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Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation

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Summary

Plants, compared to animals, exhibit an amazing adaptability and plasticity in their development. This is largely dependent on the ability of plants to form new organs, such as lateral roots, leaves, and flowers during postembryonic development. Organ primordia develop from founder cell populations into organs by coordinated cell division and differentiation. Here, we show that organ formation in *Arabidopsis* involves dynamic gradients of the signaling molecule auxin with maxima at the primordia tips. These gradients are mediated by cellular efflux requiring asymmetrically localized PIN proteins, which represent a functionally redundant network for auxin distribution in both aerial and underground organs. PIN1 polar localization undergoes a dynamic rearrangement, which correlates with establishment of auxin gradients and primordium development. Our results suggest that PIN-dependent, local auxin gradients represent a common module for formation of all plant organs, regardless of their mature morphology or developmental origin.

Introduction

Plasticity and adaptability of development represent the most important life strategy of plants. Unlike animals, which can escape unfavorable conditions by behavioral responses, plants compensate for their sessile lifestyle by changing their development in response to external cues. Whereas animals establish the entire body organization during embryogenesis, the adult life form of plants is largely dependent on postembryonic development. Self-maintaining stem cell systems—the meristems, which are established at both ends of the apical-basal embryonic axis—are able to perpetuate existing organs and initiate new organ primordia (Steeves and Sussex, 1989). Some primordia develop into determinate organs with finite cell numbers, whereas others develop into growing shoots or roots bearing a secondary meristem at the tip. Aerial organs such as leaves and secondary shoots, including flowers, originate from primordia on the flanks of the shoot meristem. Flower primordia give rise to lateral organs, such as sepals, petals, stamens, and carpels. Carpels fuse to form the gynoecium, which produces organs—ovules—along the placenta of the ovary. Ovules, in turn, initiate integuments as lateral primordia (Esau, 1977). The situation is less complex in the root where lateral root primordia are initiated from localized cell groups within the pericycle (Dubrovsky et al., 2000).

The morphological diversity of plant organs depends on the developmental origin of their primordia: shoot meristems generate leaves, floral meristems floral organs, pistils ovules, ovules integuments, and roots make lateral roots. The difference between leaves and floral organs, for example, can be attributed to the expression of floral homeotic genes in the latter but not the former organs (Honma and Goto, 2001). A common feature of all shoot-derived, as opposed to root-derived, organ primordia is the expression of the *AINTEGUMENTA* transcription factor (Elliott et al., 1996). Another example is the involvement of the *WUSCHEL* homeobox protein in both flower and ovule development (Gross-Hardt et al., 2002). By contrast, underground organs such as lateral roots are developmentally distinct from aerial organs in terms of primordium initiation, subsequent development as well as regulatory gene expression (Malamy and Benfey, 1997). These observations suggested at least two different regulatory mechanisms for postembryonic organ formation in plants.

Studies focused on the initiation and positioning of organs, such as leaves (Reinhardt et al., 2000), flowers (Okada et al., 1991; Oka et al., 1999), and lateral roots (Laskowski et al., 1995), have implicated the plant-signaling molecule auxin. Auxin is unique among plant hormones in being actively and directionally transported from the place of synthesis in young apical parts. Current models propose that members of the PIN protein family of auxin efflux regulators represent an important part of a network for auxin distribution throughout the plant (reviewed in Friml, 2003). PIN proteins have been shown to mediate various developmental processes such as vascular tissue and flower development (PIN1; Gälweiler et al., 1998), tropisms (PIN2, PIN3; Müller et al., 1998, Friml et al., 2002b), as well as patterning of the root (PIN4; Friml et al., 2002a). Cellular polarities of PIN protein localization correlate with directions of auxin flux (reviewed in Friml, 2003). The flexibility of the transport system may result, at the cellular level, from the continuous cycling of PIN proteins between the plasma membrane and endosomes (Geldner et al., 2001, 2003a), allowing for rapid changes in PIN polar localization and hence redirection of auxin fluxes in development. So far, this has been demonstrated for PIN3 relocation in response to gravity stimulation (Friml et al., 2002b). It remains to be determined whether PIN relocation for auxin redistribution could also provide a general mecha-

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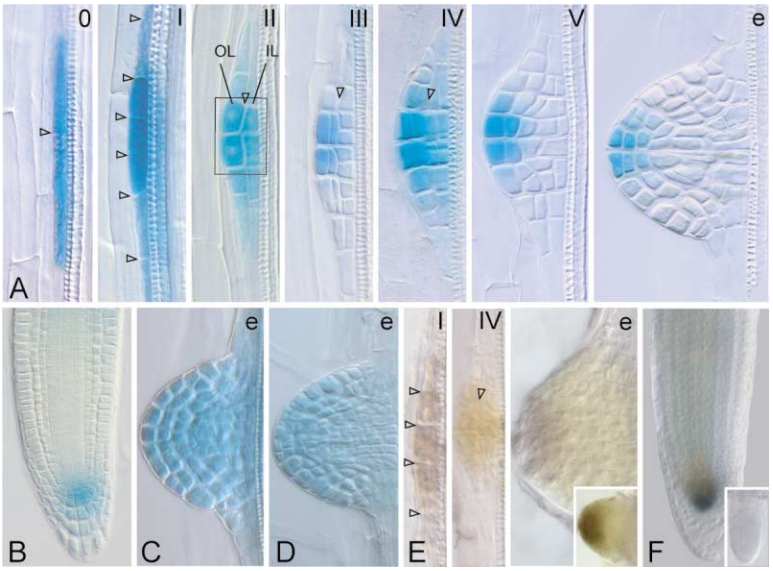

Figure 1. Distribution of Auxin and Its Response in Lateral Root Primordia

(A) Spatial pattern of *DR5::GUS* expression during primordium development: the DR5 activity gradient with the maximum at the primordium tip is gradually established. OL and IL, outer and inner layers; central cells outlined.

(B) *DR5::GUS* in columella initial region of the mature lateral root.

(C) 2,4-D treatment: loss of DR5 gradient and induction of DR5 activity in the entire primordium.

(D) NPA treatment: impaired DR5 gradient after inhibition of auxin efflux.

(E) Immunolocalization of IAA during primordium development: the IAA gradient with the maximum at the tip is gradually established. Inset shows increased signal, however no change of the gradient after the IAA treatment.

(F) IAA accumulation in columella initial region of the mature lateral root. Inset shows negative control, no signal without IAA fixation.

Division planes marked by arrowheads and developmental stages indicated in the upper right corner: I, II, III, IV, V, and e – emergence. GUS signal in blue (A–D), IAA signal in brown (E and F).

nism for other developmental processes including organ formation.

Here, we analyze the role of auxin in the formation of diverse organs during *Arabidopsis* development. In each case, developmentally regulated changes in PIN polar localization redirect auxin fluxes to create local auxin gradients required for the establishment of primordia and their development into mature organs. Our data suggest that efflux-mediated auxin gradients represent a common module that operates in the formation of all plant organs, regardless of their developmental origin and fate.

