Asymmetric gibberellin signaling regulates vacuolar trafficking of PIN auxin transporters during root gravitropism

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Gravitropic bending of plant organs is mediated by an asymmetric signaling of the plant hormone auxin between the upper and lower side of the respective organ. Here, we show that also another plant hormone, gibberellic acid (GA), shows asymmetric action during gravitropic responses. Immunodetection using an antibody against GA and monitoring GA signaling output by downstream degradation of DELLA proteins revealed an asymmetric GA distribution and response with the maximum at the lower side of gravistimulated roots. Genetic or pharmacological manipulation of GA levels or response affects gravity-mediated auxin redistribution and root bending response. The higher GA levels at the lower side of the root correlate with increased amounts of PIN-FORMED2 (PIN2) auxin transporter at the plasma membrane. The observed increase in PIN2 stability is caused by a specific GA effect on trafficking of PIN proteins to lytic vacuoles that presumably occurs downstream of brefeldin A-sensitive endosomes. Our results suggest that asymmetric auxin distribution instructive for gravity-induced differential growth is consolidated by the asymmetric action of GA that stabilizes the PIN-dependent auxin stream along the lower side of gravistimulated roots.

DIRECTIONALITY IN GROWTH

Directional growth as a response to light or gravity (i.e., tropisms) is a key adaptation response adjusting plant growth to the environment. Tropisms are accomplished by asymmetric elongation of cells within responding organs, which is mediated by differential distribution of the signaling molecule auxin (1). Following a gravitropic stimulus, the intercellular transport of auxin is redirected toward the lower sides of organs, where, in the stem, the higher auxin concentration stimulates cell elongation causing upward bending and, in roots, high auxin concentrations inhibit elongation; as a consequence, roots bend downward (2). The directional cell-to-cell auxin transport depends on the activity of AUXIN RESISTANT1 (AUX1) influx (3) and PIN efflux transporters (4), as well as on auxin transporters from the ATP BINDING CASSETTE SUBFAMILY B (ABC B) family (5). The directionality of auxin flow is determined by polarity of PIN subcellular localization at the plasma membrane (PM) (6, 7). A mechanism for initial redirection of auxin fluxes involves gravity-induced changes in the polarity of PIN localization as observed for PIN3 in root columella (8–10) or shoot endodermis cells (11). The initial auxin asymmetry is further propagated from the root tip by asymmetric degradation of PIN2 at the upper vs. the lower side of the gravistimulated root (12, 13).

PIN proteins undergo constitutive subcellular dynamics involving endocytic recycling to different domains at the PM or rerouting via the prevacuolar compartment to the lytic vacuole for degradation (12–15). Thus, PIN abundance can be regulated by changes in trafficking or transcription (16). Several other hormonal signaling pathways have been shown to affect directionality and throughput of PIN-dependent auxin fluxes. These include feedback regulation of PIN internalization (17, 18) and polarity (19) by auxin; brassinosteroid (20), cytokinin (21, 22), and ethylene (23, 24) effects on PIN transcription; as well as cytokinin effects on PIN degradation (25). Moreover, gibberellic biosynthesis mutants show increased PIN degradation (26).

With respect to the gravitropism, the main focus of the previous research has been on auxin and, in particular, its asymmetric distribution. However, in some monocot species, including rice, maize, and barley, asymmetric accumulation of auxin and gibberellic acid (GA) at the lower side of gravistimulated organs has been reported (27–29), but general validity and physiological significance of these observations remain unclear.

Here, we show an asymmetric distribution of GA and GA signaling during root gravitropic growth. Furthermore, we observed that GA increases the levels of PIN auxin transporters at the PM by inhibiting PIN vacuolar trafficking. These observations suggest that an interplay between asymmetric auxin and gibberellin activities modulates and stabilizes auxin fluxes for root gravitropic responses.

Results and Discussion

Gravity Stimulation Induces Asymmetric GA Distribution and Response. To test for a role of GA in root gravitropism in Arabidopsis thaliana, we examined GA distribution following gravistimulation. As direct measurements of GA distributions are technically not feasible in intact Arabidopsis roots, we established whole-mount immunodetection of GA in Arabidopsis root tips by using an antibody raised against BSA-conjugated GA (for details see Materials and Methods). Control experiments showed specific binding of the anti-GA antibody to the epidermal cells of cell division and elongation zones of the root tip (Fig. S1 A–G). Much weaker anti-GA signals were detected in the seedlings in which GA production was inhibited genetically in the ga1–3 mutant (30) or pharmacologically by uniconazole (Uni) treatment (31) (Fig. S1 H–J), confirming that a specific labeling of GA had been detected. As our immunolocalization method is only reliable for root tips (32), we cannot assess the GA distribution in the more differentiated parts of the root or make

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a quantitative comparison between different root tissues such as endodermis, in which GA is important to control root growth (33). Following gravistimulation, the originally symmetric GA labeling became asymmetric with markedly stronger signal at the lower side of the root and a weaker signal at the upper side after approximately 3 h (Fig. 1 A–C). This result suggests that gravity induces asymmetry in GA distribution in gravistimulated root tips.

