Current Biology 23, 817–822, May 6, 2013 ©2013 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2013.03.064

Report

An Auxin Transport Mechanism Restricts Positive Orthogravitropism in Lateral Roots

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Summary

As soon as a seed germinates, plant growth relates to gravity to ensure that the root penetrates the soil and the shoot expands aerially. Whereas mechanisms of positive and negative orthogravitropism of primary roots and shoots are relatively well understood [1–3], lateral organs often show more complex growth behavior [4]. Lateral roots (LRs) seemingly suppress positive gravitropic growth and show a defined gravitropic set-point angle (GSA) that allows radial expansion of the root system (plagiotropism) [3, 4]. Despite its eminent importance for root architecture, it so far remains completely unknown how lateral organs partially suppress positive orthogravitropism. Here we show that the phytohormone auxin steers GSA formation and limits positive orthogravitropism in LR. Low and high auxin levels/signaling lead to radial or axial root systems, respectively. At a cellular level, it is the auxin transport-dependent regulation of asymmetric growth in the elongation zone that determines GSA. Our data suggest that strong repression of PIN4/PIN7 and transient PIN3 expression limit auxin redistribution in young LR columella cells. We conclude that PIN activity, by temporally limiting the asymmetric auxin fluxes in the tip of LRs, induces transient, differential growth responses in the elongation zone and, consequently, controls root architecture.

Results and Discussion

Differential Growth Dynamics Define GSA Formation in LRs

The gravitropic set-point angle (GSA) defines the angular growth of an organ with respect to the gravity vector [4] (GSA = 0° for positive and 180° for negative orthogravitropism; see inset of Figure 1C). Lateral roots (LRs) initially emerged at a 90° angle from the main root, but they eventually slightly inclined rootward (Figures 1A–1C; see also Figures S1A and S1B available online). A gravity stimulus of 180° induced emerging LRs to grow shootward, toward the new gravity vector, suggesting that we investigate earliest GSA formation in emerging LRs (Figure 1B). The angular growth toward gravity of young LRs is a highly conserved pattern and, accordingly, 70%–80% of emerged LRs display an initial GSA between 51° and 70° (Figure 1C). This initial angular growth away from the main root is maintained for a certain period (Figure 1A; Figure S1C; [5, 6]).

To investigate how LRs initially suppress positive orthogravitropism, we concentrated on GSA establishment of emerging LRs. For this, we used light sheet-based fluorescence microscopy (LSFM), an approach that maintains seedling growth close to physiological conditions [7]. During GSA establishment, cellular elongation at the upper epidermal cell files was more pronounced as compared to the lower ones (Figures 1D and 1E). Freshly emerged LRs initially do not display a defined elongation zone, and asymmetric growth coincides with its de novo establishment (Movie S1). Based on these observations, we classified young LRs according to their differentiation status. Stage I LRs lack an elongation zone, whereas the establishment of cellular elongation and GSA is associated with stage II LRs (Figure 1D; Figures S1A and S1B). Appearance of the differentiation zone (e.g., root hairs) marks stage III, which is furthermore characterized by straight (constant GSA) growth (Figure S1C). Our data show that, in young LRs, GSA is defined by differential growth response in the elongation zone.

Asymmetric Auxin Signaling Correlates with the Formation of GSA in Young LRs

Although asymmetric auxin signaling regulates differential growth responses in various tissues [8], it was previously not observed in LRs [9]. We reexamined the highly responsive auxin promoter element DR5 transcriptionally fused to GFP (DR5rev::GFP; [10]) and observed an asymmetric distribution of DR5-based auxin signaling in LRs (Figure 1F). Higher auxin signaling in lower epidermal cell files (1) preceded the formation of the elongation zone (stage I), (2) was highest at the peak of differential growth response (stage II), and (3) was less frequently observed in more mature LRs (stage III) (Figure 1G). We assume that the transient nature of this asymmetry limited its detection in the previous study [9] and conclude that asymmetric auxin signaling is stage-dependent and correlates with differential growth during GSA formation.

