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Report

# Auxin Controls Gravitropic Setpoint Angle in Higher Plant Lateral Branches

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## Summary

**Lateral branches in higher plants are often maintained at specific angles with respect to gravity, a quantity known as the gravitropic setpoint angle (GSA) [1]. Despite the importance of GSA control as a fundamental determinant of plant form, the mechanisms underlying gravity-dependent angled growth are not known. Here we address the central questions of how stable isotropic growth of a branch at a nonvertical angle is maintained and of how the value of that angle is set. We show that nonvertical lateral root and shoot branches are distinguished from the primary axis by the existence of an auxin-dependent antigravitropic offset mechanism that operates in tension with gravitropic response to generate angled isotropic growth. Further, we show that the GSA of lateral roots and shoots is dependent upon the magnitude of the antigravitropic offset component. Finally, we show that auxin specifies GSA values dynamically throughout development by regulating the magnitude of the antigravitropic offset component via TIR1/AFB-Aux/IAA-ARF-dependent auxin signaling within the gravity-sensing cells of the root and shoot. The involvement of auxin in controlling GSA is yet another example of auxin's remarkable capacity to self-organize in development [2] and provides a conceptual framework for understanding the specification of GSA throughout nature.**

## Results and Discussion

### Primary and Lateral Shoots Are Distinguished by the Existence of an Angle Offset Mechanism

To begin to analyze the mechanism that generates gravity-dependent nonvertical growth, we performed decapitation experiments in *Arabidopsis thaliana* (*Arabidopsis*) and *Pisum sativum* (pea) shoots. The removal of the primary shoot apical meristem (SAM) in many species results in the branch of the subapical node becoming the new main shoot apex [3]. We observed that this assumption of a leading, primary role is accompanied by a shift to a near vertical gravitropic setpoint angle (GSA) in the subapical branch, a transition occurring over the course of 48 to 72 hr (Figure 1B). This change in GSA is suppressed by the application of auxin (1 mM indole-3-acetic acid [IAA] in lanolin) to the apical stump (Figures 1B and 1C), indicating the existence of a developmental switch that shares features with the mechanism regulating bud outgrowth [3]. These data demonstrate that the GSA of nonvertical lateral branches and the approximately vertical primary axis is determined principally on the existence of, or lack of, some kind of angle offset mechanism.

### Stable Nonvertical Growth of Shoot and Root Branches Is Sustained by an Antigravitropic Offset Mechanism

Upon being displaced either above or below its GSA, an organ will rapidly undergo a gravitropic response to return to its original angle of growth [1,4] (Figures S1A–S1D and the Supplemental Introduction available online). For lateral root and shoot branches, this requires reorientation both with and against the gravity vector, demonstrating unequivocally that the mechanistic basis for the robust maintenance of nonvertical growth cannot lie solely in differences in gravitropic competence between primary and lateral organs. At the same time, the fact that a lateral branch can switch to a vertical GSA state where the maintenance of growth angle is accounted for by Cholodny-Went-based gravitropic response (reviewed in [5]) suggests that parsimonious hypotheses for the nature of the angle offset mechanism should include Cholodny-Went gravitropism as the means of reorientating lateral organs upon displacement from their GSA. Thus there are two classes of offset mechanism to consider initially. In the first, perception of the orientation of the gravity vector is shifted such that despite a branch being inclined from the vertical, no asymmetry in auxin response is generated, while in the second, the perception of the gravity vector in the branch is the same as in the primary axis. Here, the anisotropic growth that would otherwise develop in response to radial asymmetry in auxin response must be counteracted by an opposing, antigravitropic anisotropy to generate net isotropic, nonvertical growth. To distinguish these two classes of mechanism, we used a horizontal one-dimensional clinostat to subject shoots and roots to omnilateral gravitational stimulation. We reasoned that if nonvertical GSAs were the product of balancing gravitropic and antigravitropic components, then clinorotation might reveal the effects of the latter. In these experiments, *Arabidopsis* plants were rotated horizontally at speeds from 4 revolutions per hour (rph) to 1 revolution per minute (rpm). After 8 hr of clinorotation, we observe that cauline branches grow with a marked outward/downward anisotropy (assessed in relation to the vertical in upright plants) consistent with the action of an antigravitropic growth component operating in the absence of balancing gravitropic response (Figures 1D and 1E). This effect was the same regardless of whether shoot branches were on or off the axis of rotation (Figures S1E and S1F). We observed a similar pattern of outward/upward growth in *Arabidopsis* lateral roots subjected to clinorotation, again, either on or away from the axis of rotation, indicating that a common mechanism might underlie these similar root and shoot responses (Figures 1G, 1H, S1E, and S1F).

