and Sachs, 2001). Recently, auxin levels in Arabidopsis roots have been inferred, at cellular resolution, from the expression of the uidA (GUS) reporter gene driven by the synthetic auxin-responsive promoter DRS. Elevated GUS activity levels, corresponding to an “auxin maximum,” were observed in the columella initials of the root cap, whereas auxin-induced changes of GUS activity correlated well with perturbations in patterning (Sabatini et al., 1990).

Conceptually, cellular auxin levels can be regulated by several processes such as production, degradation, (de)conjugation, or directional transport. Auxin is unique among plant hormones in being polarly transported through the vascular system away from the source tissues toward the root tip (Lomax et al., 1995). Auxin is then in part redirected back up to the distal elongation zone, thereby regulating root gravitropism (Rashotte et al., 2000). The concept of auxin transport, as described by the chemiosmotic hypothesis (Rubery and Sheldrake, 1974; Raven, 1975), proposes that transport is driven
by a proton motive gradient and mediated by the action of influx and efflux carriers. The direction of auxin flux was proposed to be determined by asymmetric cellular localization of the efflux carrier. In search for these carriers, genetic approaches have been used to identify several auxin transport mutants in *Arabidopsis*, e.g., *aux1* (Bennett et al., 1996), *tir* (Ruegger et al., 1997), *rcn* (Garbers et al., 1996), and *pid* (Bennett et al., 1995). Another of these mutants, named *pin1* for its characteristic pin shape, showed a drastic reduction in basipetal auxin transport (Okada et al., 1991). The *AtPIN1* gene encodes a membrane protein with similarity to various transporter proteins from bacteria, consistent with a function of *AtPIN1* as a component of the auxin efflux (Gälweiler et al., 1998). Even more strikingly, AtPIN1 was polarly localized to the basal ends of xylem parenchyma cells of *Arabidopsis* inflorescence axes in accordance with classical concepts. A homolog of *AtPIN1*, the *AtPIN2* gene, was shown to be involved in regulation of root gravitropism and auxin redistribution after gravity stimulation, presumably by facilitating upward auxin transport in the root epidermis (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998). However, neither *Atpin1* and *Atpin2* nor mutants in the putative auxin influx carrier *AUX1* have revealed a role of auxin transport in regulating both auxin distribution and patterning.

Here, we describe the molecular analysis of the *AtPIN4* gene, a novel member of the *PIN* gene family. We demonstrate that *AtPIN4* is expressed below the *AtPIN1* domain in both developing and mature root meristems. We provide evidence that *AtPIN4*, but not *AtPIN1*, is essential for the correct establishment of an auxin gradient and is important for pattern formation in the root tip. Our results suggest a unique role for *AtPIN4* in regulating both auxin homeostasis and patterning through sink-mediated auxin distribution in root tips.

Results

Isolation of the *AtPIN4* Gene

To identify additional members of the *Arabidopsis* PIN gene family, we screened the IGF bacterial artificial chromosome (IGF BAC) library with probes derived from conserved regions of *AtPIN1*. Eight positive BAC clones mapped to the same region near the top of chromosome 2 and contained the *AtPIN4* gene, which consists of six exons and five introns (Figure 1A). Using a gene-specific probe, we isolated a full-length cDNA. The cDNA is 2310 bp long and contains an open reading frame encoding a deduced protein of 616 amino acid residues with a predicted molecular mass of 66.7 kDa. A BLAST-based computer comparison revealed 65% and 64% identity with *AtPIN1* and *AtPIN2*, respectively (Figure 1B). *AtPIN4* has a three-domain topology characteristic of PIN proteins. Two N- and C-terminal hydrophobic regions each containing 4–6 putative transmembrane segments (amino acid residues 1–179 and 454–616) are linked by a hydrophilic region of 275 amino acids. In contrast to the highly conserved hydrophobic regions, this linker region is less conserved (Figure 1B).

To investigate the function of the *AtPIN4* gene, we isolated three mutant alleles termed *Atpin4-1*, -2, and -3 and constructed antisense (*CaMV35S::asAtPIN4*) transgenic plants (Figure 1A; for details see Experimental Procedures). Northern blot analysis using an *AtPIN4*-specific probe identified a transcript of ~2 kb in roots, seedlings, siliques, stems, leaves, and cotyledons (Figure 1C). No *AtPIN4*-specific signal was observed in seedlings of *Atpin4-1*, -2, and -3 homozygous mutants, suggesting that these mutants were null (Figure 1D).