Auxin Determines GSA of Young LRs

To further assess the role of auxin in LR GSA, we treated seedlings with auxins. NAA (napthaleneacetic acid) treatments strongly enhanced differential elongation in LRs (Figure S2A) and consequently enhanced bending (reduced GSA) toward the main root (Figures 2A, 2B, and 2D). This finding indicates that auxin levels or signaling in the elongation zone is a limiting factor and could explain the lack of positive orthogravitropism in young LRs.

Next we performed main root meristem ablation experiments to remove the sink tissue and to locally increase auxin accumulation/signaling above the cut [11]. Three days after

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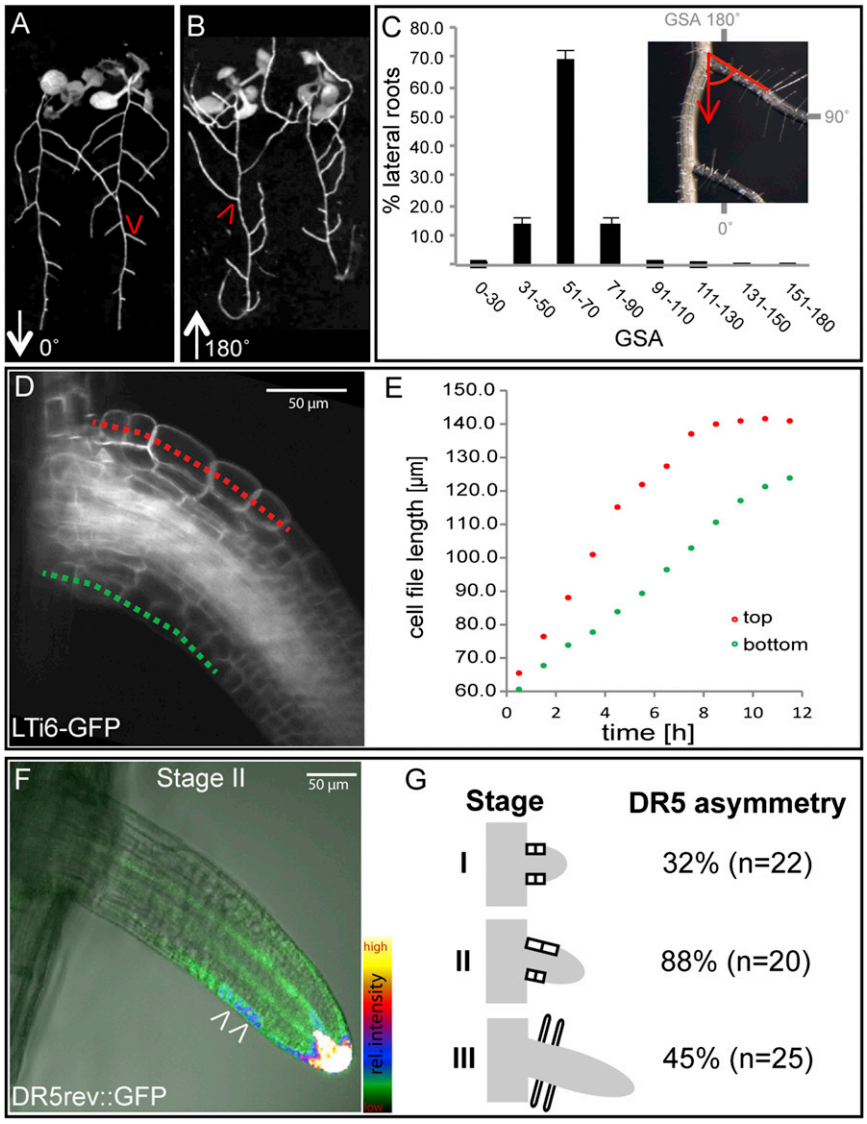

Figure 1. GSA Formation in *Arabidopsis thaliana*

(A) Representative 14-DAG WT seedlings.

(B) Plates with WT seedlings were turned (180°) 7 days after germination. Arrow denotes the gravity vector. Red arrowheads depict representative LRs.

(C) GSA distribution of LRs. Bars represent SEM (n = 7 experiments; 40–60 LRs per experiment).