This idea of an antigravitropic growth component is a very old one: de Vries in 1872, in some of the earliest clinorotation experiments, noted a similar outward bending of lateral organs from several species, a phenomenon he named epinasty [6]. Subsequent researchers have confirmed these observations in species including *Coleus blumei* Benth., wheat, pea, and *Capsicum annuum*, and remarkably, these so-called epinastic

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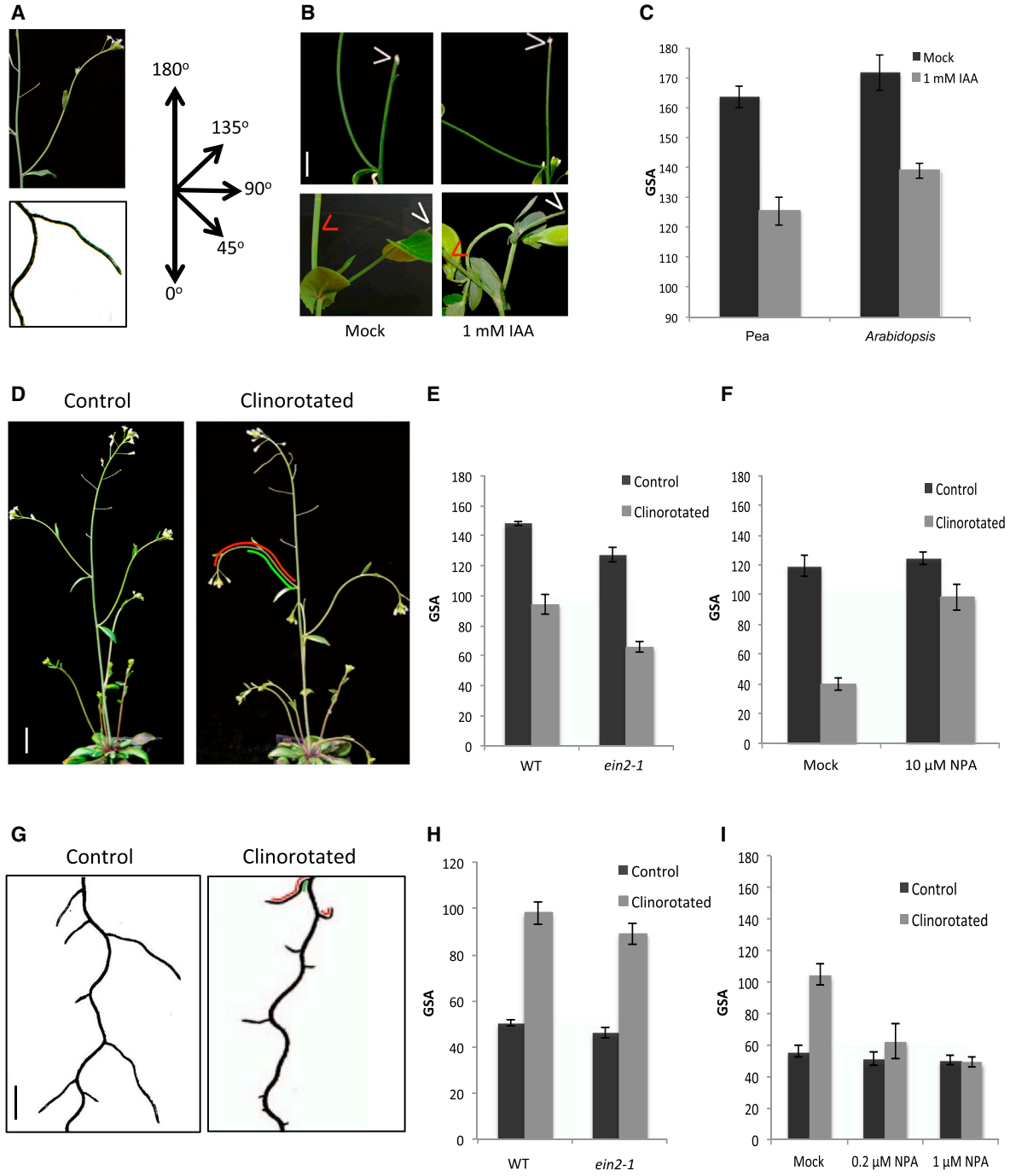


Figure 1. The Nonvertical GSA of Lateral Shoots and Roots Is Driven by a Lateral Branch-Specific Auxin-Dependent Antigravitropic Offset Mechanism

(A) Typical GSA profiles of *A. thaliana* (Col. 0) shoot and root branches with diagram of GSA designations.

(B and C) Changes in the GSA of *Arabidopsis* and pea subapical branches after removal of the shoot apex and application of 1 mM IAA or mock treatment to the apical stump (white arrowheads, decapitated apices; red arrowheads, subapical lateral branches) (B). Quantitative analysis of branch GSA is shown ($p < 0.05$; error bars indicate the SEM) (C).