Results

Auxin and Auxin Response Gradients in Lateral Root Primordia

The synthetic auxin responsive promoters such as DR5 (Ulmasov et al., 1997) have become useful tools for monitoring auxin response in planta. It has been shown that activity of these reporters correlates with auxin content in roots (Casimiro et al., 2001). We examined auxin response during lateral root formation using *DR5::GUS* (Figure 1A) and *DR5rev::PEH A*, which gave identical results. DR5 activity was absent from the entire differentiated root and was detected only within the pericycle in the presumptive founder cells of primordia (stage 0). After the formation of short initial cells by anticlinal divisions (stage I), staining was detected in all these cells. When outer and inner layers were established by periclinal divisions (stage II), staining was confined to the central cells of both layer derivatives. During the progression to later stages (III to emergence, e), a gradient in DR5 activity with its maximum at the primordium tip was gradually established. In the mature lateral root, DR5 activity was highest in columella initials and surrounding cells (Figure 1B), identical to the reported pattern in primary roots (Sabatini et al., 1999).

The activity of the DR5 reporter does not necessarily reflect auxin levels in all cases because of its own sensitivity threshold and saturation of the auxin-signaling pathway. Therefore, we examined whether DR5 can be used in lateral root primordia as a reporter of endogenous auxin levels. The exogenously applied naturally occurring auxin IAA or its synthetic analog NAA (up to 10 μM, 12 hr) increased the intensity of the DR5 signal in the primordia without changing its spatial pattern (data not shown) similar like it has been shown in the primary root (Friml et al., 2002a). In contrast, the same treatment with another auxin analog 2,4-D, which can be only poorly transported by auxin efflux (Delbarre et al., 1996) or metabolized, induced DR5 activity in all lateral root primordium cells (Figure 1C). In addition, inhibition of efflux by the phytotropin NPA interfered with the auxin response gradient (Figure 1D). These data demonstrate an active auxin efflux in lateral root primordia, which can handle exogenously applied auxin and in this manner maintain the endogenous auxin distribution. It also suggests that the spatially restricted signals in untreated roots reflect differences in auxin levels. To obtain additional confirmation, the distribution of auxin itself was visualized using an anti-IAA antibody, which is specific to IAA (Caruso et al., 1995). In developing lateral roots, IAA was detected already at the earliest primordium stage in short initial cells, subsequently, the staining was confined to central cells, and later the maximum in IAA accumulation was established at the primordium tip (Figure 1E). In the mature lateral root, IAA accumulated around the columella initials (Figure 1F). The incubation of roots with IAA led to a visible increase in

the signal intensity (Figure 1E, inset), whereas experiments omitting either the primary antibody or the IAA fixation by EDAC (Figure 1F, inset) did not yield any distinct staining pattern. Thus, the IAA accumulation pattern mirrored the DR5 activity pattern. In summary, these results suggest a dynamic auxin gradient during lateral root primordia development that is mediated by auxin transport.

PIN Expression and Localization in Lateral Root Primordia

The best-characterized molecular components of auxin transport are the PIN auxin efflux regulators. The entire *PIN* family in *Arabidopsis* consists of 8 members. *PIN5* and *PIN8* lack the entire middle hydrophilic region present in all other characterized PIN proteins and might be therefore functionally divergent. To examine which PIN proteins may play a role in auxin distribution during primordia development, we first analyzed *PIN* expression in lateral roots using *PIN1, 2, 3, 4, 6::GUS* and *PIN7:GUS* transgenic plants and confirmed the staining patterns by protein localization experiments (Figure 2). All analyzed *PIN* genes were expressed from earliest developmental stages on, except *PIN2*, which was expressed only after primordium emergence (e) with PIN2 localized in epidermis cells toward the base of the lateral root (Figures 2B and 2C). In contrast, *PIN1* expression was detected from stage II on only in the derivatives of inner layer cells (Figure 2A). *PIN3* was expressed at the base of the primordium and from stage V on additionally in columella precursors of the newly forming meristem (Figures 2D and 2E). The *PIN4* expression pattern was partially overlapping, although more restricted to the margins (Figures 2F and 2G). Interestingly, a newly isolated *PIN* gene, the primordia-specific *PIN6*, was also expressed from the earliest stages on. Later, its expression was detected exclusively in primordium margins (Figures 2H and 2I). *PIN7* displayed a similar expression pattern in the margins; however at later stages, *PIN7* expression became restricted to inner, provascular cells, mirroring *PIN1* expression (data not shown). Thus, at least 6 *PIN* genes are expressed during lateral root primordium development in specific, partially overlapping patterns. This represents the molecular basis for a transport system that can mediate auxin redistribution during primordium development.

DR5 Activity Gradients and Lateral Root Formation in *pin* Mutants

To test whether differentially expressed PIN proteins play any role in auxin gradients and lateral root formation, we analyzed primordium initiation and development as well as the spatial pattern of DR5 activity in *pin* mutants and in *35S::PIN1* transgenic plants. *35S::PIN1* overexpressed PIN1 in all cells of the lateral root primordium (Figure 4C). The density of initiated lateral root primordia (from stage I on) in 5- to 10-days old seedlings was scored. *Pin3* (54%; Student's t-test: $P < 0.002$), *pin7* (170%; $P < 0.008$) and *35S::PIN1* (153%; $P < 0.02$) seedlings showed significant changes in number of initiated lateral roots compared to control (100%; $n = 147$), *pin1* (91%; $n = 76$) or *pin2* (93%; $n = 32$) seedlings. This suggests the requirement of various PIN proteins for the lateral root initiation.

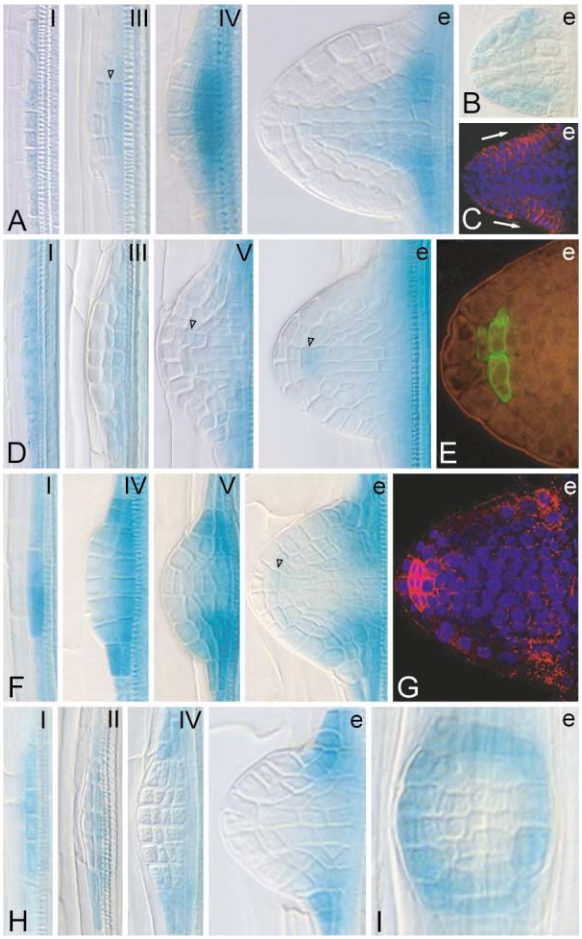

Figure 2. PIN Expression and Localization Patterns during Lateral Root Formation

(A) *PIN1::GUS* expression in derivatives of inner cells. Arrowhead marks the border between inner and outer cells.
(B) *PIN2::GUS* in outer cells.
(C) PIN2 polar localization pointing away from the tip (arrows).
(D) *PIN3::GUS* at the base and later appearing in the forming meristem (arrowhead).
(E) PIN3 localization in the columella precursors of forming lateral root meristem.
(F) *PIN4::GUS* at the base, in the margins, and later in the meristem (arrowhead).
(G) PIN4 localization in margins and the meristem.
(H) *PIN6::GUS* in margins.
(I) *PIN6::GUS*, face view.
Developmental stages are indicated in the upper right corner.
GUS staining in blue (A, B, D, F, H, and I), protein localization signal in red (C and G) or green (E), nuclear counterstain blue (C and G).