(D and E) Light sheet-based live imaging of an emerging LR (D). The red and green data points denote top and bottom epidermal cell files, respectively (E).

(F) DR5-driven GFP signal in a representative stage II LR (confocal microscopy). Arrowheads denote asymmetry.

(G) Percentage of LRs displaying asymmetric DR5 in stage I–III LRs ($n \geq 20$ LRs from > 6 independent seedlings).

See also Figure S1.

ablation, we observed the expected enhancement of DR5 above the cut but also higher DR5 (auxin) signaling in the tip of LRs emerging closest to the cut (Figures S2B–S2F), possibly due to increased cellular auxin levels. Higher auxin signaling in the main and LR coincided with a reduction in GSA of the respective LR (Figures 2C and 2E). Together with the exogenous auxin applications, our data suggest that local increase in auxin levels/signaling in the main root or in the LR columella (tip) induces LR bending toward gravity.

We subsequently analyzed whether auxin signaling is required for GSA determination. The auxin receptors TIR1/AFBs and ABP1 mediate most of the auxin responses via distinct mechanisms [12]. *tir1-1* knockout and *abp1-5* partial loss-of-function mutants showed a GSA shift closer to 90° (Figures 2A, 2F, 2G, and 2I). In consonance with reduced auxin signaling, auxin depletion by the l-kynurenine (KYN) inhibitor of auxin biosynthesis [13] also resulted in significantly reduced LR bending (GSA closer to 90°) (Figures 2A, 2H, and 2J).

We conclude that high and low auxin levels/signaling control GSA in emerging LRs and result in an axial or radial root system, respectively.

Polar Auxin Transport Is Required for GSA Formation in Young LRs

It is surprising that exogenous auxin enhances differential growth in LRs. This may suggest that an asymmetric auxin perception mechanism is differentially regulated at the upper and lower sides of the LR. Alternatively, exogenous auxin is efficiently transported and asymmetrically redistributed toward the elongation zone in the LR. To discriminate between these two scenarios, we used the synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid), which is an active auxin-signaling molecule but a weaker substrate for auxin efflux carriers [14].

Low 2,4-D (25 nM) concentration lowered LR GSA similar to NAA (100 or 500 nM; Figure 2D). In contrast, increased exogenous 2,4-D (100 nM) inhibited LR bending (GSA closer to 90°) (Figures 3A, 3B, and 3H). This concentration-dependent effect suggests that auxin requires redistribution (auxin transport) to determine GSA. In accordance, treatments with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) similarly increased GSA (Figures 3A, 3C, and 3I) and abolished asymmetric auxin signaling in stage II LRs (Figures 3N, 3O, and 3R).

These findings suggest that an auxin transport mechanism is required for asymmetric auxin signaling and GSA determination of LRs.

LR Columella Cells Function in GSA Establishment

Sedimentation of starch-containing statoliths provides positional information in columella cells [2, 15]. Morphological changes and maturation of gravity-sensing columella cells have been proposed as playing a role in LR tropism [6]. Using a columella-specific reporter line, we observed that molecular columella identity was already present in stage I LRs (Figure S3A), and the formation of starch-loaded statoliths correlated with the establishment of an asymmetric elongation zone (Figures S3B and S3C). In accordance, starchless mutant *pgm1* showed reduced bending (GSA closer to 90°) of