**AtPIN4 Is Localized in Root Meristem Precursor Cells of the Embryo**

To determine the localization of *AtPIN4*, we performed whole-mount in situ immunolocalization experiments using an *AtPIN4*-specific antiserum. No *AtPIN4* signals were detected in early-globular embryos (Figure 2A). In late-globular embryos, strong *AtPIN4* staining (indicated by red) was observed along the surface of the hypophysis and at the basal end of the adjacent suspensor cell (Figure 2B). During the triangular and subsequent developmental stages, suspensor staining gradually disappeared and additional signals appeared around the vascular cells with a maximum of the staining at the basal end (Figures 2C and 2D). In addition, the endodermis initials stained prominently at the heart stage (Figure 2E). The weak staining occasionally observed in epidermis of the apical embryo part (Figures 2A–2E) corresponds to a crossexaction with another *AtPIN* protein, since it can be observed also in *Atpin4* mutant embryos (data not shown). In situ hybridization with an *AtPIN4*-specific antisense riboprobe labeled the basal end of the embryo, suggesting that spatial accumulation of *AtPIN4* protein was determined by transcriptional control (Figure 2F, compare with Figure 2E). This was confirmed by the expression pattern of a transcriptional fusion of the *AtPIN4* promoter with the GUS reporter gene that labeled the basal region corresponding to the quiescent center precursors and surrounding cells (Figure 2G). We also identified a marker line, originally designated *LENNY*, with an insertion of *Ds-GUS* (Sundaresan et al., 1995) downstream of *AtPIN4* promotor that exhibited a staining pattern identical to the *AtPIN4* expression pattern (Figure 2H).

**Disruption of *AtPIN4* Affects DR5-Driven Reporter Expression in Embryos**

We studied auxin accumulation in wild-type and *Atpin4* mutant, using a synthetic auxin-responsive promotor (*DR5rev*) fused to the reporter gene PEH A (Dotson et al., 1996). A similar construct (*DR5::GUS*) has been used to visualize auxin accumulation at cellular resolution in *Arabidopsis* roots (Sabatini et al., 1999). Wild-type embryos expressed PEH A very weakly in the basal part of the embryo from the early-heart stage on (Figures 3A–3E). The staining intensity was increased in late-heart and torpedo-stage embryos and the signal remained restricted to the basal part, with a maximum of PEH A activity in columella precursor cells (Figures 3D and 3E). In contrast, *Atpin4-1* (34 of 52), *Atpin4-2* (24 of 50), and *Atpin4-3* (68 of 81) mutant embryos showed a significantly different PEH A activity pattern (Figures 3F–3J). Strong staining was observed in all subepidermal cells of globular proembryos (Figure 3F). At triangular and heart stages, the domain of PEH A activity was located mostly in the presumptive vascular tissue and
Figure 1. Genomic Organization and Expression of the \textit{AtPIN4} Gene

(A) Structure of the \textit{AtPIN4} genomic region. Adjacent markers and genetic distances are shown on the chromosomal map. Below, an enlarged view of the \textit{AtPIN4} gene is presented with exons (black boxes) and introns (empty boxes). \textit{En-1} transposon insertion sites and the \textit{DsE} insertion site are marked by gray triangles with nucleotide position from ATG indicated.

(B) Comparison of \textit{AtPIN1}, \textit{AtPIN2}, and \textit{AtPIN4} protein sequences. The amino acid number is shown on the left. Identical amino acids are indicated as black boxes and gaps as dots. Bars above the sequence blocks denote transmembrane domain segments as predicted by the Kyte and Doolittle method.

(C) Northern blot analysis of \textit{AtPIN4} mRNA in different tissues. Total RNA extracted from different \textit{Arabidopsis thaliana} tissues and hybridized to an \textit{AtPIN4} probe. (1) Wild-type suspension culture cells; (2) roots from \textit{Arabidopsis} plants grown in liquid culture; (3) cauline leaves; (4) inflorescence; (5) siliques; (6) seedlings grown on MS agar for 6 days; (7) rosette leaves; (8) roots; (9) flowers; (10) cotyledons. All blots were rehybridized to a constitutively expressed \textit{ACTIN} gene to control for loading.

