A Molecular Framework for Plant Regeneration

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Plants and some animals have a profound capacity to regenerate organs from adult tissues. Molecular mechanisms for regeneration have, however, been largely unexplored. Here we investigate a local regeneration response in *Arabidopsis* roots. Laser-induced wounding disrupts the flow of auxin—a cell-fate–instructive plant hormone—in root tips, and we demonstrate that resulting cell-fate changes require the PLETHORA, SHORTROOT, and SCARECROW transcription factors. These transcription factors regulate the expression and polar position of PIN auxin efflux–facilitating membrane proteins to reconstitute auxin transport in renewed root tips. Thus, a regeneration mechanism using embryonic root stem-cell patterning factors first responds to and subsequently stabilizes a new hormone distribution.

Among vertebrates, salamanders represent a rarity in their ability to regenerate lost structures, such as limbs. In contrast, the ability to regenerate organs is wide-spread in the plant kingdom. Here we investigate regeneration in *Arabidopsis* roots. In normal root development, the root meristem produces root cap cells on its distal face and produces various cell types on its proximal face (fig. S1). The quiescent center (QC), a mitotically inactive organizing center required for stem-cell maintenance (*1*), lies between the stem cells for these cell populations. The GRAS family transcription factors—SHORTROOT (SHR) and SCARECROW (SCR) (*2*–*5*)—and the auxin-responsive AP2/EREBP (APETALA2/ethylene responsive element binding protein) family transcription factors PLETHORA1 (PLT1) and PLT2, contribute to QC and stem cell patterning (*6*). *PLT* mRNA distribution is regulated by an auxin maximum that is distal to the vascular precursors (*6*, *7*). PIN transmembrane proteins localize asymmetrically at plasma membranes of auxin-transporting cells, correlated with the direction of auxin flow (*8*–*11*). PIN proteins restrict *PLT* transcription and maintain the auxin response maximum by mediating polar auxin transport (PAT) (*8*). Therefore, auxin redistribution after wounding may induce organ regeneration. Here we analyze regeneration after auxin modulation by using patterning and polarity markers in wild type (WT) and key patterning mutants to reveal the interaction network that reestablishes a new root tip.

QC laser ablation results in complete respecification of the root cap and QC from distal provascular tissue (*12*). Root cap and QC markers appeared in the respecified region 24 hours or more after proximal displacement of an auxin response maximum (*7*). To explore the role of auxin in this process, we monitored auxin response and the expression of patterning genes after QC ablation in real time.

A shift in auxin response occurred proximal to ablated QC cells as early as 3 hours after ablation, when the auxin distribution marker DR5rev::GFP (green fluorescent protein) (*10*) (Fig. 1A) appeared in vascular stem cells, cortex/endodermis stem cells, and their daughters (Fig. 1B, inset). DR5rev::GFP formed a new maximum within 16 hours after ablation (Fig. 1B, bracket) and expanded into more cell layers above the ablated QC (Fig. 1, C and D), where starch granules marked distal root cap respecification (Fig. 1D, inset).

pPLT1::ERCFP (endoplasmic reticulum–targeted cyan flourescent protein) predominantly marks the QC and surrounding stem cells (Fig. 1E). pPLT1::ERCFP expression relocalized rapidly around 6 hours after ablation, in line with the auxin response factor dependency of *PLT* genes (Fig. 1F, bracket) (*6*). A more pronounced proximal shift of the pPLT1::ERCFP expression domain was observed from day 2 after ablation onwards (Fig. 1, G and H), tightly following the auxin maximum.

The QC-specific marker pWOX5::ERGFP (Fig. 1I) (*8*) appeared in former cortex/endodermis stem cells and their daughters and in 1 to 2 cell layers proximal to the ablated QC around 16 hours after ablation (Fig. 1J, bracket). pWOX5::ERGFP was gradually confined to central former provascular cells, which acquired other QC markers later (Fig. 1, K and L).

pSHR::SHRGFP is nuclear and cytoplasmic in provascular cells but exclusively nuclear in endodermis and QC (Fig. 1M) (*13*). pSHR::SHRGFP became restricted to the nucleus of provascular cells about 16 hours after ablation (Fig. 1, N to P, arrows).