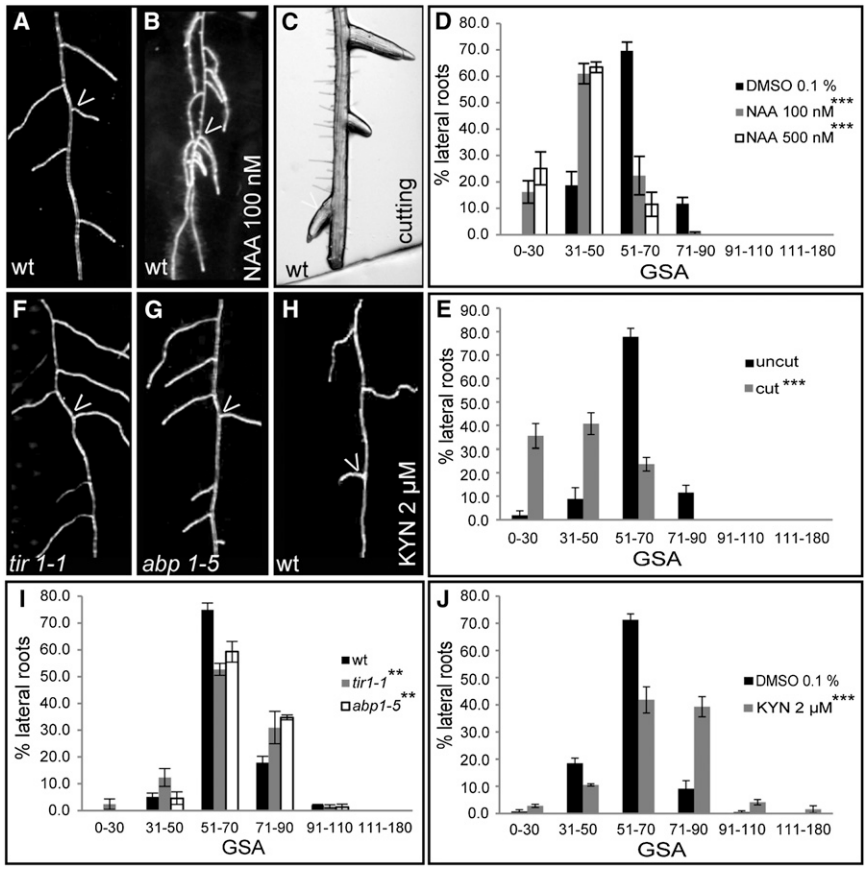

Figure 2. Auxin Plays a Central Role in LR GSA Formation

(A–E, H, and J) Representative images (A–C and H) and GSA distributions (D, E, and J) of untreated (A), auxin-treated (B and D), main root ablated (C and E) and l-kynurenine-treated (H and J) WT seedlings.

(F, G, and I) *tir1-1* (F) and *abp1-5* (G) mutant seedlings and their corresponding LR GSA distributions (I). Error bars represent SEM (n = 3 experiments; 40–60 LRs per experiment). ** denotes $p < 0.001$ Kolmogorov-Smirnov (KS) test; *** denotes $p < 0.0001$. Arrowheads depict representative LRs.

See also Figure S2.

emerging LRs (Figures S3D–S3F). We therefore assume that both gravity perception in columella cells (Figure S3; [6]) and polar auxin transport (Figures 3A–3C, 3H, and 3I) affect GSA of emerging LRs. Next, we focused on PIN3, PIN4, and PIN7 auxin efflux carriers, because they function in main root columella (Figure 3L; [8, 16]). pPIN3::PIN3-GFP-expressing seedlings showed strong signal in columella cells of young LRs (Figure 3M). In contrast, PIN4-GFP and PIN7-GFP were found in the stele, but not at all or only as a very faint signal in columella cells (Figure 3M; Figure S3G). Notably, *PIN4*, but not *PIN7*, showed promoter activity in stage II LRs (Figures S3I–S3L), indicating posttranscriptional and transcriptional regulation of PIN4 and PIN7, respectively.

Consequently, we conclude that, compared to main roots, PIN4 and PIN7 activity is particularly suppressed in LR columella cells, possibly pinpointing lower auxin transport rates.

PIN Auxin Efflux Activity Is Required for the Establishment of GSA in Young LRs

Although PIN3 is expressed in freshly emerged LR columella cells, *pin3* mutant LRs showed a slightly stronger response (Figures 3A, 3D, and 3J). In agreement with this, more mature LRs of *pin3* mutants orient downward faster than wild-type (WT) [9]. Notably, PIN4 and PIN7 showed ectopic upregulation in *pin3* mutant stage II LRs (Figures S4A and S4B), which possibly accounts for the enhanced LR bending compared to WT. This finding supports our assumption that PIN4 and PIN7 repression leads to reduced LR bending in WT.