(D) Northern blot analysis of \textit{AtPIN4} mRNA from \textit{Atpin4} mutant seedlings. Seedlings with genotype (1) \textit{Atpin4-1} het; (2) \textit{Atpin4-1}; (3) \textit{Atpin4-2}; (4) \textit{Atpin4-3}.

was thus enlarged and shifted upward relative to the position in corresponding wild-type embryos (Figures 3G–3I, compare with Figures 3B–3D). At the torpedo stage, \textit{Atpin4} mutant embryos displayed more restricted staining which, in contrast to wild-type, had its maximum in basal provascular cells (Figure 3J, compare with Figure 3E). Thus, lack of \textit{AtPIN4} function raised auxin levels as indicated by increased PEH A reporter activity. Moreover, the basally localized auxin response maximum of wild-type embryos was shifted apically in \textit{Atpin4} embryos. Together, these changes suggest that \textit{AtPIN4} is involved in both regulating auxin levels and proper positioning of the auxin response maximum in \textit{Arabidopsis} embryogenesis.

**Disruption of \textit{AtPIN4} Affects Patterning in the Developing Root Meristem**

To determine functional requirement of \textit{AtPIN4} during embryogenesis, we compared the development of wild-type and \textit{Atpin4} mutant embryos. No difference was observed prior to the globular stage \((n = 91)\), at which stage cell divisions started to deviate from the stereotypic pattern of wild-type in the region of the future root meristem (Figure 4). In wild-type, the hypophysis divides...
Figure 2. Expression and Localization of the AtPIN4 Protein during Embryogenesis

(A) Early globular stage. No AtPIN4 protein was found.

(B) Globular stage prior to hypophysis division: the hypophysis cell boundary is marked for AtPIN4. AtPIN4 is also found in the neighboring suspensor cell at the basal cell boundary.

(C) Late globular stage. The hypophysis cell has divided. The lens-shaped and provascular initial cells are stained for presence of AtPIN4. Weaker AtPIN4 signal marks the basal side of basal cell.

(D) Triangular stage: the lens-shaped cell has divided and AtPIN4 is distributed equally at the boundaries of QC cells. The provascular initials show label at their basal sides.

(E) Heart stage: additional AtPIN4 signal appears in the endodermis and provascular initials with the majority of signal at the basal boundaries.

(F) Heart stage: AtPIN4 mRNA localization by in situ hybridization in the root meristem region.

(G) Globular stage: GUS staining of transgenic plants carrying AtPIN4::GUS construct in the root meristem region.

(H) Heart stage: GUS staining in the root meristem region of enhancer trap line LENNY.

AtPIN4 protein localization is indicated by red (CY3-conjugated secondary antibody; [A], [B], [C], and [D]) or by green (FITC-conjugated secondary antibody; [E]). The nuclei were stained with DAPI (A–D), indicated with blue. Basal staining indicated by arrowheads. Scale bars, 10 μm.
Asymmetrically to give an upper lens-shaped cell and a larger lower cell (Figure 4A). Whereas the former gives rise to the quiescent center of the root meristem, the latter produces the single layer of columella initials. In wild-type embryos, the division of the basal cell occurred only rarely before the triangular stage (4/149) (Figure 4A) and the division of the lens-shaped cell never took place before the heart stage (0/149) (Figure 4B). In Atpin4 mutant embryos, however, these cells divided prematurely at the globular stage (Figure 4C). These premature cell divisions occurred frequently in mutant embryos for each of the three alleles (Atpin4-1: 18/119; Atpin4-2: 20/122; Atpin4-3: 29/133) and in AtPIN4 antisense embryos (12/48). Later in embryogenesis, the normally nondividing quiescent center (QC) cells showed divisions, and columella precursor cells underwent supernumerary divisions (Figure 4F, compare with Figure 4E). In addition, abnormal planes of cell division were observed, e.g., in columella precursors (Figure 4F). Furthermore, periclinal divisions of the uppermost suspensor cell (Figure 4D) occurred in Atpin4 mutant (16/83) and AtPIN4 antisense (12/56) heart-stage embryos, whereas wild-type suspensor cells divided anticlinally before mitotic quiescence at this stage. The aberrant cell divisions correlated with an altered expression pattern of the QC-specific marker, QC25, described previously for the postembryonic root (Sabatini et al., 1999). We determined that wild-type embryos expressed QC25 exclusively in QC cells already at the heart stage (Figure 6N). In contrast, Atpin4 embryos expressed QC25 in a much broader domain (Figure 6O). Thus, a molecular marker confirms morphological observations, suggesting cell fate changes of the meristem cells in developing root.