In order to assess the developmental rate of primordia, we determined the proportion of primordia, which had reached emergence stage in 6-day-old plants. About 20% of primordia in control plants (21%; $n = 159$) or *pin2* mutants (29%; $n = 296$) reached the emergence stage, whereas this proportion was significantly lower in *pin1* mutants (7%; $P < 0.001$) and *35S::PIN1* (14%; $P < 0.003$), indicating slower primordia development. Also, the pattern of DR5 activity was analyzed in primor-

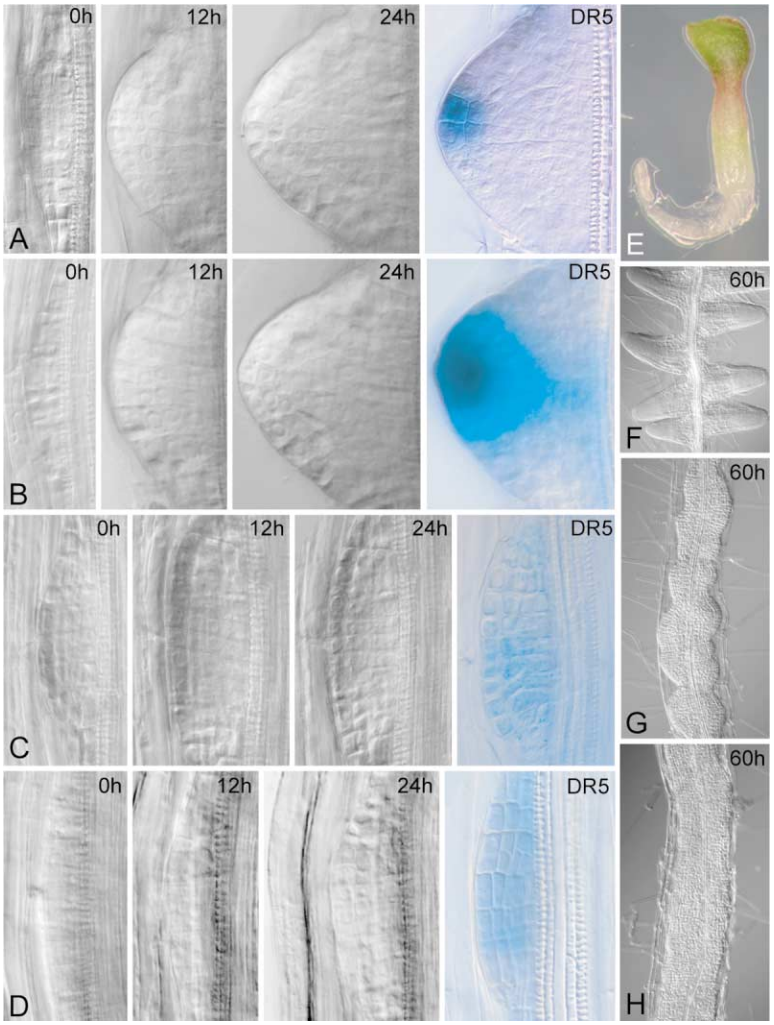

Figure 3. In Vivo Monitored Correlation between Individual Primordium Development and the DR5 Gradient

(A and B) Wild-type (A) and *pin2* (B) primordium development with correctly established DR5 gradient.

(C) Retarded *pin1* primordium development without established DR5 gradient.

(D) Retarded *35S::PIN1* primordium development, no DR5 gradient.

(E) Postembryonic phenotype of *pin1 pin3 pin4 pin7* mutant: very short root and cotyledon defects.

(F–H) Induction of lateral root development by NAA treatment. Normal primordia development in wild-type (F) seedlings. Affected primordia development in *pin3 pin7* (G) and *pin1 pin3 pin4* (H) mutants.

Numbers indicate time points during primordium development (A–D) or period of NAA treatment (F–H). GUS staining in blue (A–D).

dia between stage IV and emergence. In control plants, most primordia at these stages displayed the typical DR5 pattern with maximum at the tip (see Figures 1A and 3A). However, some primordia (27%; n = 138) did not establish the DR5 gradient at this stage. Interestingly, in *pin2* seedlings the DR5 gradient was formed in all primordia at these stages (100%; n = 24; Figure 3B). By contrast, more than half of the *pin1* (62%, n = 95) or *35S::PIN1* primordia (77%, n = 56) displayed rather diffuse DR5 patterns (Figures 3C and 3D). These data demonstrate that the differentially expressed PIN proteins play distinct roles in both the initiation and development of primordia as well as in the establishment of the auxin gradient during lateral root formation.

In Vivo Analysis of Lateral Root Development

We established an experimental system for in vivo analysis of primordium development, which enabled us to study lateral root formation in relation to the PIN-dependent auxin distribution in more detail. Four hours time-lapse observation of individual primordia revealed that they typically developed within 24 hr from stage II–III to stage VII or emergence (Figure 3A). A smaller proportion of primordia (16%, n = 91) developed more slowly. Few primordia (6%) displayed an irregular shape. In *pin1* mutants (Figure 3C; 43%, n = 88) and *35S::PIN1* (Figure 3D; 65%; n = 54), highly increased frequencies of primordia with retarded growth were found. In addition, in *pin1* (29%) but not in *35S::PIN1* (7%) plants the proportion of primordia with irregular shape was increased. Interestingly, *pin2* primordia (Figure 3B; 8%; n = 36) typically developed faster than those of control plants. Regardless of seedling genotypes, the individual primordia, which developed normally, displayed normal DR5 activity distribution, whereas abnormal development was accompanied by defects in DR5 activity distribution (Figures 3A–3D). In conclusion, these findings support a correlation between PIN-dependent auxin gradients and lateral root primordia development. Notably, also in control plants certain variability in the rate of lateral root development was observed, with some primordia showing less DR5 signal accumulation at the tip and slower development. It is well known that the initiation of the lateral roots as well as their outgrowth is variable and largely depends on the availability of the nutrients and minerals (Dubrovsky et al., 2000). It is possible that the adaptation mechanism regulating the rate of lateral roots development is based on the balanced auxin supply to the tip and its PIN2-dependent retrieval through outer layers of the primordium.