(D, E, G, and H) Effect of horizontal clinorotation on lateral shoot (D and E) and lateral root (G and H) GSA. Note: for clinorotated plants, a nominal GSA was derived by measurement of the growth angle of the final 5 mm of cauline branches and 2 mm of lateral roots with respect to the vertical in upright plants. Green and red lines represent the action of gravitropic and antigravitropic growth components, respectively. Lateral shoot GSA (E) and lateral root GSA (H) in wild-type and *ein2-1* mutant plants are shown.

(F and I) Effect of local application of the auxin transport inhibitor NPA and subsequent clinorotation on lateral shoot (F) and lateral root GSA (I). Clinorotation at 4 rph (D–F) and 1 rpm (G–I). Scale bars represent 1 cm (D) and 0.5 cm (G). Error bars indicate the SEM. See also Figure S1.

phenomena have also been observed in early space flight-based experiments in the late 1960s [7–11]. The nature of these growth responses in lateral organs has, however, been the subject of forceful debate with arguments advanced that the observed outward anisotropy is merely an ethylene-mediated stress response to clinorotation rather than an effect of the

withdrawal of normal gravitational stimulation [10–14]. To address this crucial question, we performed clinorotation experiments with the *Arabidopsis* mutant *ein2-1*, which is completely insensitive to ethylene ([15] and data not shown). Horizontal clinorotation of *ein2-1* induces outward anisotropic growth of cauline branches and lateral roots as observed in wild-type Col-0, confirming that these patterns of branch growth are not ethylene-mediated responses to physical movement while horizontal (Figures 1E, 1H, S1H, and S1I). We performed several additional experiments to ensure that the patterns of branch growth observed on the clinostat reflect the underlying biology of GSA control rather than artifacts of clinorotation (Figures S1E–S1G and the accompanying legend). Together, these data are not consistent with a model for the maintenance of nonvertical lateral branch GSAs based on an altered gravity perception, which we predict would respond to omnilateral gravitational stimulation with isotropic growth as in the primary shoot and root (data not shown). Rather, they support the idea that standard Cholodny-Went gravitropic response, which would otherwise cause bending to the vertical, is balanced by an antigravitropic offset component.

### The Antigravitropic Offset Mechanism Is Auxin Dependent

To test whether or not auxin transport is required for the action of the antigravitropic offset, we performed clinorotation experiments in the presence of the auxin transport inhibitor NPA. For shoot experiments, NPA or mock treatments were applied to branches via a thin layer of lanolin. Cauline branches treated with 10 μM NPA in this manner 2 hr before clinorotation display a significant reduction in outward anisotropic growth (Figure 1F). For root system experiments, plants were grown on media containing 0.2 μM or 1 μM NPA, representing treatments where the gravitropism of primary and lateral roots is either mildly (0.2 μM) or more severely (1 μM) affected ([16, 17] and data not shown). Both of these NPA treatments are sufficient to dramatically reduce outward/upward bending in clinorotated lateral roots, indicating that auxin transport is also required for the activity of the antigravitropic offset in the root (Figure 1I).

These auxin transport experiments are consistent with classical data on auxin distribution in clinorotated lateral organs derived from the *Avena* bioassay and the use of radiolabelled IAA [10, 18, 19]. Together, they suggest that the antigravitropic growth component that opposes gravitropic responses in lateral branches is also driven by auxin. The action of these gravitropic and antigravitropic auxin components is illustrated in Figures 1D and 1G: under normal upright growth conditions, gravitropism (green line) that would otherwise move the branch toward the vertical is counteracted by antigravitropism (red line), resulting in net symmetry in auxin response and hence isotropic but angled growth. Under clinorotation and in the absence of a stable gravity reference, auxin movement to the lower side of the shoot or root branch is lost while continued antigravitropism sustains auxin levels on the upper side, driving outward anisotropic growth.

This model for the maintenance of gravity-dependent nonvertical growth retains the central element of the Cholodny-Went hypothesis in that isotropic growth at any given GSA is the result of symmetrical auxin distribution and response between opposite sides of an organ while asymmetry in response leads to anisotropic growth. Thus in a given nonvertical GSA state the antigravitropic offset can be thought of as largely constant regardless of the actual orientation of the branch. In contrast, the gravitropic component is continuously variable according to branch orientation within the gravity field. For a shoot branch that is shifted to a more vertical angle, an initial decrease in the magnitude of the gravitropic component would prompt outward/downward anisotropic growth driven by the constant antigravitropic offset. This downward bending would diminish as the magnitude of the gravitropic component increased with the increasing displacement of statoliths as the branch moves back away from the vertical. For a shoot branch displaced to an angle less vertical than its GSA, the reverse would apply, with an increased magnitude of gravitropic response consistent with the general principles of sine law (see the Supplemental Introduction) [20, 21].