In contrast to *pin3*, multiple columella *pin* mutant combinations, such as *pin3 pin4* and *pin3 pin7* double mutants and *pin3 pin4 pin7* triple mutant showed strongly reduced bending of young LRs (GSA closer to 90°) (Figures 3A, 3E–3G, and 3K), confirming functional redundancy. In accordance with this, the asymmetric auxin response was visibly reduced in *pin3 pin7* mutant LRs (Figure 3P–3R).

To exclude that the observed changes in GSA are related to general growth defects, we gravistimulated stage III, *pin* mutant LRs. In agreement with previous studies on *pin3* single mutant LRs [9, 17], multiple *pin3 pin7* or *pin3 pin4 pin7*, showing defects in GSA formation (Figure 3K), remained responsive to changes in gravity (Figures S4C and S4D).

Our data indicate that a redundant, PIN-dependent auxin flux mechanism is a decisive factor in setting asymmetric auxin response and GSA formation in LRs.

Auxin Determines GSA via the Regulation of PIN-Dependent Asymmetric Auxin Flux

PIN auxin efflux carriers determine the rate and direction of auxin transport [18, 19]. Therefore, we analyzed the spatial expression and membrane localization of PIN3 during gravitropic LR growth. PIN3 was detectable throughout stages I and II (Figure 4A) but declined during later LR growth (Figure 4J) [20]. Emerging LRs (stage I) showed asymmetric PIN3 expression or membrane distribution in 33% of the analyzed seedlings (Figure 4A; Figure S3H). PIN3 asymmetry at the plasma membrane (lower level at the apical/basal compared to lateral sides) was most pronounced in stage II (71%) (Figure 4A; Figure S3H; Movie S2) and subsequently declined in stage III (Figure S3H). The simultaneous visualization of PIN3-GFP and DR5rev::RFP illustrated the correlation between the polarization of PIN3-GFP and the asymmetry of auxin signaling (Figures 4A and 4B; Movies S2 and S3), both preceding differential growth.

We consequently further investigated the PIN dependency of the auxin-determined GSA. Although PIN4-GFP and PIN7-GFP remained robustly repressed in stage II LRs, NAA treatment or main root cutting led to increased PIN3-GFP signal in columella cells (Figures 4C–4H). Furthermore, we treated 6-day-old to 7-day-old WT and (due to redundancy) *pin3 pin7* double mutant seedlings with low auxin concentrations. Notably, the positive effect of auxin on young LR bending (shift in mean GSA) was reduced in *pin3 pin7* mutants (Figure 4I).