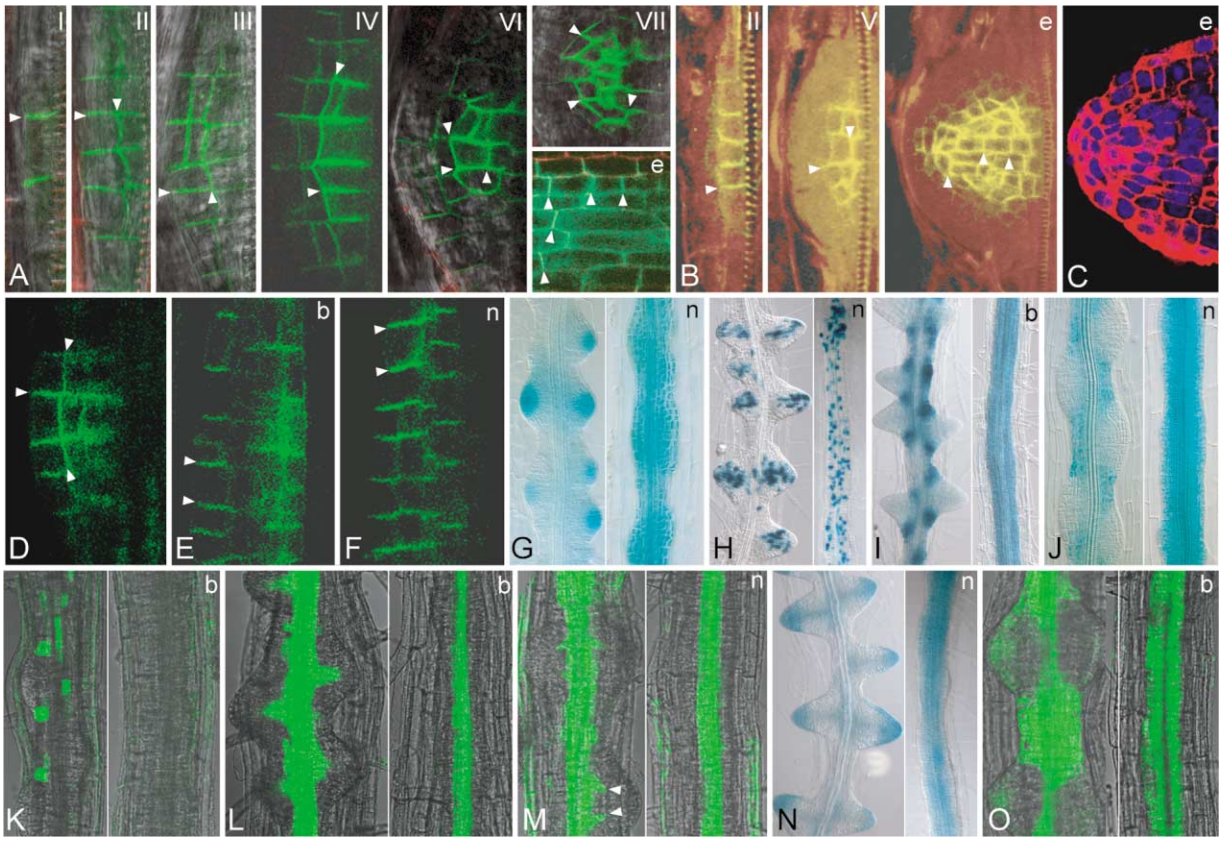

Figure 4. Correlation of PIN1 Relocation, DR5 Gradients, and Primordium Development

(A and B) PIN1:GFP (A) and PIN1 (B) localization in developing lateral root primordium with gradual establishment of PIN1 at the lateral cell membranes.

(C) Nonpolar PIN1 localization in all cells of *35S::PIN1* lateral root primordium.

(D–F) PIN1:GFP rearrangement in primordium initiated after IAA treatment (D), no relocation to lateral sides after IAA + BFA (E) or IAA + NPA treatments (F).

(G–O) Misexpression of molecular markers after inhibition of PIN1 relocation. Left image after treatment with IAA, right image after IAA + NPA (n) or BFA (b). Auxin response: *DR5rev::PEH A* (G); cell division: *CycB1:GUS* (H); margins: *CUC3::GUS* (I); *PIN6::GUS* (J) and M0223 (K); inner layer: Q0990 (L); pericycle (arrowheads): M0171 (M); columella and protophloem: *AUX1::GUS* (N); stele of the primary root: Q0680 (O).

Arrowheads indicate PIN1 polar localization (A,B, D–F). GUS and PEH A signal in blue (G–J and N), GFP signal in green (A, D–F, K–M, and O), PIN1 signal in yellow (B) or red (C), nuclear counterstain blue (C).

PIN Functional Redundancy in Lateral Root Formation

The fact that defects in lateral root formation are only mild in single *pin* mutants together with the partially overlapping expression pattern of various PIN proteins suggested functional redundancy between PIN proteins. Therefore, we analyzed multiple *pin* mutants with respect to the lateral root development. The lateral root phenotypes of the multiple mutants were either additive or difficult to interpret, since also the primary root development was impaired few days after germination. For example, *pin1 pin3* roots initiated fewer primordia (58%, $P < 0.01$), which developed more slowly (only 6% emerged roots compared to 21% in controls, $P < 0.001$). On the other hand, *pin1 pin3 pin4* primary roots displayed defects a few days after germination (data not shown) and the *pin1 pin3 pin4 pin7* quadruple mutants showed strong defects already in embryonic development (Figure 3E). To analyze specific defects in lateral root formation in multiple *pin* mutants and to better distinguish defects in initiation and development, lateral root formation was artificially initiated by exogenous NAA application on impaired primary roots. In control roots, lateral root formation was initiated throughout the whole pericycle; regularly spaced primordia were established and developed into mature lateral roots (Figure 3F). By contrast, in *pin3 pin7* (Figure 3G), *pin4 pin7*, and increasingly strong in *pin1 pin4 pin7* or *pin1 pin3 pin7* (data not shown) mutants, less well-defined primordia developed. In *pin1 pin3 pin4* seedlings (Figure 3H), only massive division of pericycle cells without any trace of defined primordia was detected. Exactly the same defects were found in wild-type seedlings upon inhibition of auxin transport (Figures 4G–4O) or in weak *gnom* mutant alleles (Geldner et al., 2003b). These increasingly stronger defects in multiple *pin* mutants demonstrate that auxin efflux, which is dependent on functionally redundant PIN proteins, is essential for primordium development.

Rearrangement of PIN1 Polarity in Lateral Root Primordia

Asymmetric subcellular localization of various PIN proteins has been shown to correspond to the direction of

auxin flux (reviewed in Friml, 2003). We examined the polarity of PIN1 localization during primordium development. To gain insight into its dynamics, we constructed transgenic plants carrying functional PIN1:GFP, which showed a pattern of expression and localization identical to the endogenous PIN1 (Figures 4A and 4B). At the earliest stage, PIN1:GFP was detected exclusively on the transverse (anticlinal) sides of the short initial cells. From stage II on, the GFP signal was found in addition at lateral (periclinal) sides and later the polarity pointed more and more toward the primordium tip (Figure 4A). In the emerged lateral roots, PIN1 localization pointed predominantly toward the root apex, giving the same localization pattern, which was reported for the primary root (Friml et al., 2002a). The same relocation of PIN1 occurred also in primordia, which were initiated after exogenous IAA or NAA applications (Figure 4D). Interestingly, the PIN1 relocation to the lateral sides was prevented by substances, which have been shown to interfere with the subcellular movement of PIN proteins (Geldner et al., 2001), such as the protein trafficking inhibitor brefeldin A (BFA) or phytotropins such as NPA (Figures 4E and 4F). It has been shown previously that GNOM, a regulator of BFA-sensitive vesicle trafficking, mediates the establishment of PIN1 polarity in embryogenesis (Steinmann et al., 1999). We tested whether GNOM also mediates the PIN1 relocation during lateral root formation. This is indeed the case since in transgenic plants expressing an engineered BFA-resistant version of GNOM (*GNOM^{M696L}-myc*), BFA did not perturb PIN1 relocation (data not shown, similar to Figure 4D). Thus, GNOM-dependent rearrangement of PIN1 localization occurs during lateral root development suggesting a gradually established auxin flux through the inner cells toward the primordium tip.