AtPIN1 and AtPIN4 Are Expressed in Partially Overlapping Regions of the Seedling Root

Transcripts and proteins of AtPIN1 and AtPIN4 were localized in primary roots by whole-mount in situ hybridization and immunolocalization, respectively (Figure 5). AtPIN1 mRNA accumulated in the vascular cylinder but was absent from epidermis, cortex, quiescent center (QC), and root cap (Figure 5A). No staining was detected with the AtPIN1 sense control (Figure 5A, inset). AtPIN1 protein accumulated in the same cells as the mRNA, and was detected mostly at their lower (basal) ends (Figure 5B). In addition, a weaker AtPIN1 signal was also observed in endodermis tissue. AtPIN4 mRNA was localized in the QC and the surrounding cells of the root meristem (Figure 5C), whereas no signal was observed with the sense probe (Figure 5C, inset). AtPIN4 protein was detected in the same tissues, including the QC, the surrounding initials, and their daughter cells (Figure 5D). Higher magnification revealed both polar and nonpolar localization of AtPIN4 (Figure 5E). Whereas columella initials and columella cells displayed a nonpolar localization of AtPIN4, the cells of the proximal meristem, including endodermis, cortex, vascular initials, and their daughter cells, had AtPIN4 localized at their lower (basal) ends. The QC cells had most AtPIN4 label basally, although this polarity was less pronounced (Figure 5E). No label was observed in the roots of Atpin4 null mutants and AtPIN4 antisense seedlings (Figure 5F), although at higher antibody concentrations, crossreaction with another PIN protein was observed (data not shown). The spatial distributions of AtPIN1 and AtPIN4 partially overlapped in vascular initials and their derivatives (compare Figures 5B and 5D). The distinct patterns of
AtPIN1 and AtPIN4 localization suggest a basally directed auxin flow in roots through the vascular cylinder toward the central root meristem, where it focuses into columella initials and columella.

AtPIN1 Mutant Roots Display No Defects
AtPIN1 plays a role in long-distance basipetal auxin transport within the inflorescence axis (Galweiler et al., 1998). Our immunolocalization studies suggest a comparable role in mediating auxin flow to the root meristem region. However, we did not observe any obvious differences in root growth or gravitropic responsiveness of AtPIN1 mutant seedlings, as compared to wild-type (n = 286). Nor did we find any aberrations in root patterning between AtPIN1 (Col-0 ecotype) and Col-0 wild-type roots (n = 286). These results were in contrast to previously reported patterning defects and changes in DR5::GUS expression in the AtPIN1 mutant (Sabatini et al., 1999). Detailed inspection of the progeny from the original cross between DR5::GUS (Col-0) and AtPIN1 (Enkheim) revealed that the reported aberrations appear to involve an Enkheim-specific modifier. Moreover, the analysis of another cross between AtPIN1 (Col-0) and DR5rev::GUS (Col-0) did not reveal the previously reported abnormalities (data not shown). Taken together, these data show that AtPIN1 mutation may only in certain
circumstances affect root development and may reflect functional redundancy between different members of the AtPIN gene family.

**Disruption of AtPIN4 Affects DR5-Driven Reporter Expression and Auxin Levels in Roots**

To determine the physiological consequences of AtPIN4 disruption, we examined the auxin-responsive expression of DR5rev::PEH A in roots of transgenic wild-type and Atpin4 mutant seedlings. Wild-type roots displayed PEH A reporter activity in the initials and the first cell layer of the columella (43 of 43) (Figure 6A). Treatment with 1–5 μM NPA, which impairs auxin transport, increased PEH A reporter activity, and also expanded its expression domain (39 of 41) (Figure 6B). Atpin4 mutant roots resembled NPA-treated roots in their pattern of PEH A expression (Atpin4-1: 49 of 71; Atpin4-2: 24 of 36; and Atpin4-3: 46 of 62). The PEH A activity was increased compared to wild-type and its maximum was centered on cells that corresponded to the QC and vascular initials in wild-type (Figure 6C). These results suggested that in the Atpin4 root meristem, auxin levels were elevated, and the maximum was shifted to cells that display polar localization of AtPIN4 protein in wild-type (compare Figure 6C with Figure 5E).