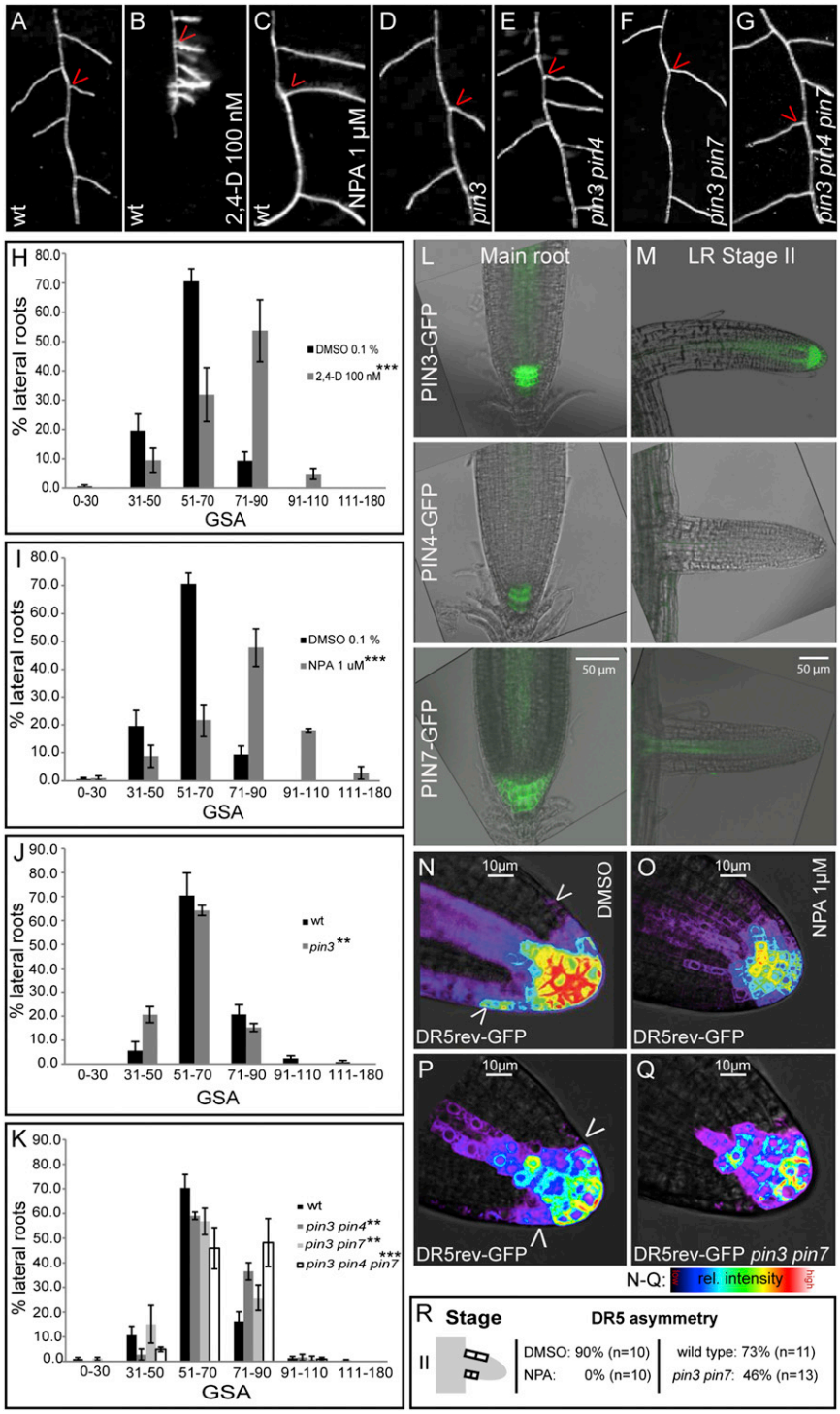

Figure 3. Columella PINs Regulate GSA Formation in Young LRs

(A–K) Representative images (A–G) and GSA distributions (H–K) of untreated (A, D–G, J, and K) and 2,4-D- (B and H) or NPA- (C and I) treated 14-day-old WT (A–C) and *pin3* (D and J), *pin3 pin4* (E and K), *pin3 pin7* (F and K), or *pin3 pin4 pin7* (G and K) mutant seedlings. Error bars represent SEM (n = 3 experiments; 40–60 LRs per experiment). ** denotes $p < 0.001$ Kolmogorov-Smirnov (KS) test; *** denotes $p < 0.0001$. Red arrowheads depict representative LRs.

(L and M) Expression of PIN3-GFP, PIN4-GFP, and PIN7-GFP in the columella of main root (L) and second stage LRs (M).

(N–Q) DR5-GFP signal in a representative second stage LR of WT controls (N and P), 1 µM NPA treatments (O), and *pin3 pin7* double mutants (Q). White arrowheads denote asymmetry in DR5.

(R) Percentage of DMSO control and NPA-treated, or WT and *pin3 pin7* mutant LRs displaying asymmetric DR5-GFP (n ≥ 10 stage II LRs). Confocal microscopy (L–Q). See also Figures S3 and S4.

Our findings show that both PIN3 polarization and expression correlate with asymmetric auxin responses and that PIN3-like function is required for the auxin effect on GSA in young, emerging lateral roots.

PIN Activity in Columella Cells Correlates with GSA in Mature LRs

Next, we addressed whether PIN3, PIN4, and PIN7 might also play a role in GSA of older LRs (beyond stage II). PIN3, PIN4, and PIN7 expression were jointly reduced in stage III lateral roots (Figure 4J). This low PIN activity in columella cells and, hence, low PIN-dependent redistribution of auxin correlated with reduced asymmetry in auxin signaling (Figure 1G) and constant GSA (non differential growth).