This mechanistic model for the maintenance of nonvertical growth predicts that lateral branches with the least vertical GSA should display the greatest abaxial bending upon clinorotation. We tested this idea by recording the response of lateral roots and cauline branches of different lengths to the withdrawal of a stable gravity reference; both lateral roots and cauline branches emerge at very shallow GSAs, becoming increasingly vertical as they grow longer (Figures 1A and S1A–S1D). Consistent with this manifest relationship between branch length and GSA, we observed that the shortest root and shoot branches exhibited the greatest degree of curvature upon clinorotation and the longest branches the least (Figures 2A and 2B). These data indicate that the GSA of a given organ is determined by the magnitude of its antigravitropic offset (which may be a zero or nonzero value) and provide confirmation that the model for GSA maintenance presented here can account for the observed GSA biology of lateral root and shoot branches.

### Auxin Specifies the Magnitude of the Antigravitropic Offset and Hence the GSA of Lateral Shoots and Roots

In exploring the involvement of auxin in regulating the transition nonzero to zero antigravitropic offset states in decapitation experiments, we also noticed that mutants with defects in auxin homeostasis or response have altered lateral organ GSA (Figure S2, summarized in Figure 2E). Mutants with higher levels of auxin (*yucca1-1D* [22], *yuc1D* [23]) or a predicted higher level of auxin response (*axr3-10* [24], *arf10-3 arf16-2* [25], *arf10-3 arf16-2 axr3-10*), have lateral shoots or roots that are more vertical than wild-type. In contrast, mutants with lower levels of auxin (*wei8 tar2* [23]) or auxin response (the auxin receptor mutants *tir1-1* [26], *afb4-2 afb5-5* [27]) have lateral branches that are less vertical (Figures 2D and S2A–S2D). We confirmed that these changes in root and shoot branch growth angle were bona fide GSA phenotypes by performing reorientation assays with *tir1-1*, *axr3-10*, and *arf10-3 arf16-1* (Figures S3A and S3B). Consistent with all of these genetic data, lateral roots cultured on media containing IAA and 2,4-dichlorophenoxyacetic acid (2,4-D) in the nanomolar range grow at increasingly vertical GSA values (Figures 2C and S2F). We observed a similar effect of auxin on lateral root GSA in bean (*Phaseolus vulgaris*) and rice (Figure S2H).

Only one set of mutants ran counter to this simple relationship between auxin response and GSA phenotype; *NPH4/ARF7* and *ARF19* are from the subclade of auxin response factors (ARFs) that have been characterized as transcriptional activators [27]. Cauline branches of *nph4-1 arf19-1* [28] are considerably less vertical than the wild-type (Figures 2D, 2E, and S2G). After decapitation, the subapical branch in *nph4-1 arf19-1* undergoes the same shift toward the vertical observed in wild-type, suggesting that the GSA phenotype in this mutant