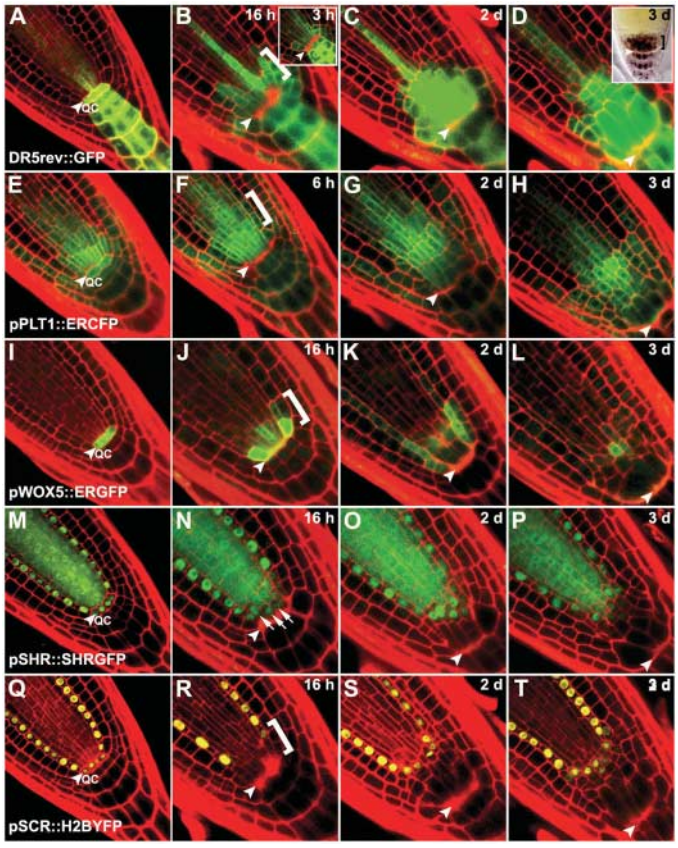

Fig. 1. Dynamic changes of auxin distribution and cell-fate identity markers after QC ablation. (**A** to **D**) DR5rev::GFP. (**E** to **H**) pPLT1::ERCFP. (**I** to **L**) pWOX5::ERGFP. (**M** to **P**) pSHR::SHRGFP. (**Q** to **T**) pSCR::H2BYFP. The left-most column shows controls before ablation. The inset in (B) is 3 hours after ablation. (F) is 6 hours after ablation. (B), (J), (N), and (R) are 16 hours after ablation. The column third from the left shows images 2 days after ablation. The images in the right-most column are 3 days after ablation. Arrowheads indicate QC cells in the left-most column and the position of ablated QC elsewhere. Arrows in (N) point to restricted pSHR::SHRGFP expression in the nucleus of former vascular stem cells. The inset in (B) shows up-regulation of DR5rev::GFP 3 hours after ablation. The inset and bracket in (D) show provascular-columella identity switches by starch granule staining. The brackets in (B), (F), (J), and (R) indicate expression changes of markers. The red signal is propidium iodide (PI) staining. The green signal is GFP except for [(E) to (H)], where it is cyan fluorescent protein (CFP). The yellow signal is yellow fluorscent protein (YFP).

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Endodermis and QC marker pSCR::H2BYFP (Fig. 1Q) (*5*) were downregulated in cortex/endodermis stem cells and their daughters within 16 hours after ablation (Fig. 1R, bracket). pSCR::H2BYFP expression appeared in the respecified region in the following 2 days to mark the new QC, as reported earlier (Fig. 1, S and T) (*7*).

Together, our results reveal that disruption of auxin flow by QC ablation rapidly upregulates the auxin response in 1 to 2 cell layers proximal to the ablated QC, which is followed by cell fate changes. Ablation of columella stem cells provoked similar effects, indicating that regeneration occurs when auxin flow is disrupted (fig. S2).