To directly correlate the PEH A signal with auxin levels, we measured the auxin content in root segments by mass spectrometry. We observed significantly elevated free IAA levels (P < 0.005) in the first mm of root tips in Atpin4 mutant seedlings, as compared to wild-type (Figure 7A). By contrast, no significant differences in free IAA levels were observed in the second mm of root tips, which is farther away from the root meristem (data not shown). Thus, direct measurement of free IAA content confirmed the DR5-based observations, supporting the notion that AtPIN4 is involved in the regulation of auxin levels and gradients in the root meristem.

**AtPIN4 Is Involved in Maintenance of Auxin Gradient and Auxin Canalization in Roots**

To test how roots regulate auxin levels, we challenged wild-type and AtPIN4 seedlings by incubation with 10 μM IAA and subsequently analyzed DR5rev::PEH A expression. In wild-type roots, the maximum of PEH A activity was restricted to a few cells, including the columella initials (42 of 43) (Figure 6D). This pattern was qualitatively similar to untreated roots, implying that the roots have homeostatic mechanisms that can handle the elevated IAA levels and maintain endogenous auxin gradients. In contrast, wild-type roots exposed to IAA in the presence of the auxin transport inhibitor NPA exhibited a general increase of PEH A activity that, in addition, was expanded across the whole root tip (38 of 41) (Figure 6E). This result suggests that polar auxin transport is the major component for auxin canalization and maintenance of IAA gradients in the root tip. Atpin4 mutant roots treated with exogenous IAA resembled NPA-treated wild-type roots, displaying highly increased levels of PEH A activity across the whole root tip (Atpin4-1: 23 of 33; Atpin4-2: 22 of 35; and Atpin4-3: 18 of 29). (Figure 6F). In addition, the maximum of PEH A staining was shifted to a zone apical to the QC, with elevated levels also in the endodermis (Figure 6F). These results suggest that Atpin4 roots are not able to maintain endogenous IAA gradients, fail to mediate flow of exogenously applied IAA to the columella region, and to neutralize it.

To confirm our DR5-based observations and to gain insights into the dynamics of IAA turnover, a study was performed with mass spectrometry to measure the relative turnover of IAA after its exogenous application (Figure 7B). The amount of IAA taken up by the root tips measured immediately after a feeding period of 30 min was significantly lower (P < 0.05) in Atpin4 mutant than in wild-type roots. This reduced ability of Atpin4 roots to accumulate exogenous IAA is in accordance with our notion that AtPIN4 mutants display impaired IAA transport, which probably prevents feeding of the columella region. We also observed that the decrease of free IAA content within the first three hours in Atpin4 roots was significantly smaller (P < 0.005) than in wild-type roots, confirming our DR5-based observations. This observation suggests a reduced capacity of Atpin4 roots to downregulate the IAA pool in this tissue, probably due to a defect in canalization IAA fluxes (Figure 7B).