As mentioned above, the GSA of older LRs further decreases after the plateau phase (stage III). We defined such an additional onset of differential growth as stage IV LRs (Figure S1D). LRs displaying a GSA below 30° (stage IV) showed higher frequency of mainly PIN4- and PIN7-GFP signal in LR columella cells (Figure 4J). Intriguingly, lateral roots in plateau (stage III) display reduced PIN3, PIN4, and PIN7 levels (Figure 4J) but show onset of differential growth when exposed to gravistimulation (Figures S4C and S4D). We therefore assumed that changes in gravity might reactivate PIN proteins in stage III LR columella cells. In agreement with this, stage III LRs showed gravity-induced upregulation of PIN3, PIN4, and PIN7 (Figure 4K).

We conclude that the cooperative activity of PIN3, PIN4, and PIN7 in more mature LR columella cells correlates with the onset of environmentally and developmentally controlled LR bending.

Concluding Remarks

Charles Darwin was one of the first to scientifically document that roots show positive and stems show negative orthogravitropism [1]. Although this general concept appears to be true for primary organs, lateral organs have a completely different growth behavior toward gravity, accounting for their radial expansion [4].

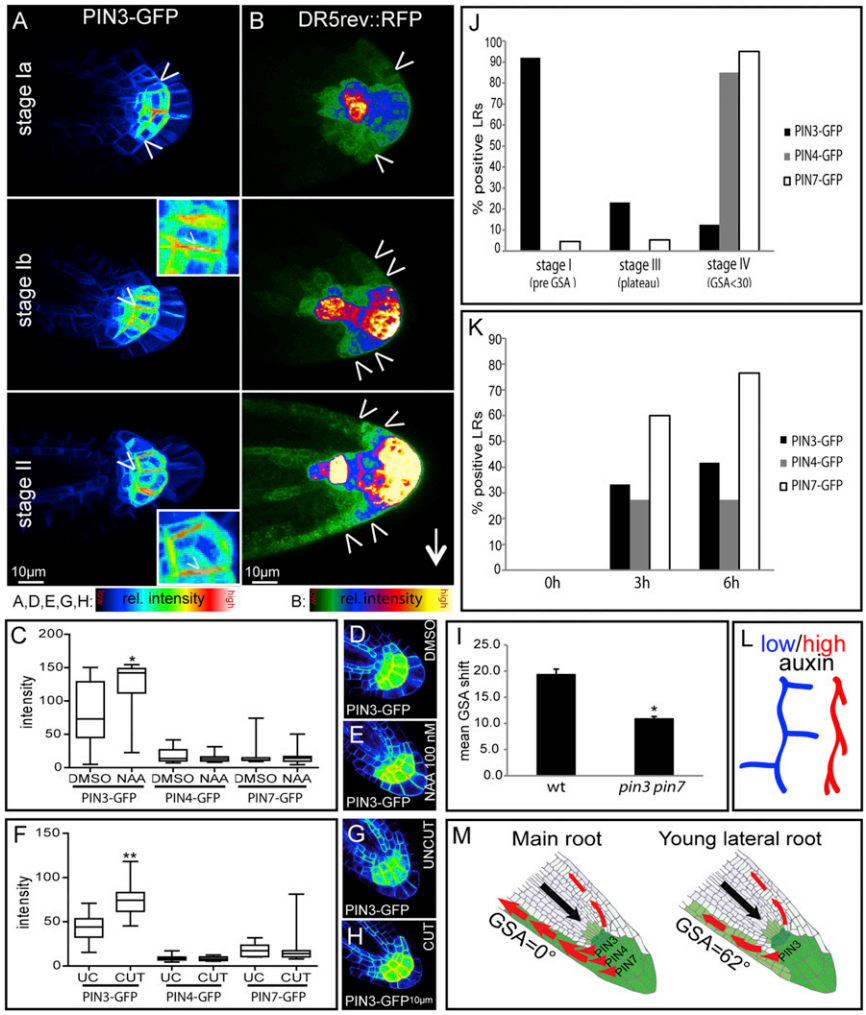

Figure 4. PIN3 Expression and Polarization Determine Auxin-Dependent GSA Formation in Young LRs

(A and B) PIN3-GFP (A) and pDR5::RFP signal (B) in stage I and II LRs. Stages Ia and Ib correspond to an earlier (Ia) and later momentum (Ib) of the same LR. White arrowheads denote asymmetric signal. Arrow denotes the direction of the gravity vector.