To reveal whether auxin distribution influences PIN localization, we monitored functional PIN-GFP fusions after QC ablation. pPIN1::PIN1EGFP mainly resides at basal and lateral sides of vascular cells, but a weak GFP signal can be detected in epidermis, cortex, QC, and in columella stem cells (Fig. 2A). No obvious changes of pPIN1::PIN1EGFP polarity appeared within 16 hours after ablation (Fig. 2B); however, pPIN1::PIN1EGFP expression decreased in 1 to 2 cell layers proximal to the ablated QC, associated with the proximally shifted auxin maximum (Fig. 2B, bracket). Over the following 2 days, pPIN1::PIN1EGFP expression was lost in these cells (Fig. 2, C and D).

pPIN2::PIN2EGFP localizes apically in epidermal and lateral root cap cells and predominantly basally in cortex cells (Fig. 2E) (*14*). pPIN2::PIN2EGFP expression in cortex and epidermis approached the ablated QC after 16 hours and resumed its original expression site relative to the new QC at later stages (Fig. 2, F and G, bracket). No obvious changes in pPIN2::PIN2EGFP polarity were observed after a proximal shift of the auxin maximum (Fig. 2, F to H).

pSCR::PIN2EGFP clearly visualizes polar PIN localization in QC and endodermal cells (Fig. 2I). pSCR::PIN2EGFP localizes to the basal-lateral membrane of endodermal cells and is preferentially distributed on the basal-lateral plasma membrane of QC cells, consistent with the normal PIN localization of PIN1 and PIN4 in these cells (*15*). pSCR::PIN2EGFP expression was downregulated in the cortex/endodermis stem cells and their daughter cells after ablation (Fig. 2J, bracket), similar to pSCR::H2BYFP (Fig. 1R, bracket). pSCR::PIN2EGFP expression reappeared in cells at the position where the new QC cells were expected to be specified at day 2 after ablation, and expression was weakly and uniformly distributed on the plasma membrane (Fig. 2K, arrows). Around day 3, however, pSCR::PIN2EGFP expression became preferentially localized to the lateral and basal membranes (Fig. 2L, arrows), suggesting that QC-specific PIN polarity is reestablished at later stages.

Immunolocalization of PIN1 and PIN2 in WT roots after ablation confirmed that the observed changes of PIN-GFP expression and localization genuinely reflect the in vivo dynamics of PIN expression and localization after ablation (fig. S3) (*16*). PIN4 immunolocalization reveals predominant expression in the QC, stem cells, and their abutting daughters, with a polar localization oriented toward the QC and columella stem cells (Fig. 2M) (*15*). PIN4 could no longer be detected in columella stem cells and at the basal ends of the former vascular stem cells 16 hours after ablation (Fig. 2N, bracket), and the PIN4 expression domain shifted proximally in the following 2 days (Fig. 2, O and P), in correlation with the proximally shifted *PLT1* expression domain and auxin maximum.

Our data demonstrate that disruption of auxin flow by QC ablation alters expression but not polar localization of PIN proteins. The timing of PIN protein distribution changes and the intermediate polarization patterns in respecifying cells suggest that PIN proteins become correctly polarized in response to auxin distribution changes only after renewed cell specification.

To test directly whether redistribution of auxin affected PIN polarity only after changes in cell identity, we exogenously applied indoleacetic acid (IAA) to the root and locally induced auxin biosynthesis in the QC, which enhances endogenous auxin levels and flux (*8*). Four-hour treatments neither changed expression level nor polarity for pPIN1::PIN1EYFP and pPIN2:PIN2EGFP fusions in the central root meristem (fig. S4, A to F). However, prolonged auxin biosynthesis in the root meristem completely eliminated PIN protein expression, consistent with previous reports on auxin- and AUXIN RESISTANT1-dependent PIN degradation (fig. S4, G to I) (*17*, *18*).

Our data indicated that cell specification was necessary for new PIN protein expression and localization after ablation. We therefore sought to investigate the role of key patterning genes in the induced regeneration process. The *PLT1* and *PLT2* genes are auxin-inducible and convey root identity when ectopically expressed (*6*). Double *plt1 plt2* mutants have a misspecified QC (*6*), although pSCR::H2BYFP and nuclear pSHR::SHRGFP are detected at its position (Fig. 3; A, B, E, and G inset).