PIN1 Relocation Correlates with DR5 Gradients and Primordium Development

The PIN1 polarity rearrangement from the transverse to the lateral sides of cells correlated with the establishment of the auxin gradient with its maximum at the primordium tip. To analyze this relationship and its consequences for primordium development, we induced synchronized initiation of lateral root primordia by exogenous IAA or NAA application. In control experiments, primordia were initiated, PIN1 relocated (Figure 4D), DR5 gradients properly formed (Figure 4G, left), and primordia developed normally (up to stage VII within 48 hr). In contrast, when PIN1 relocation was prevented either by BFA or NPA treatment (Figures 4E and 4F), initiation still occurred, nonetheless, no DR5 gradients were established (Figure 4G, right) and only a massive division of pericycle cells without clear separation into primordia was observed similar to multiple *pin* mutants (see Figure 3H). In the *GNOM^{M696L}-myc* line, however, only NPA (data not shown, but identical to Figures 4F and 4G, right) but not BFA (data not shown, identical to 4D and 4G, left) interfered with PIN1 relocation and primordium development. This data shows that under all testable conditions the coordinated rearrangement of PIN1 correlates with DR5 gradients and correct primordium development.

For a detailed analysis of the NPA- or BFA-induced defects in primordium development, we made use of various molecular markers. All these markers showed in the IAA-induced primordia the same expression pattern as in untreated seedlings with exception of margin marker M0223, which was undetectable in untreated roots (data not shown). The cell division marker *CYCB::GUS* confirmed that in differentiated root parts after IAA treatment, cell division activity was confined exclusively to developing primordia (Figure 4H, left). In contrast, in NPA or BFA treated seedlings, *CYCB::GUS* expression was detected along the whole pericycle (Figure 4H, right). Stele markers Q0680 (Figure 4O) and Q1630 (data not shown) discontinued expression in the pericycle of the primary root at positions of developing primordia, but were homogeneously expressed upon NPA and BFA treatment. The establishment of primordium margins was analyzed using three different markers—*CUC3::GUS*, *PIN6::GUS*, and M0223. After IAA treatment, these markers were specifically expressed only in primordium boundaries (Figures 4I–4K, left). After concomitant NPA or BFA treatment, however, *CUC3::GUS* (Figure 4I) or *PIN6::GUS* (Figure 4J) showed a homogenous expression, whereas M0223 expression was not initiated (Figure 4K). Also the coordinated differentiation of various cell types in developing primordia, such as stele (Q0990; Figure 4L), pericycle (M0171; Figure 4M), or vasculature and lateral root cap (*AUX1::GUS*; Figure 4N), did not occur in NPA- or BFA-treated seedlings. Thus, molecular markers further confirmed that in absence of coordinated PIN-dependent auxin transport, dividing pericycle cells do not develop into lateral root primordium-like structures, they do not define boundaries and various primordium-specific cell types.

PIN-Dependent Auxin Distribution in Cotyledon Formation

We analyzed whether a PIN-mediated auxin gradient dependent mechanism operates in other organogenetic processes. In *Arabidopsis*, two symmetrically positioned embryonic leaves (cotyledons) are initiated at the triangular stage of embryogenesis and develop subsequently (Mayer et al., 1991). From the triangular stage on, beside an apical-basal auxin gradient, additional sites of *DR5rev::GFP* activity (Figure 5A) as well as IAA accumulation (Figure 5B) were detected at the tips of incipient cotyledons. As reported, PIN1 was localized to the apical ends of the cells in outer embryo layers (Steinmann et al., 1999). Analysis of PIN1:GFP revealed that its polar localization pointed exactly toward the position of auxin accumulation in incipient cotyledon tips (Figure 5N). Inside the primordium, PIN1 localization was gradually established at the basal side of future vasculature cells during cotyledon development (Figures 5H–5L). In analogy to lateral root development, NPA and BFA interfered with the gradual establishment of PIN1 polarity (Figure 5M) as well as with DR5 gradient and cotyledon establishment in in vitro cultured embryos (Figure 5D). Similar defects were observed also in *gnom* mutant embryos (Figure 5C). As expected in *GNOM^{M696L}-myc* embryos, the BFA effect on PIN1 localization was not observed and hence the DR5 gradients and cotyledons formed normally (Figure 5E compare to 5D). Moreover, *pin1* (Figure 5F; Okada et al., 1991), *pin4 pin7* (Figure 5G), and *pin1 pin3 pin4 pin7* (Figure 3E)