(C–H) Images (D, E, G, and H) and signal quantification (C and F) of pPIN3::PIN3-GFP, pPIN4::PIN4-GFP and pPIN7::PIN7-GFP expression after treatments with 100 nM NAA (C–E) or after main root meristem ablation (F–H) ($n \geq 10$ individual LRs).

(I) Mean GSA shift in 100 nM NAA treated WT and *pin3 pin7* seedlings. Error bars represent SEM (n = 3 experiments; 40–60 LRs per experiment).

(J and K) Percentage of PIN3-GFP, PIN4-GFP, and PIN7-GFP positive root tips of stage I, III, and IV LRs (8-, 9-, and 10-day-old seedlings; binocular fluorescence microscope; $n \geq 30$ LRs) (J) and in 90° gravity stimulated young stage III LRs (K) ($n \geq 15$ individual LRs).

(L) Low and high auxin levels lead to radial and axial root systems, respectively.

(M) Working model depicts differences in auxin redistribution between main and lateral roots and its effect on GSA.

Confocal microscopy (A, B, D, E, G and H). UC, uncut. * denotes $p < 0.05$ and ** denotes $p < 0.001$ student t test. See also Figure S4.

Our genetic, pharmacologic, and physiologic studies show that high and low auxin levels/signaling determine the angular growth of LRs, ultimately leading to a more axial or radial root system, respectively. We propose a model in which, in absence of PIN4 and PIN7, the PIN3-dependent redistribution of auxin in the columella cells represents a bottleneck for early GSA determination. Unlike in the main root, where the joint activity of PIN3, PIN4, and PIN7 enables a full gravitropic response and elicits a switch-like behavior [21, 22], the solitary expression of PIN3 in young LRs appears to limit the degree of (1) asymmetric auxin flux, (2) differential growth response in the elongation zone and, ultimately, (3) GSA (Figures 4L and 4M). Here we mainly focused on emerging lateral roots as a stable system for LR GSA establishment. However, during later LR growth, the possibly coordinated activation of PIN3, PIN4, and/or PIN7 in LR columella cells correlates with LR GSA closer to 0°. Similarly, gravity-induced changes in PIN occurrence in columella cells herald the onset of differential growth responses in LRs, suggesting that PIN proteins also play a role in GSA of more mature lateral roots.

In conclusion, we assume that modulation of asymmetric auxin transport rates in LR columella cells suppresses positive orthogravitropism and, hence, could be sufficient to manipulate root system architecture.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.03.064.

Acknowledgments

We are grateful to Jim Haseloff, Ben Scheres, Jiri Friml, and Ykä Helariuta for sharing published material; Stefan Kepinsky, Chris Wolverton, and KV group members for helpful comments; David Whittaker for help in preparing the manuscript; and the BOKU-VIBT Imaging Center for access and expertise. This work was supported by the Vienna Science and Technology Fund (WWTF) (to M.R.R., E.B., and J.K.-V.); the Agency for Innovation by Science and Technology (IWT) (predoctoral fellowship to E. Barbez); Land Baden-Württemberg, the Chica und Heinz Schaller Stiftung, and the CellNetworks cluster of excellence (to A.M.); the ERC starting independent research grant ERC-2007-Stg-207362-HCPO (to P.M., E. Benková); the cluster of excellence "Macromolecular Complexes" (DFG EXC115) (to D.v.W., E.H.K.S.).

Received: November 30, 2012
Revised: March 6, 2013
Accepted: March 26, 2013
Published: April 11, 2013

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