DR5rev::GFP expression in *plt1-4 plt2-2* double mutants spread throughout root vascular tissues 16 hours after ablation (Fig. 3C). However, only faint expression of DR5rev::GFP was detected in 1 to 2 cell layers proximal to the ablated QC cells (Fig. 3C, bracket), which disappeared at day 2 (Fig. 3D, bracket). Moreover, these cell layers lacked starch granules (Fig. 3D, inset and bracket), indicating that no respecification of columella cells occurs in *plt1-4 plt2-2* double mutants. Furthermore, pSHR::SHRGFP expression was lost in former vascular stem cells and their daughters, and nucleus-restricted pSHR::SHRGFP expression was undetectable in their abutting GFP-expressing provascular cells (Fig. 3F, bracket). These findings indicated defects in nuclear SHR targeting as well as QC respecification, both associated with root regeneration.

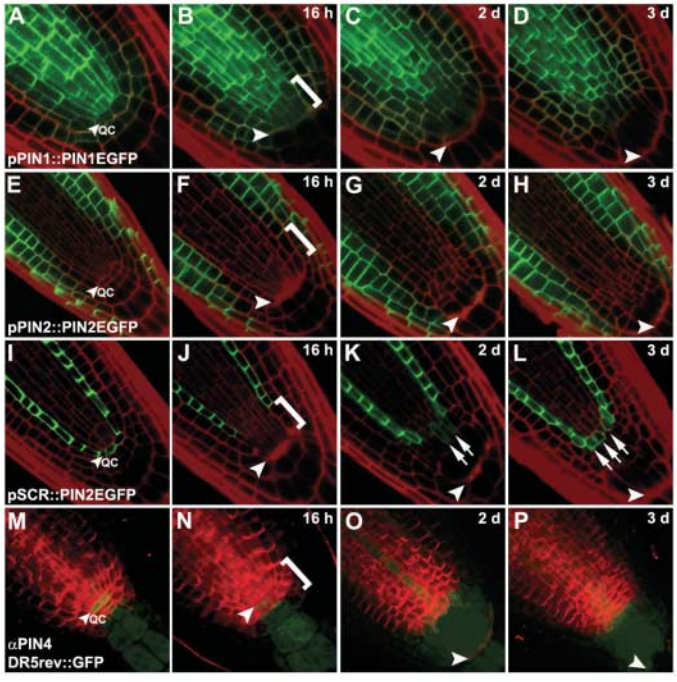

Fig. 2. PIN expression and polarity after QC ablation. (**A** to **D**) pPIN1::PIN1EGFP and (**E** to **H**) pPIN2::PIN2EGFP. (**I** to **L**) *SCR* promoter drove PIN2EGFP protein fusion. (**M** to **P**) PIN4 immunolocalization in WT roots containing DR5rev::GFP. The leftmost columns shows control before ablation. The images in the column second from the left are 16 hours after ablation. The column third from the left is 2 days after ablation. The right-most column is 3 days after ablation. Arrowheads indicate QC cells in the left-most column and the position of ablated QC elsewhere. Arrows in (K) and (L) show localization changes of pSCR::PIN2EGFP in cells at the position where the new QC cells were expected to be specified. The brackets in (B), (J), and (N) indicate down-regulation of markers and PIN expression. The bracket in (F) shows pPIN2::PIN2EGFP expression in cortex and epidermis approached the ablated QC. The red signal in [(A) to (L)] is PI staining; in [(M) to (P)] is Cy3 signal. The green signal is GFP.

No obvious defects in PIN1 expression and distribution were observed in *plt1-4 plt2-2* double mutants (Fig. 3G), suggesting no role for *PLT* genes in PIN1 localization. Down-regulation of PIN1 expression followed ablation as in WT (Fig. 3H, bracket), indicating that *PLT* genes are not required for this response.