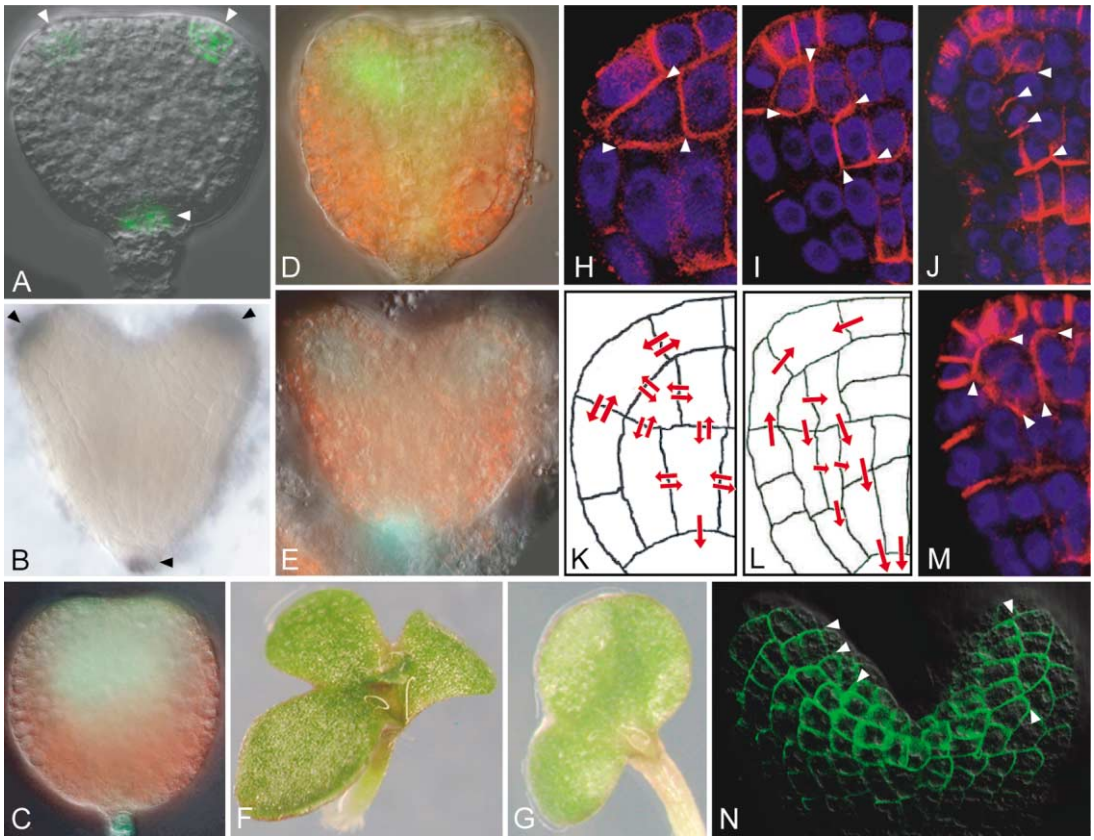

Figure 5. PIN-Dependent Auxin Gradients in Cotyledon Formation

(A) *DR5rev::GFP* in triangular embryo. Signals (arrowheads) visible in the basal region and in the tips of incipient cotyledons.
(B) IAA immunolocalization mirroring the DR5 pattern in heart stage embryo.
(C) Defects in the DR5 gradients and cotyledon formation in the *gnom* embryo.
(D and E) BFA treatment: defects in DR5 gradient and cotyledon formation in control embryo (D). No BFA effects on DR5 gradients and cotyledon formation in BFA resistant *GNOM*^{M696L}*-myc* embryo (E).
(F and G) Defects in cotyledon formation in *pin1* (F) and *pin4 pin7* (G) mutants.
(H–J) PIN1 localization in embryos. In the globular embryo stage, the basal PIN1 localization in cells below incipient cotyledons is not established yet (H). Gradual establishment of PIN1 basal localization at triangular (I) and early heart (J) stages.
(K and L) Schematics of putative PIN1-dependent auxin fluxes (arrows) at globular (K) and triangular (L) stage.
(M) NPA treatment prevents the establishment of PIN1 basal localization in provascular cells.
(N) PIN1:GFP polarity in the epidermis of heart embryos pointing toward the incipient cotyledon tips.
Arrowheads indicate PIN1 polar localization (H–J, M, and N). GFP signals in green (A, C–E, and N). IAA signal in brown (B) and PIN1 signal in red (H–J, and M). Nuclear counterstain in blue (H–J, and M).

mutants displayed increasingly stronger defects in cotyledon formation. These data demonstrate, in analogy with lateral roots, a correlation between the GNOM-dependent establishment of PIN1 polar localization, auxin gradients, and cotyledon formation. However, the direction of auxin flux is opposite to that in lateral root primordia. In incipient cotyledons, the auxin gradient is established by transport through the outer layer toward the primordium tip. From there auxin appears to be "drained" into the primordium interior and through the future vascular system toward the basal embryo pole.

PIN-Dependent Auxin Distribution in Shoot-Derived Organogenesis

Various shoot-derived organs such as leaves, flowers with different floral organs, and ovules are formed post-embryonically, contributing largely to the final shape of the mature plant. We analyzed the spatial pattern of DR5 activity, PIN1 localization as well as consequences of auxin efflux inhibition on these processes. Using *DR5rev::GFP* plants, we detected local DR5 activity gradients with a maximum at the tip of primordia in all inspected organs, such as leaves or flowers (Figure 6A), all floral organs (Figure 6C), ovules (Figure 6L), as well as integuments (Figure 6N). Also PIN1 and PIN1:GFP localizations were detected in all primordia, such as those flanking the shoot (Figure 6E) or floral (Figure 6F) meristems, as well as those of floral organs (Figures 6G and 6H), ovules (Figures 6M and 6P), or ovules with integuments (Figure 6O). In all cases, PIN1 was expressed in the outer cell layer with its polarity pointing toward the primordium tip (Figures 6H and 6F, insets). In the inner future vascular cells, PIN1 localization was gradually established toward the basal ends during primordium development (Figure 6E, inset). Inhibition of auxin efflux interfered with the DR5 gradients in the

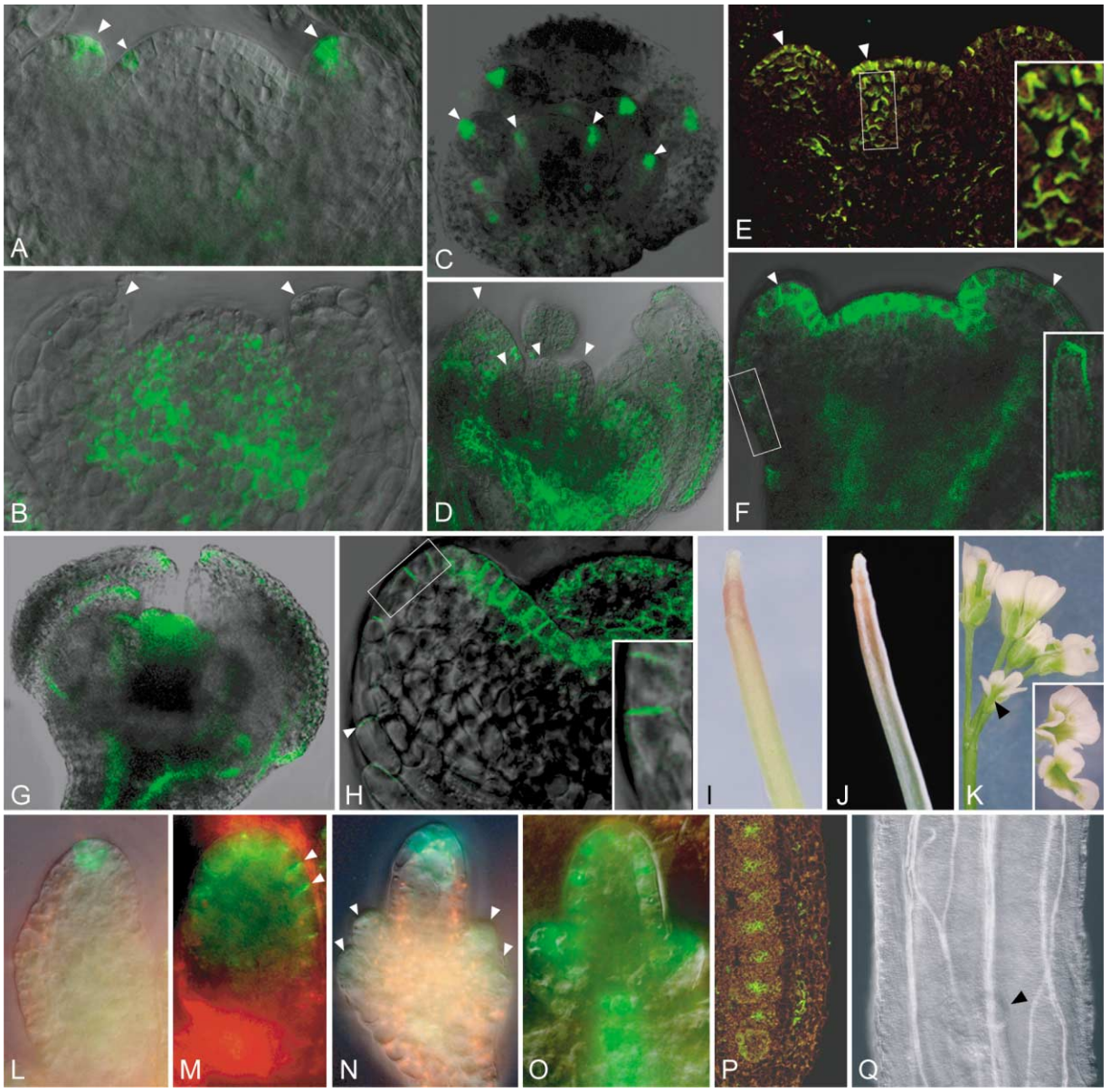

Figure 6. PIN-Dependent Auxin Gradients in Shoot-Derived Organ Formation

(A and B) *DR5rev::GFP* in the apical meristem. Accumulation of signals visible at the position of incipient primordia and their tips (A). Auxin efflux inhibition by NPA interferes with the DR5 signal at tips (B).