**Disruption of AtPIN4 Affects Root Meristem Patterning**

To determine functional requirement of AtPIN4 in postembryonic development, we compared the growth and pattern of wild-type and Atpin4 mutant roots. By comparing root meristems between wild-type and Atpin4 mutants (Figures 6G–6K), we observed several distinct aberrations in cell patterns of four-day-old seedling roots (Figure 6H, compare with Figure 6G). Abnormal roots were observed in 95%–97% of the seedling progeny of Atpin4 homozygous mutant plants, regardless of the allele examined (Atpin4-1: 40 of 42; Atpin4-2: 55 of 57; Atpin4-3: 47 of 48). Identical pattern aberrations occurred in 38 of 38 AtPIN4 antisense seedlings (Figure 6I). The well-defined QC of mitotically quiescent cells was replaced by cells that divided in irregular planes (Figure 6H, arrowheads). In addition, the mutant displayed two, instead of one, tiers of columella initials (Figure 6I, arrows) and supernumerary cells in the differentiated columella layers with often aberrant shapes (Figures 6H and 6I). Endodermis cells of AtPIN4 mutant roots also behaved abnormally, undergoing periclinal divisions (Figure 6H, inset, arrow). As seedling development progressed, aberrations became more pronounced, and the root meristem often completely lost its regular pattern (Figures 6J and 6K). The columella formed up to eight additional layers of root cap cells (Atpin4: 34 of 92; AtPIN4 antisense: 59 of 112; Figures 6I and 6J) and massive periclinal divisions in the proximal root meristem led to a characteristic swelling of the root tip (Atpin4: 19 of 92; AtPIN4 antisense: 23 of 112; Figure 6K). The many features of AtPIN4 root meristem phenotype, including QC divisions, extra columella cell columns, endodermis periclinal divisions, and extra root cap and swelling, were mimicked by growing wild-type seedling on 1 μM NPA (Figure 6B, data not shown), further supporting a role of AtPIN4 in auxin efflux.

To gain further insight into the changes of cell identities in Atpin4 mutant roots, we analyzed expression patterns of cell-type-specific markers. The expression domains
of QC cell fate marker QC25 (Figures 6L–6O) and QC/columella marker COL148 (Figures 6P and 6Q) were expanded, suggesting that root meristem initials and their daughter cells displayed some features of QC cells. Conversely, cells at the QC position in Atpin4 mutant seedlings display much weaker and frequently (12/13), no detectable expression of pSCR::GFP, in contrast to wild-type (1/34) QC cells (Figure 6S, compare with Figure 6R). This observation was further confirmed by Northern blot analysis with an SCR-specific probe (data not shown). In addition, endodermal cells originating from periclinal divisions displayed weak or no expression of pSCR::GFP, confirming changes in cell behavior (Figures 6R and 6S, insets). The columella differentiation
defect in Atpin4 mutant seedlings was reflected in the abnormal expression of the columella initial marker J2341 in the supernumerary tier of columella initials (Figure 6U, arrows; compare with Figure 6T). In addition, the cells located at the position of wild-type QC also expressed the marker J2341 (Figure 6U, arrowhead; compare with Figure 6T), suggesting that these cells adopted at least some features of columella initials. The staining pattern of five different cell type markers, in conjunction with changes in cell morphology and behavior, strongly suggest changes in cell fate, with QC and surrounding cells probably adopting mixed cell fates. Taken together, loss of AtPIN4 function affects rate and orientation of cell divisions, polar expansion, and cell fates within the root meristem.

Discussion

AtPIN4 Is a Novel Regulator of Auxin Efflux
The AtPIN4 gene encodes a member of the PIN family of putative auxin efflux carriers (Friml, 2000) that all share limited structural similarity with transport proteins of the major facilitator group (Marger and Saier, 1993). Although auxin transport has not been biochemically demonstrated for any PIN protein, our findings suggest that AtPIN4 functions either as an auxin carrier or a regulator of auxin transport. As shown previously for other PIN proteins, AtPIN4 is polarly localized, and the mutant phenotype can be mimicked by chemical inhibition of auxin efflux in wild-type plants. In addition, we observed defects in Atpin4 mutants outside of the AtPIN4 expression domain, suggesting non-cell autonomous action of AtPIN4 consistent with a role in long-range signaling. We also detected a shift in auxin-responsive gene expression, which spatially correlated with the loss of AtPIN4 expression. Taken together, our observations support the notion that AtPIN4 represents an important regulatory component of polar auxin transport, possibly an auxin efflux carrier.

Localized AtPIN4 Expression Is Required for Graded Auxin Distribution
Direct auxin measurements have demonstrated auxin gradients in Scots pine (Uggla et al., 1996). In the Arabidopsis root tip, locally elevated auxin levels (auxin maximum) have been inferred for the columella initials and toward the columella, suggesting that auxin is supplied by the root tip, locally elevated auxin levels (auxin maximum) have been inferred for the columella initials and toward the columella, suggesting that auxin is supplied by the auxin efflux carrier. The activity of AtPIN4 in the supernumerary tier of columella initials (Figure 6U, arrows; compare with Figure 6T), suggests that these cells adopted at least some features of columella initials. The staining pattern of five different cell type markers, in conjunction with changes in cell morphology and behavior, strongly suggest changes in cell fate, with QC and surrounding cells probably adopting mixed cell fates. Taken together, loss of AtPIN4 function affects rate and orientation of cell divisions, polar expansion, and cell fates within the root meristem.