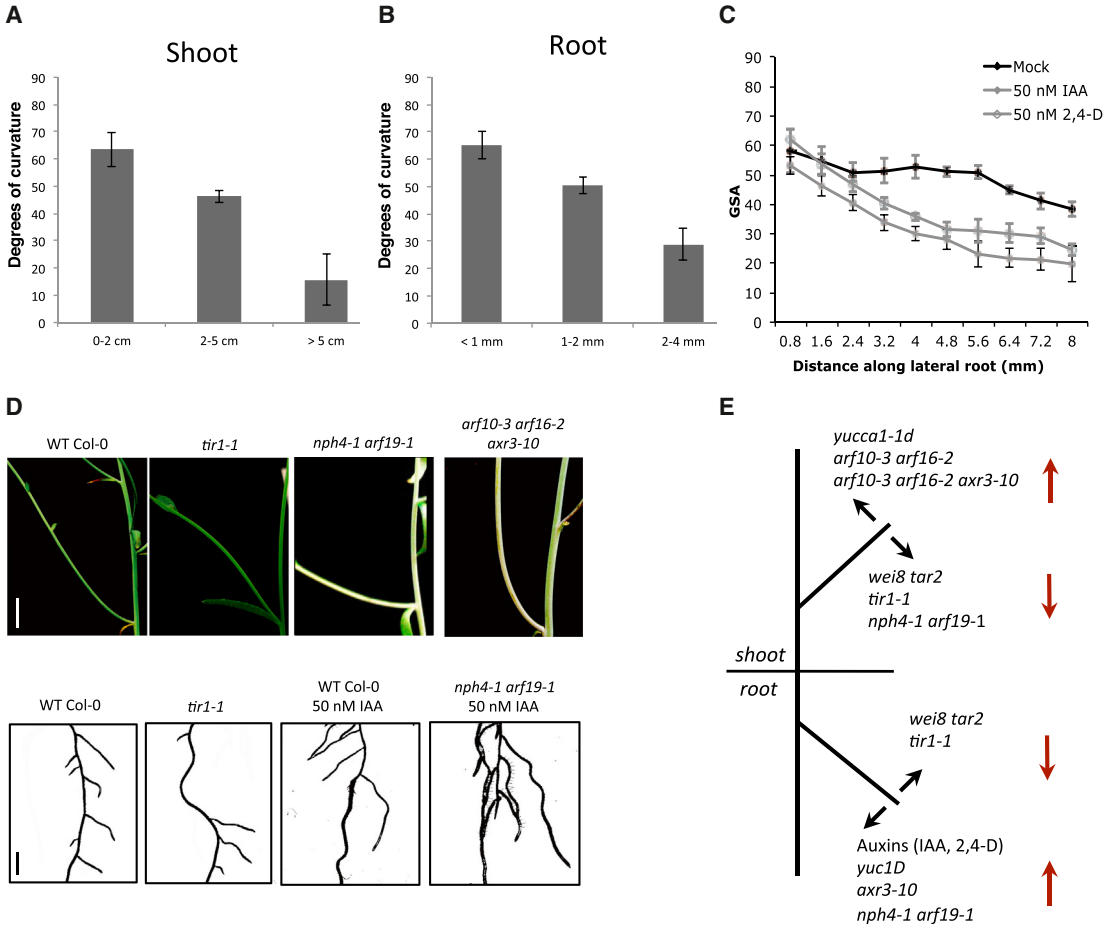


Figure 2. Auxin Regulates Shoot Branch and Lateral Root GSA

(A and B) Magnitude of outward anisotropic growth in cauline branches (A) and lateral roots (B) of increasing ages (defined as branches length size classes) after 8 hr of clinorotation, 4 rph (A) and 1 rpm (B).

(C) Effect of auxin (IAA, 2,4-D) on wild-type (Col-0) lateral root GSA; changes in GSA as measured along lateral roots in successive 0.8 mm segments (mean of 12–15 lateral roots, one-way ANOVA, $p < 0.05$ for data points 4–10).

(D) Typical GSA profiles of lateral shoots and roots of wild-type and auxin signaling mutants (*tir1-1*, *nph4-1 arf19-1*, *arf10-3 arf16-2 axr3-10*) with or without IAA treatment.

(E) Schematic representation of the effect of auxin synthesis and signaling mutants on lateral shoot and root GSA (data presented in Figures S3 and S4). Black lines represent the primary axis and lateral branches, black arrows changes in branch GSA and red arrows higher and lower levels of auxin or auxin response, respectively.

Scale bars represent 1 cm (D, top) and 0.5 cm (D, bottom). Error bars indicate the SEM. See also Figure S2.

represents a bona fide defect in GSA control rather than one in gravitropic response (Figure S2I). In the root, loss of *NPH4/ARF7* function has the opposite effect with *arf7-201* [29] lateral roots growing at a more vertical GSA (Figure S2J). For examination of the effects of the combined loss of ARF7 and ARF19 function on lateral root GSA, it was necessary to perform the analysis on media containing auxin in order to induce lateral root formation. Under these conditions, *nph4-1 arf19-1* lateral roots have a GSA that is significantly more vertical than that of wild-type plants grown at the same concentration of IAA (Figures 2D, 2E, and S2K).

Thus, with the exception of NPH4/ARF7 and ARF19, the removal of negative regulators of auxin signaling (e.g., repressing ARFs and Aux/IAAs) causes lateral branch GSA to be more vertical, while the removal of positive regulators of auxin signaling (e.g., TIR1) induces a less vertical phenotype. In addition to any alterations in basic gravitropic response in these mutants, these data indicate that TIR1 is a negative regulator of the antigravitropic offset, while ARF10, ARF16, and AXR3 are positive regulators of this mechanism.

To test this hypothesis, we performed clinorotation experiments with mutants or treatments that induce more or less vertical lateral GSA phenotypes. In both the shoot and root, mutants with a less vertical GSA (*tir1-1* in both root and shoot, *nph4-1 arf19-1* in the shoot only) exhibit a greater degree of outward anisotropy, while those with a more vertical GSA (*arf10-3 arf16-2* in the shoot, *axr3-10* and auxin treatment in the root) exhibit less bending (Figures 3, S3C, and S3D). We conclude that auxin acts to alter the GSA of a lateral organ by changing the magnitude of the antigravitropic offset.

## The Auxin-Mediated Specification of Shoot Branch GSA Is Effected in the Gravity-Sensing Cells of the Shoot and the Root

To identify the cell types in which changes in auxin sensitivity can affect GSA, we altered auxin signaling in a