(C and D) *DR5rev::GFP* in developing flowers. Signals visible at the tips of all floral organ primordia (C). Auxin efflux inhibition by NPA interferes with the DR5 signals at tips (D).

(E and F) PIN1 localization in apical (E) and secondary floral (F) meristems. In the incipient vasculature below the primordia, PIN1 basal localization is gradually established (inset in E). PIN1 localizes in the epidermis cells toward primordia tips (inset in F).

(G) PIN1:GFP in epidermis and incipient vasculature in developing flowers.

(H) PIN1:GFP polar localization (inset) pointing toward the tips of floral organ primordia.

(I) Pin-like inflorescence in wild-type plant grown on NPA.

(J) *pin1* inflorescence without the initiated lateral organs.

(K) Defective flowers in *pin3 pin7* mutants. Missing stamens and carpels indicated (arrowheads). Inset shows example with missing sepals, petals, and fused petals.

(L and M) Young ovule primordium. *DR5rev::GFP* signal at the tip (L). PIN1:GFP polar localization in outer layer (M).

(N and O) Older ovule primordium. *DR5rev::GFP* signal at the tip and at tips of developing integuments (arrowheads) (N). PIN1:GFP polar localization in outer layer and in inner cells (O).

(P) PIN1 signals in developing ovule primordia in wild-type gynoecium.

(Q) *pin1* gynoecium, no or entirely misshaped (arrowhead) ovules develop.

Arrowheads mark incipient primordia and their tips (A–E, and N) or PIN1 polar localization (F, H, and M). GFP signal in green (A–D, F–H, and L–O) and PIN1 signal in yellow (E and P).

primordia. For example, treatment of the shoot apical meristem (Figure 6B) or developing flowers (Figure 6D) with NPA or BFA resulted in withdrawal of the DR5 signal from primordium tips and its accumulation in central parts below the apex. The long-term inhibition of auxin efflux by these substances led to the formation of a pin-like inflorescence (Figure 6I)—a plant strikingly similar to the *pin1* mutant (Figure 6J; Okada et al., 1991). Loss of PIN1 function was shown to cause defects in all post-embryonic organogenetic processes such as development of leaves, flowers, and floral organs (Okada et al., 1991). We found also ovule defects, since in flowers, which occasionally develop, the gynoecium resembled hollow tubes with no ovules or few malformed structures (Figure 6Q, compare to 6P). The strong defects in the *pin1* mutant after transition to flowering render PIN1 as the main and nonredundant regulator of organogenesis at this stage. Nonetheless, flower defects were found also in other *pin* mutants, such as *pin3 pin7*, which developed abnormal flowers with fused petals, no stamens, and occasionally no sepals (Figure 6K). All these observations suggest that also in shoot-derived organ formation, PIN-dependent efflux mediates primordium development by supplying auxin to the tip through the outer layer, from where auxin is drained through the primordium interior into the vascular network.

Discussion

The architecture of the adult plant is largely dependent on the formation of a variety of new organs during post-embryonic development. These organs are initiated as lateral primordia and subsequently attain diverse morphologies, due to the expression of different sets of regulatory genes. Thus, developmental fates of organ primordia are conditioned by their developmental history, ultimately going back to the fundamental decision of shoot versus root in embryogenesis.

We have addressed the developmental issue of organ formation in general to identify common underlying mechanisms, regardless of the developmental origin and fate of individual primordia.

PIN-Dependent Auxin Gradients in Lateral Root Formation

In *Arabidopsis*, lateral roots are initiated within the pericycle, developing primordia display a nearly invariant cell division pattern and proceed through several well-defined stages. Eventually, a new meristem is formed, which is anatomically identical to the primary root meristem (Malamy and Benfey, 1997). Auxin promotes lateral root initiation, as was demonstrated by exogenous auxin application (Blakely and Evans, 1979) and by the analysis of mutants affected in auxin biosynthesis (Barlier et al., 2000) or response (Fukaki et al., 2002). We found that auxin accumulates at the position of future primordia. At later stages, an auxin gradient is gradually established with its maximum at the primordium tip. This gradient is dependent on auxin efflux mediated by differentially expressed, functionally redundant PIN proteins. Moreover, the polar localization of PIN1 gradually changes to the cell surface facing the future tip. This rearrangement correlates with and is required for the auxin gradient. Interference with either PIN-dependent auxin transport or PIN relocation causes defects in both auxin gradient and lateral root formation. All the available correlative evidence from both genetic and physiological experiments taken together with what is known about the mechanism of auxin transport, suggest the following model of how primordium development is regulated. First, auxin accumulates at the position of the future primordia, cell division is activated and subsequently, endogenous signals mediate retargeting of continuously cycling PIN proteins. As a consequence, auxin transport is redirected, providing auxin from the root vasculature via the interior of the primordium into the tip from where auxin is transported away through the outer layer ("fountain" model; Figure 7A). Thus, the combined action of differentially expressed and localized PIN proteins results in the formation of the auxin gradient, which mediates proper lateral root development.

PIN-Dependent Auxin Gradients in Aerial Organs Formation

Plant aerial organs such as cotyledons, leaves, and floral organs have long been regarded as homologous structures (Esau, 1977). In all aerial organ formation, we observed similar correlations between the PIN-dependent auxin efflux, auxin gradients, and primordia development as for lateral root formation. However, in contrast to the "fountain" flow in the root, the direction of auxin transport appears to be reversed. In aerial organs, auxin is supplied through the outer layer and accumulates at the primordium tip, from which it is drained into the interior of the primordium and transported downward through its middle ("reverse fountain"; Figure 7B). Following this route, new vasculature differentiates and thus, the newly formed organ is connected to the existing vascular strands in more mature parts of the plant. Another distinguishing feature seems to be a dominant role of PIN1 as regulator of both local auxin distribution and organogenesis in the shoot apex, based on the strong defects in the *pin1* mutant after transition to flowering. Despite these differences, a common auxin gradient-dependent mechanism seems to underlie primordium development of both aerial and underground organs.

A Common Developmental Module for Organogenesis in Plants

During organ formation, first a site of primordium initiation has to be selected and then a new growth axis has to be established. Our results suggest that redirection of auxin flow and local accumulations of auxin play a fundamental role in these processes. As a first step, auxin accumulates at the site of initiation. Already this step might be mediated by PIN-dependent auxin transport, as evidenced by organ initiation and positioning defects observed in *pin* mutants or caused by manipulation of local auxin distribution (Reinhardt et al., 2000; Laskowski et al., 1995). In a second step, cell division is activated and the auxin transport machinery reorganizes. Regardless of the type of primordia, the new direction of PIN-mediated auxin transport determines the growth axis of the developing organ, establishing an auxin gradient with its maximum at the tip.