AtPIN4-Dependent Auxin Gradients are Sink-Driven
Our data suggest that a graded distribution of auxin with a maximum basal to the QC of the root correlates with correct patterning. The extension of this maximum to the root base after loss of AtPIN4, which is polarly localized toward the columella, suggest that auxin is supplied by the more basal tissue and transported into the maximum region in an AtPIN4-dependent fashion. The auxin maximum in the root is established against the slope of the gradient away from the source of auxin production. The continuous auxin supply through the stele to the maximum region raises the question of what happens to auxin there. Atpin4 mutant plants, as well as plants with chemically inhibited auxin transport, are defective in local auxin inactivation, implying that auxin is downregulated in root tip. However, other members of the PIN family (e.g., AtPIN2 and AtPIN3), together with the auxin permease AUX1, apparently facilitate lateral and basipetal rerouting of a portion of auxin from columella cells through root cap, epidermal, and cortical cell layers back up to the distal elongation zone thereby regulating gravitropism (Friml et al., 2002; Rashotte et al., 2000, 2001; Swarup et al., 2001). Available evidence suggests...
Figure 6. Meristem Pattern, DR5 Auxin Response Reporter, and Cell Marker Expression in Wild-Type and Atpin4 Mutant Roots

(A–C) DR5rev::PEH A expression in root meristem of wild-type (A and B) and Atpin4 mutant (C) transgenic plants. The maximum of DR5rev::PEH A expression in wild-type is detected in columella initials and first columella tier (A). In contrast, in NPA-treated plants (B) and in Atpin4 mutants (C), the DR5rev::PEH A expression maximum is shifted into QC cells and vascular initials, and the overall PEH A activity in root tip is elevated.

(D–F) DR5rev::PEH A expression in root meristem of wild-type (D and E) and Atpin4 mutant (F) plants after four hours of IAA treatment. In
that the region of the auxin maximum below the QC acts as an AtPIN4-dependent sink for auxin. We propose that this basal sink controls the auxin distribution and thereby patterning, since chemical or genetic interference with the sink abolishes this gradient. By contrast, the auxin distribution is maintained in the presence of additional external auxin sources. Our analyses of the Atpin4 embryo phenotype and AtPIN4 embryo localization pattern suggest a similar mechanism for controlling auxin levels and patterning in embryogenesis.

Our model of a sink-driven morphogenetic auxin gradient in the Arabidopsis root tip contrasts with the well-established morphogen gradients in animals that are source driven (reviewed in Telesman et al., 2001), such as the anterior-posterior gradient of Bicoid in the Drosophila preblastoderm embryo (Driever and Nüsslein-Volhard, 1988) or the spreading of the DPP gradient from the anterior-posterior compartment boundary in the Drosophila wing imaginal discs (Basler and Struhl, 1994). Nonetheless, the principle of cell fate determination based on the relative concentrations of a morphogen at specific positions along the gradient may apply in plants as well as in animals.