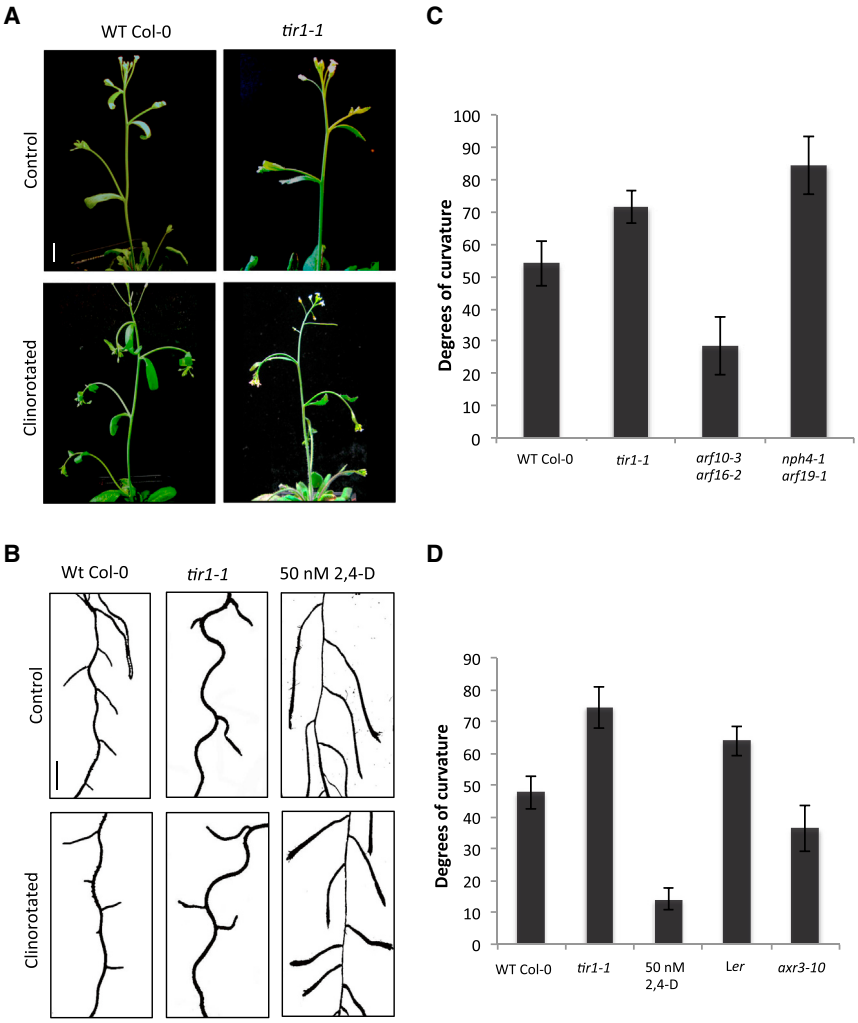


Figure 3. Auxin Regulates GSA by Modulating the Magnitude of the Antigravitropic Offset

Changes in lateral shoot GSA (A and C) and lateral root GSA (B and D) after clinorotation (4 rph and 1 rpm, respectively, 8 hr) in wild-type and auxin response mutants and auxin-treated seedlings. Shoot branch GSA, *tir1-1* (A and C), *nph4-1 arf19-1* and *arf10-3 arf16-2* (C); lateral root GSA, *tir1-1*, 50 nM 2,4-D (B and D) *axr3-10* with corresponding wild-type controls (D).

(A and B) Control untreated plants (top panels) and a clinorotated plants (bottom panels).

(C and D) Graphs comparing the magnitude of bending (degrees of curvature) in different auxin response mutants, and in response to 2,4-D treatment, upon clinorotation ($p < 0.05$, one-way ANOVA, n = 15–20).

Scale bars represent 1 cm (A) and 0.5 cm (B). Error bars indicate the SEM. See also Figure S3.

cell-type-specific manner beginning with the gravity-sensing cells from which auxin fluxes associated with graviresponse are directed. In the shoot, these statocytes are located in endodermis, a tissue marked by the expression of the *Arabidopsis* gene *SCARECROW* (*SCR*) [30]. Previous work on auxin signaling in embryogenesis included the characterization of a transgenic line expressing the mutant stabilized Aux/IAA corepressor protein mutant bodenlos/iaa12 (bdl) under the control of the *SCR* promoter (*SCR::bdl*) [31]. Only one postembryonic phenotype of *SCR::bdl* was described, that of a defect in cauline branch gravitropism. We examined this line and established that the defect is not a principally one of gravitropism but rather a change in GSA control; the primary inflorescence displays a normal vertical GSA, while the cauline branches have an extreme nonvertical GSA (Figures 4A and 4C). We demonstrated that this was a bona fide defect in GSA control by confirming that upon decapitation of the primary SAM, subapical cauline branches in *SCR::bdl* transition from an extreme nonvertical GSA to a near vertical one, reflecting the loss of antigravitropic offset and confirming that the cauline branches retain gravitropic competence but have an particularly strong antigravitropic offset (Figure 4A). To provide additional evidence of the importance of variation in auxin signaling in shoot statocytes for GSA control, we generated *Arabidopsis* plants expressing an inducible *SCR::bdl:GR* construct. Dexamethasone treatment of these lines induces a less vertical GSA state in shoot branches, again, without affecting the GSA of the primary shoot (Figure S4). This induced GSA state, consistent with the activity of a strong antigravitropic offset, is lost 72 hr after the cessation of dexamethasone induction (Figure S4B).