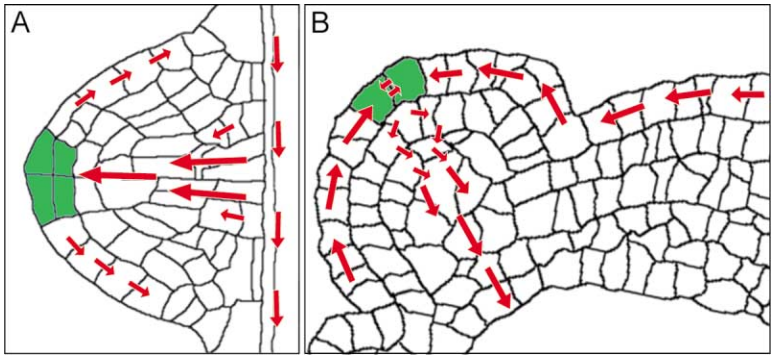

Figure 7. Model for Auxin Transport and Distribution in Root- and Shoot-Derived Organ Primordia

(A) Lateral root primordium: auxin is provided by PIN-dependent auxin transport through the primordium interior toward the tip, where it accumulates. From here, part of the auxin is retrieved by a PIN2-dependent auxin route through the outer layers.

(B) Aerial organ primordium: PIN1 is a major component for auxin distribution. Auxin is provided to the primordium tip through the outer layers. From the tip, auxin is drained through a gradually established transport route toward the vasculature.

Places of auxin accumulation are depicted in green. Presumptive routes of auxin transport are depicted by red arrows.

Local, efflux-dependent auxin gradients as a common module for organ development may also apply to other plants. Higher plants form an evolutionarily closely related group of species, they share the same set of organs (Esau, 1977), and organ development is affected by auxin and auxin efflux inhibitors treatment in all species tested (for overview see Lomax et al., 1995). Moreover, PIN-related sequences have been found in both dicot and monocot plant species. Therefore, it is likely that the auxin transport-dependent mechanism, which we demonstrated for *Arabidopsis*, also operates in organ formation in other higher plants.

Although developmental strategies differ markedly between plants and animals, both groups of organisms make use of developmental modules. In *Drosophila*, for example, imaginal discs give rise to morphologically diverse organs, such as antennae, legs, wings, or genitals. Organs of one type can be transformed into another type, either by mutation or transdetermination, revealing the developmental similarity of organ primordia (Maves and Schubiger, 1999). The underlying patterning process involves gradients of morphogens, such as Decapentaplegic or Wingless, that regulate development of all organs, regardless of their origin and fate (Cadigan, 2002). Thus, modular development in organ formation and patterning seems to be a general principle of higher life forms.

Experimental Procedures

Used Materials

The *DR5::GUS* (Ulmasov et al., 1997); *gnom* (Mayer et al., 1993); *GNOM^{M696L}-myc* (Geldner et al., 2003a); *CYCb::GUS* (Ferreira et al., 1994); *CUC3::GUS* (Vroemen et al., 2003); *AUX1::GUS* (Marchant et al., 1999); *pin1-1* (Okada et al., 1991); *pin1::En134* (Gälweiler et al., 1998); *pin2* (Müller et al., 1998); *PIN2::GUS* (Friml et al., 2003); *PIN3::GUS, pin3-2*, *pin3-3* (Friml et al., 2002b); *DR5rev::PEH A, PIN4::GUS*, and *pin4-3* (Friml et al., 2002a) have been described. The *pin1-1* and *pin2* (*eir1-1*) mutant lines with introduced *DR5::GUS* have been generated previously (Sabatini et al., 1999). *DR5rev::GFP* has been generated by replacing *PEH A* in *DR5rev::PEH A* by ER-targeted eGFP coding sequence (Clontech). GFP-based marker lines were isolated from the library based on the GAL4/*UAS* transactivation system (Haseloff, 1999). *PIN1::GUS* and *PIN6::GUS* (AF087819) constructs were generated by fusing PCR-amplified fragments (nucleotides −1289 to −5 and −1794 to −1) with the *uidA* gene. The *PIN7:GUS* (AF087820) translational fusion was generated by fusing *uidA* gene to the C terminus of the *PIN7* coding sequence. *pin7-1* (http://genetrap.cshl.org/) and *pin3-5* (http://signal.salk.edu/cgi-bin/tdnaexpress) mutant lines have sequence-indexed insertions at positions 1349 and −51 from ATG, respectively. The *pin1*, *3*, *4*, *7* multiple mutants were generated from *pin1*, *pin3-5*, *pin4-3* and *pin7-1* single mutant alleles. *35S::PIN1* plants were generated by fusion of the CaMV35S promoter with the *PIN1* coding sequence. PIN1:GFP was generated by insertion of mGFP4 into the *PIN1* genomic fragment (nucleotides −2320 to 3508 from ATG) at position 1510. Its functionality was verified by crossing of three independent transgenic lines into *pin1* mutants, in total 350 F2 plants were analyzed for rescue. The anti-IAA (1: 500; agdia, http://www.agdia.com), -PIN1 (1: 300; Gälweiler et al., 1998), -PIN2 (1: 200; Müller et al., 1998), -PIN3 (1: 50), and -PIN4 (1: 400; Friml et al., 2002a, 2002b) antibodies have been described. Alkaline phosphatase-conjugated antimouse (Novagen), FITC-, and CY3-conjugated antirabbit secondary antibodies (Dianova) were diluted 1:1000, 1:200, and 1:600, respectively.

Growth Conditions

Arabidopsis plants and seedlings were grown as described (Friml et al., 2002a). Exogenous drug application was performed by incubation of 5 days old seedlings in 0.5 × MS liquid medium supplemented with 1–25 μM IAA, NAA, 2,4-D, NPA, and BFA for up to 60 hr. NPA treatment of floral primordia was described (Nemhauser et al., 2000). For in vitro embryo culture, excised ovules were placed on 0.5 × MS media containing 2% sucrose, 400 mg/l glutamine, and 0.3% Phytagel supplemented with 10–20 μM BFA or NPA. Plates were kept in the dark at 22°C for up to 4 days. For ovule development analysis, carpels were dissected, fixed in ethanol/acetic acid (3:1), and mounted in chloralhydrate. For phenotypic analyses of lateral root initiation and development, at least 20 seedlings were processed (Malamy and Benfey, 1997) and inspected. For in vivo analysis of individual lateral root primordia development, 5-day-old seedlings were transferred on slides with a thin layer of 0.5 × MS, 0.5% agarose medium, covered, supplemented with 0.5 × MS liquid medium, and incubated over night in a humid chamber. For all comparisons, at least three independent experiments were performed giving the same statistically significant results. Data were statistically evaluated using ProStat 1.02 (PolySoftware International).

Expression and Localization Analysis

Histochemical staining for GUS activity, whole-mount PIN immunolocalization (Friml et al., 2003; Steinmann et al., 1999) as well as immunolocalization at sections (Gälweiler et al., 1998) were performed as described. IAA immunolocalization was done after prefixation with 3% EDAC for 1 hr. GFP was visualized in 5% glycerol without fixation. Microscopy was done on a Zeiss Axiophot equipped with an Axiocam HR CCD camera. For confocal laser scanning microscopy, a Leica TCS SP2 was used. Images were processed in Adobe Photoshop 7.0 (Adobe Inc.).

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