SCR is cell-autonomously required for specification of QC cells and thereby required for maintenance of the surrounding stem cells (*4*). DR5rev::GFP and pWOX5::ERGFP are properly expressed in the *scr-4* null allele (*19*) before the root ceases growth (Fig. 3, I and M), although several QC-specific markers are absent in *scr* mutants (*4*). DR5rev::GFP expression accumulated above the ablated QC cells within 16 hours after ablation in *scr-4* roots (Fig. 3J, bracket) but remained restricted to about 2 layers proximal to the ablated QC cells (Fig. 3, K and L), where specification of columella cells occurred as indicated by starch granule staining (Fig. 3L, inset and bracket). However, no fate changes occurred in the proximally abutting layer, which should become the new QC, and pWOX5::ERGFP expression was lost 3 days after ablation (Fig. 3P), despite an initial response (Fig. 3, N and O, bracket). These data suggest that the role of SCR in QC specification is critical during regeneration.

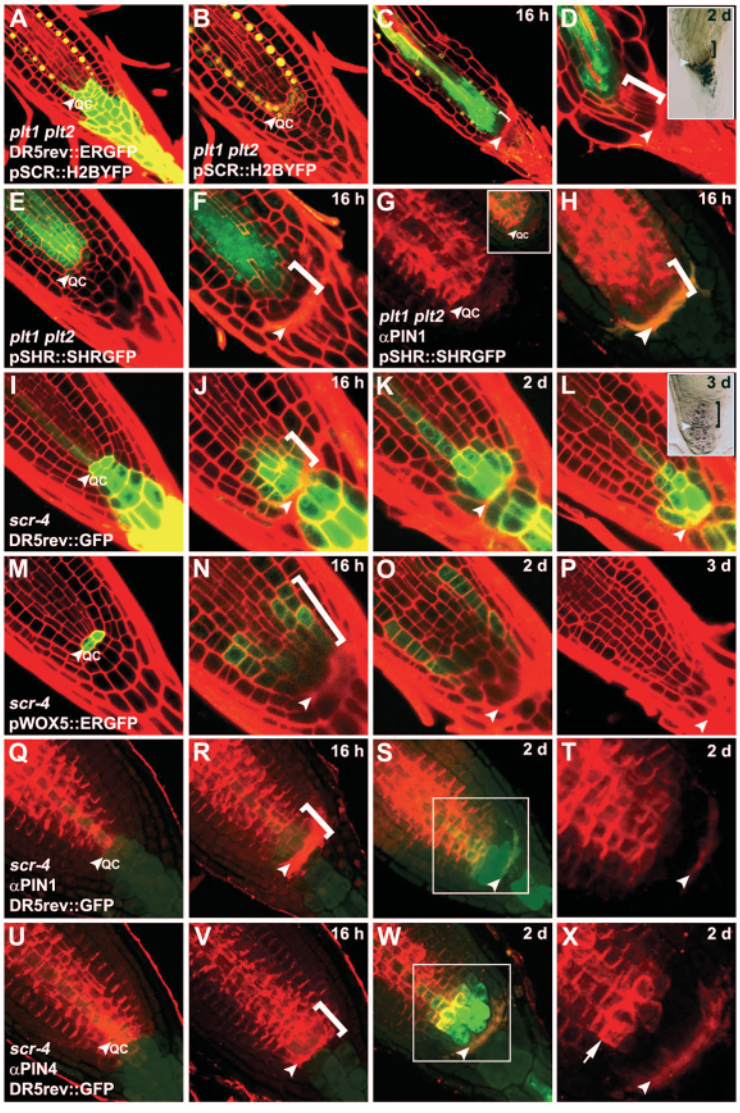

Fig. 3. The roles of *PLT*s and *SCR* are critical during regeneration. (**A** to **D**) DR5rev::GFP and pSCR::H2BYFP expression in *plt1-4 plt2-2* roots. (**E** and **F**) pSHR::SHRGFP expression in *plt1-4 plt2-2* roots. (**G** and **H**) PIN1 immunolocalization in *plt1-4 plt2-2* roots containing pSHR::SHRGFP. (**I** to **L**) DR5rev::GFP and (**M** to **P**) pWOX5::ERGFP expression in *scr-4* roots. (**Q** to **T**) PIN1 and (**U** to **X**) PIN4 immunolocalization in *scr-4* roots containing DR5rev::GFP. (T) and (X) show the enlarged box regions in (S) and (W), respectively. (A), (B), (E), (G), (I), (M), (Q), and (U) show control before ablation. (C), (F), (H), (J), (N), (R), and (V) are 16 hours after ablation. (D), (K), (O), (S), (T), (W), and (X) are 2 days after ablation. (L) and (P) are 3 days after ablation. Arrowheads indicate QC cells in (A), (B), (E), (G), (I), (M), (Q), and (U) and the position of ablated QC elsewhere. The inset and bracket in (D) show no provascular-columella identity switches in *plt1-4 plt2-2*. Brackets in (C) and (D) show faint or no expression in 1 to 2 cell layers proximal to the ablated QC. The bracket in (F) shows down-regulation of pSHR::SHRGFP and no nuclear-restricted pSHR::SHRGFP expression. The inset and bracket in (L) shows provascular-columella identity switches in *scr-4*. The brackets in (H), (J), (N), (R), and (V) indicate changes of markers and PIN expression. The arrow in (X) points to a basal-to-apical shift of PIN4 localization. The red signal in [(A) to (F)] and [(I) to (P)] is PI staining; in [(G), (H), and (Q) to (X)] is a Cy3 signal. The green signal is GFP and the yellow signal is YFP.