Experimental Procedures

Materials and Growth Conditions

The AtPIN4 gene was identified in the IGF BAC genomic library http://www.mpimp-golm.mpg.de/mpi-mp-map/) with an AtPIN1 probe (nucleotides 1–385; accession number AF089084; Gäweiler et al., 1998). The full-length AtPIN4 cDNA was isolated from cDNA libraries prepared from seedlings, leaves, and whole plant tissues. The primer pair 5’-CTCAATGCCCAGTCTCTACACT-3’ and 5’-ATC TTCTTTCAGCCTCAGTCT-3’ was used to amplify the AtPIN4 genomic sequence (accession number AF087016). AtPIN4 antisense plants were generated using a construct spanning 157–822 nucleotides of AtPIN4 cDNA in antisense orientation under the control of the CaMV35S promoter. The construct for monitoring of endoge-
ous auxin levels DR5::GUS was described by Ulmasov et al. (1997). DRiBrev::PEHA consists of nine repeats of the DRS element (TGCTTC) fused in inverse orientation to the minimal CAATSS promoter and the phosphate monoester hydrolase. PEH A (Dotson et al., 1996) gene. The AtPIn4::GUS construct was generated by fusion of a PCR-amplified fragment (nucleotides 1764 to 1 upstream of ATG and the GUS gene). The homologous AtPIn4-1, -2, and -3 mutant lines were identified by reverse genetic screening of an En-1 transposon mutagenized collection of A. thaliana (Col-0) as described (Muller et al., 1998). Six En-1 insertions were identified and flanking regions sequenced. Three introns-located insertions were not further analyzed. The other insertions, termed AtPIn4-1, -2, and -3 were located 25 nucleotides upstream of the start codon, and the first and second exons at positions 1003 and 1578, respectively. The following probes and primers were used: nucleotides 1429–2012 and nucleotides 679–1149 of the AtPIN4 cDNA, 5’-CTCAATGCCACTGTCTTCTCACT-3’ and 5’-ATCTTCTTCTGTCACTTCT-3’. The LENNY line contains the Ds-GUS transposon inserted within the third intron of the AtPIn4 gene at position 2058. Marker lines J2341, COL148, pSCHR::GFP, and QC25 were previously described (Sabatini et al., 1999).

Expression Analysis and Immunolocalization

Histochemical staining for GUS and PEH A activity was performed using modified indigogenic method with 5-bromo-4-chloro-3-indoxyl β-D-glucuronide (X-Gluc, Sigma) or 5-bromo-4-chloro-3-indoxyl phosphate (XPP, Research Organics) as substrates (Dotson et al., 1996; Sabatini et al., 1999). For all comparisons between wild-type, NPA treated wild-type, and AtPIn4 lines, identical staining conditions were used. In the case that a different transgenic insertion was used, at least four independent lines (less 40 roots each) were analyzed. Northern blot analysis and whole-mount in situ hybridization experiments were performed as described (Muller et al., 1998) with probes corresponding to a region of AtPIN4 (nucleotides 679–1149). AtPIN4-specific antibodies were generated using a recombinant protein corresponding to amino acids 243 to 400 of AtPIN4 as described (Glaweiler et al., 1998). Immunolocalization in roots and embryos were performed as described (Steissmann et al., 1999, Muller et al., 1998). Affinity-purified primary anti-AtPIN1 (Glaweiler et al., 1998) and anti-AtPIN4 antibodies were diluted 1:150 and 1:200, respectively. The secondary antibodies, FITC-conjugated and CY3-conjugated anti-rabbit antibody (Dianova) were diluted 1:200 or 1:800, respectively. Solutions during the whole-mount in situ hybridization and immunolocalization procedures were changed using a pipetting robot (Insuit Pro, Intavis).

Growth Conditions and Microscopy

Plant growth conditions were as previously described (Muller et al., 1998). Exogenous auxin application was performed by incubation of 4-day-old seedlings in 0.5× MS liquid medium supplemented by 2×10–25 μM IAA and/or 25 μM NPA for 0.5 to 6 hr. Starch granules in the central root cap were visualized by lugol staining and for histological analysis of embryos; dissected ovules were fixed and cleared as described (Sabatini et al., 1999). Both embryos and roots were viewed with Nomarski optics using a Leica DMRB microscope equipped with a video camera (Hitachi, HV-C20A). Fluorescent samples were inspected by confocal laser scanning microscopy as described (Muller et al., 1998). Images were processed using Adobe Photoshop 4.0 (Adobe Systems, Inc.) and final composition of figures was performed with CorelDRAW 7 (Corel Corporation).

Analysis of IAA Contents and Turnover Rates

For each sample, 1 mm root segments from 50 seedlings were collected and pooled. For turnover studies, seedlings were incubated in medium containing 10 μM IAA for 30 min, thoroughly washed, and transferred to MS medium for the rest of the incubation period. The samples were extracted, purified, and analyzed by combined GC-SRM-MS (gas chromatography-selected reaction monitoring-mass spectrometry) as described (Edlund et al., 1995). Calculation of isotopic dilution factors was based on the addition of 100 pg 13C6-IAA to each sample. The significance of reported differences in IAA content and IAA turnover was interpreted from the T values.

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