There are strong similarities between *SCR::bdl* and *SCR::bdl:GR* plants and *nph4-1 arf19-1* mutants, all having the least vertical shoot branch GSA phenotypes of the mutants tested here. *bdl/iaa12* is a stabilizing gain-of-function mutation, and BDL/IAA12 protein has been shown to interact with NPH4/ARF7 and ARF19 [32]. Thus, regardless of the contribution of wild-type BDL/IAA12 to GSA control, the likely bdl-mediated blocking of ARF action, including NPH4/ARF7 and ARF19, in just the shoot endodermal statocytes is sufficient to modulate the GSA of shoot branches. To test the idea that variation in NPH4/ARF7-mediated signaling in shoot statocytes alone can alter the GSA of shoot branches, we made transgenic plants expressing an inducible form of ARF7 in endodermal statocytes (*SCR::ARF7:GR*). Dexamethasone treatment of these plants induces a very vertical cauline branch GSA phenotype (Figures 4B and 4D), consistent with the hypothesis that ARF7 is a negative regulator of the antigravitropic offset in the shoot and further, that changes in auxin response in the shoot statocytes alone are sufficient to specify GSA values of shoot branches.

To test whether the modulation of auxin sensitivity in the gravity-sensing cells of the columella root cap is similarly sufficient to alter lateral root GSA, we used the GAL4 transactivation system [33]. Plants expressing a stabilized version of Aux/IAA AXR3/IAA17 under the control of the UAS promoter (*UAS::axr3-1*) were crossed to the enhancer-trap lines J1092 (driving GAL4 expression in the columella and lateral root cap [LRC]) and M0013 (driving GAL4 expression in the LRC) [33]. Expression of the hypermorphic/neomorphic axr3-1 mutant protein in the columella and LRC but not the LRC alone causes lateral roots to grow with a more vertical GSA