We asked whether the restricted expression of DR5rev::GFP proximal to the ablated QC of *scr-4* was caused by alterations in auxin re-routing. Therefore, we investigated PIN1 and PIN4 expression and localization in *scr-4* before and after QC ablation. PIN1 and PIN4 expression and distribution in *scr-4* mutants (Fig. 3, Q and U) was largely as in WT (Fig. 2M and fig. S3, A and B). Down-regulation of PIN1 and PIN4 expression in the cells abutting the ablated QC (Fig. 3, R and V, brackets) also occurred as in WT (Fig. 2N and fig. S3D, brackets), suggesting that SCR function is not required for this response. Cells at the proximal end of the proximally shifted auxin maximum, where the new QC should form, displayed a basal-to-apical shift of PIN4 localization (Fig. 3, W and X, arrow); whereas PIN1 expression and localization remained WT-like (Fig. 3, S and T). The PIN4 repolarization in the absence of *SCR* may cause auxin to be transported away from the proximally-shifted maximum and thereby explain the restricted expression of the auxin response marker DR5rev::GFP proximal to the ablated QC.

SHR, like *SCR*, is required for QC cell specification (*4*). *SHR* transcription occurs in the stele while the protein moves one cell layer outward, as shown by pSHR::SHRGFP expression (Fig. 1M) (*13*). Indeed, we found that *SHR* is also required for QC but not columella respecification and for proper PIN4 expression and localization by using the same set of fluorescent markers and antibodies in the null allele of *SHR*, *shr-2* (fig. S5) (*19*). As observed in *scr-4*, PIN4 was repolarized in provascular cells of *shr-2* roots (fig. S5P, arrow).

Our studies reveal essential roles for *PLT*, *SCR*, and *SHR* genes in respecifying pattern and polarity in the *Arabidopsis* root. They also provide further evidence that PIN proteins can become correctly polarized in response to auxin distribution changes only after renewed cell specification, rather than directly in response to auxin distribution changes. PIN4 localization is reversed in cells nearby the new auxin response maximum in the absence of *SCR* or *SHR*.

Our data suggest a model in which auxin-induced cell-fate changes depend on new expression domains and activity of PLT, SCR, and

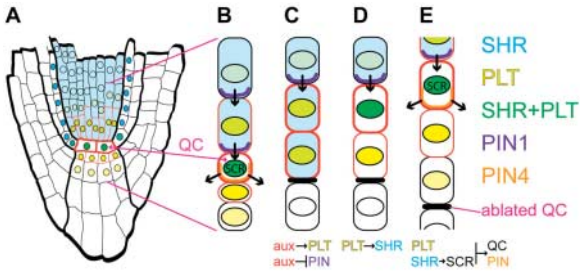

Fig. 4. A model for root regeneration. (**A**) *Arabidopsis* root meristem. (**B**) Enlarged cell file as marked in (A). (**C**) After ablation, auxin accumulation shifts, which induces *PLT* genes and downregulates PIN expression. (**D**) *PLT* genes promote SHR nuclear localization. (**E**) Nuclear SHR promotes *SCR* expression and, together with *PLT* genes, new QC specification. New, correctly polarized PIN expression depends on *PLT*, *SHR*, and *SCR* genes and reconstitutes auxin transport (black arrows). The red outline of cells marks auxin accumulation.