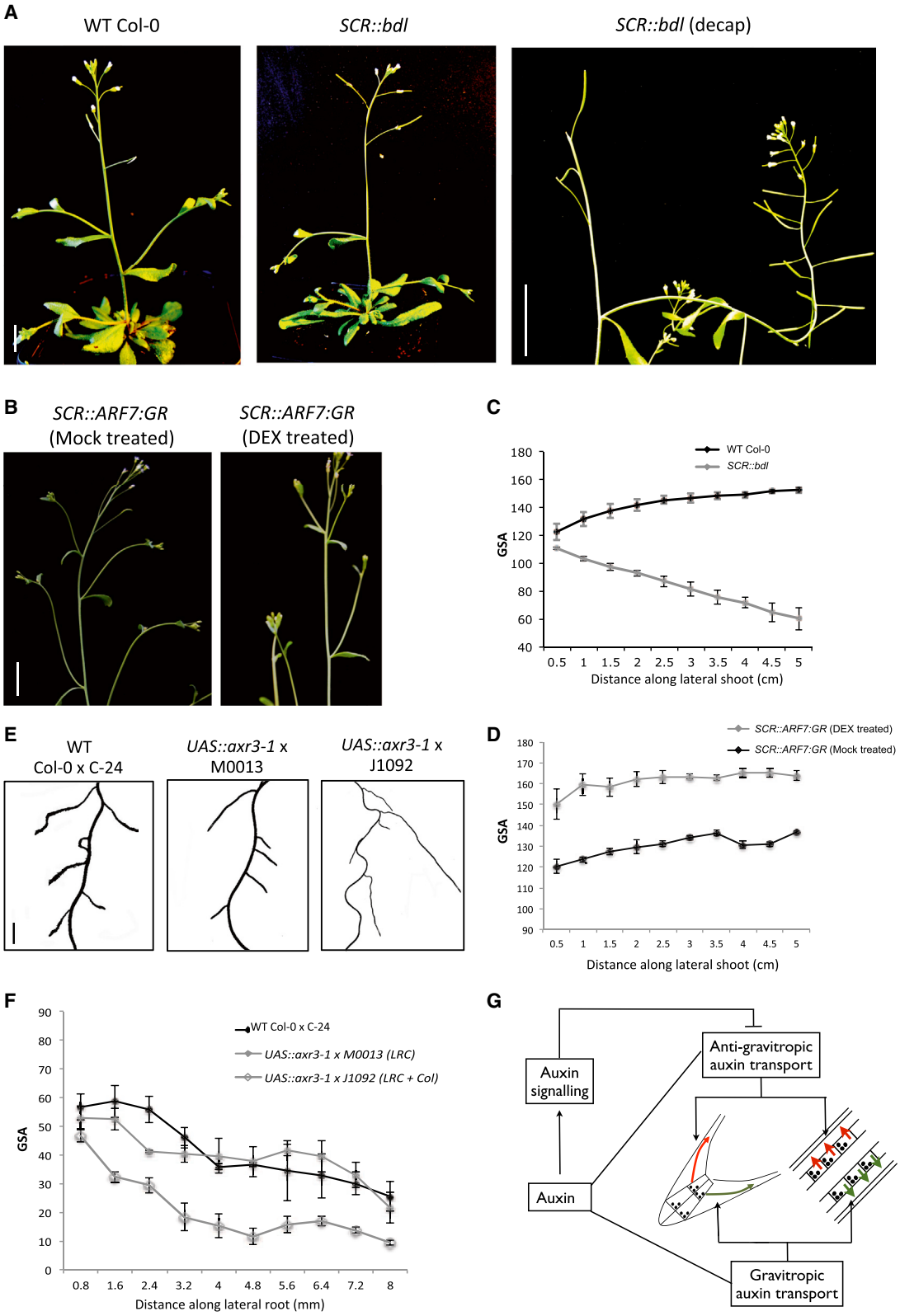


Figure 4. Auxin specifies GSA within the gravity-sensing cells of the root and shoot

(A and C) Shoot GSA phenotypes of 28-day-old intact wild-type plants and intact and decapitated *SCR::bdl* plants.

(B and D) Shoot GSA phenotypes of 28-day-old mock- and Dex-treated *SCR::ARF7:GR* plants.

(C and D) Quantification of lateral shoot GSA in (intact) wild-type and *SCR::bdl* plants (C) and in mock- and Dex-treated *SCR::ARF7:GR* plants (D).

(E and F) Root GSA phenotypes of plants expressing stabilized axr3-1/iaa17 under the control of the UAS promoter (*UAS::axr3-1*) in the GAL4 driver line backgrounds M0013 (expression in the lateral root cap [LRC]) and J1092 (expression in the LRC and columella). Quantification of lateral root GSA in

*(legend continued on next page)*

(Figures 4E and 4F). These data demonstrate that the modulation of auxin signaling specifically in the columella statocytes is sufficient to alter lateral root GSA without affecting primary root GSA, indicating that, as in the shoot, it is within the statocytes that the auxin-dependent regulation of the magnitude of the antigravitropic offset and hence GSA is effected.

## Conclusions

The data presented here have revealed four fundamental features of GSA control in *Arabidopsis*. First, the GSA states of the near-vertical primary axis and nonvertical lateral branches are distinguished by the existence of an antigravitropic offset mechanism operating in the latter. Second, the antigravitropic offset mechanism is auxin dependent and acts in tension with gravitropism to generate net symmetry in auxin levels and response between the upper and lower sides of lateral branches. Third, the GSA of graviresponsive organs is dependent on the magnitude of the antigravitropic offset and its interaction with gravitropic response. Fourth, auxin specifies nonvertical GSA values by modulating the magnitude of the antigravitropic offset component within the gravity-sensing cells of lateral roots and shoots.

The principal features of this model of GSA control are illustrated in Figure 4G, which depicts the basic relevant relationships between auxin, auxin transport, and auxin signaling within the gravity-sensing cells of the root and shoot. It is important to emphasize that there are two mechanistically distinct roles for auxin in this model: in addition to driving isotropic growth itself in the epidermis, auxin and auxin signaling within the gravity-sensing cells is negatively regulating the magnitude of the antigravitropic offset mechanism. The mechanisms we have identified also provide multiple nodes through which environmental signals relating to resource status (in particular nutrients below ground and light above) can be integrated such that changes to optimize resource capture can be effected.

The dependence of the antigravitropic offset on auxin transport is consistent with the fact that changes in auxin signaling solely within gravity-sensing cells is sufficient to bring about changes in growth in nonadjacent epidermal tissues. In establishing the molecular basis of the antagonistic interaction of gravitropic and antigravitropic offset components, an obvious hypothesis to test will be whether this antagonism lies in opposing inputs into the polarity of PIN protein localization or activity in gravity-sensing cells; PIN localization and activity depend upon PIN protein phosphorylation [34, 35], and so it will be interesting to examine the extent to which gravity-dependent, nonvertical growth might stem from competing phosphatase and kinase activities regulating the phosphorylation state, and hence net symmetry of PINs or PIN activity within statocytes. The ability to separate gravitropic and antigravitropic offset components as described here provides a powerful experimental system to test this and other hypotheses. We predict that the general principles of GSA control that we have set out here will be of wide relevance throughout the higher plants and thus provide a conceptual framework for understanding GSA variation throughout nature.

### Supplemental Information

Supplemental Information includes Supplemental Introduction, Supplemental Experimental Procedures, and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.034.

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wild-type and plants expressing *axr3-1* under the control of the two driver lines is shown (F).
(G) Schematic model of the action of the auxin-dependent gravitropic and antigravitropic offset components also showing the TIR1/AFB-signaling-mediated regulation of the antigravitropic offset by auxin that occurs within the gravity-sensing cells. Green arrows and red arrows represent the gravitropic and antigravitropic auxin transport components, respectively.
Scale bars represent 1 cm (A and B) and 0.5 cm (E). Error bars indicate the SEM. See also Figure S4.

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