SHR transcription factors (Fig. 4). *PLT* genes are first upregulated where the auxin response builds up. The rapid response of *PLT1* transcription to local alteration of auxin distribution suggests that *PLT* genes are key effectors transducing the auxin signal into a repatterning process. Buildup of a new maximum is facilitated by transcription factor–independent downregulation of PIN expression. Dependent on *PLT* genes, SHR attains nuclear localization in a single provascular cell layer, which allows *SCR* expression and new QC specification. New PIN expression depends on *PLT*, *SHR*, and *SCR* genes, and PIN polarity is set only after these patterning genes have adopted their new expression domains. New correctly polarized PIN expression in turn reconstitutes the auxin transport route in the root tip and facilitates the completion of the regeneration process.

Our finding that auxin distribution changes the polar localization of PIN proteins only in the absence of fate regulators suggests that, in the root meristem, defined cell types have intrinsic polar marks to which PIN proteins are delivered. The PINOID (PID) kinase acts as a binary switch to position PIN proteins at opposing membranes, which supports the idea that an underlying, PIN-independent mechanism sets polarity (*20*). By influencing factors such as PID, but also protein trafficking regulators such as the GNOM ARF-GEF [guanine nucleotide exchange factor for adenosine diphosphate (ADP)–ribosylation factor] (*21*, *22*), cell-fate regulators may alter PIN protein trafficking. Classical canalization hypotheses proposed feedback between auxin flow and auxin transport in auxin-dependent developmental processes (*23*–*25*). Our results show that in the root, auxin redistribution first induces complex cell-fate changes and, only consequently, induces changes in the polarity of auxin flow, indicating that the classical canalization dogma does not apply here. However, in the absence of cell-fate determinants, such as in *scr* and *shr* mutants, or in other developmental contexts, such as during shoot-derived primordium positioning (*26*) or leaf vascularization, the PIN protein localization machinery may respond to other cues such as to auxin itself.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/311/5759/385/DC1
Materials and Methods
Figs. S1 to S5
References

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Chromosomes Can Congress to the Metaphase Plate Before Biorientation

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The stable propagation of genetic material during cell division depends on the congression of chromosomes to the spindle equator before the cell initiates anaphase. It is generally assumed that congression requires that chromosomes are connected to the opposite poles of the bipolar spindle ("bioriented"). In mammalian cells, we found that chromosomes can congress before becoming bioriented. By combining the use of reversible chemical inhibitors, live-cell light microscopy, and correlative electron microscopy, we found that monooriented chromosomes could glide toward the spindle equator alongside kinetochore fibers attached to other already bioriented chromosomes. This congression mechanism depended on the kinetochore-associated, plus end–directed microtubule motor CENP-E (kinesin-7).

Successful cell division requires proper "biorientation" of chromosomes, whereby microtubule bundles (K fibers) connect sister kinetochores of each chromosome to opposite spindle poles (*1*). Biorientation errors are linked to chromosome loss and cancers (*2*). Formation of sister K fibers occurs asynchronously (*3*), and once a kinetochore captures microtubules growing from a spindle pole, the chromosome is transported toward this pole and becomes "monooriented" (*4*). Monooriented chromosomes remain near the spindle pole for variable times (*3*, *4*) until they suddenly "congress" to the spindle equator. Current models of mitotic spindle formation (*5*, *6*) postulate that chromosome congression occurs as the result of biorientation (*7*).

We followed movements of individual chromosomes in mammalian cells by differential interference contrast (DIC) time-lapse microscopy (*8*). In addition to the chromosome oscillations that occur toward and away from spindle poles, we frequently observed monooriented chromosomes making direct movements to the metaphase plate as if they were attempting to congress (fig. S1). Centromeres

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