To verify the antibody-based observations, we analyzed down-stream GA responses following gravistimulation. GA promotes degradation of downstream regulators, the DELLA proteins, which depends on cellular GA concentrations (34). Thus, quantitative analysis of the GFP-tagged DELLA protein REPRESSOR OF GA1-3 (RGA) in RGA::GFP-RGA seedlings allows monitoring of the GA response in plant tissues. We observed a significant ($P \leq 0.05$) decrease of the GFP-RGA signal at the lower side of the root tip approximately 3 h after gravistimulation (Fig. 1 D–F). This reflects an increase in GA response at the lower side of the root and is entirely consistent with increased anti-GA signals there. In summary, GA immunodetection and GA response monitoring revealed asymmetric GA distribution with higher GA levels at the lower side of the gravistimulated roots.

Our data reveal that not only auxin (35, 36) but also GA shows an asymmetric distribution in the gravistimulated root tips. To compare the kinetics of these events, we monitored the expression of GA-responsive GA20ox1 (37) and auxin-responsive GH3.1 genes (38). Following gravitropic stimulation, GA20ox1 expression showed gradual up-regulation (Fig. S2), whereas the GH3.1 gene showed faster but transient up-regulation of expression (Fig. S2). Consistently, gravity-induced auxin response asymmetry as monitored by the DR5rev::GFP (35) or DII-Venus (36) reporters was established faster than the observed GA asymmetry, suggesting a chronological order of changes in hormone responses during gravitropism, whereby GA asymmetry develops only after the initial auxin asymmetry establishment.

**GA Action Is Required for Root Gravitropism and Asymmetric Auxin Distribution.** Asymmetry in GA distribution during gravitropism implies that GA plays a role in root gravitropic responses that was also suggested by gravitropic defects in GA biosynthesis mutants (26). Therefore, we studied the consequences of manipulating GA levels and GA signaling on root gravitropism in Arabidopsis. Treatment with the GA biosynthesis inhibitor uniconazole, as well as GA depletion in the GA biosynthetic ga1-3 mutant (30) or inhibition of GA signaling in the dominant-negative DELLA mutant gaiΔ17 (39), consistently caused defects in gravitropic root bending (Fig. 2 A and B). Effects of increased GA signaling were analyzed in seedlings treated with exogenous GA and the Arabidopsis pentaple mutant carrying lesions in all five DELLA repressors acting downstream of GA (40). Also, these treatments and mutants showed a defect in root gravitropism (Fig. 2 A and B), confirming that manipulation of GA levels and signaling reduce the root gravitropic response.

Root gravitropic bending is accompanied by asymmetric auxin distribution and response with the maximum at the lower side of gravistimulated roots (35, 36) that is disrupted in GA biosynthesis mutants (26). This asymmetry can be monitored with auxin response reporters such as DR5rev::GFP (41, 42) showing increased activity at the lower side of the root (Fig. 2C). Positive (i.e., GA treatment) and negative (i.e., uniconazole treatment) manipulations of GA levels interfered with the establishment of the asymmetric auxin response in the root tip following gravistimulation. Roots treated with GA showed a symmetrically increased signal in the lateral root cap that was not detected in nontreated roots (Fig. 2 C and D). On the contrary, inhibition of GA biosynthesis by uniconazole prevented lateral accumulation of the DR5rev::GFP signal (Fig. 2E). These analyses show that balanced levels of GA are required for gravity-induced asymmetric auxin responses and for root gravitropic growth.

**GA Acts on Root Gravitropism by Regulating Levels of PIN Auxin Transporters.** Next, we assessed a possible mechanism by which GA interferes with auxin distribution and root gravitropism. Gravity-induced auxin redistribution requires directional activity of PIN auxin transporters (4, 6), of which loss-of-function (pin2/ pin1) (43) and gain-of-function (35S::PIN1) (4, 44) alleles show root gravitropic phenotypes. Notably, interfering with GA biosynthesis by uniconazole treatment amplified pin2 defects (Fig. S5), but largely normalized the severe agravitropic phenotype of 35S::PIN1 roots (Fig. 3B). Similarly, inhibiting GA signaling in the dominant-negative DELLA mutant gaiΔ17 (39) rescued normal gravitropic growth in 35S::PIN1 × gaiΔ17 double-mutant lines (Fig. 3C). These effects of GA on pin mutants suggest that GA regulates root gravitropism by a mechanism involving PIN auxin transporters.

The agravitropic phenotype of 35S::PIN1 roots is presumably caused by the ectopic expression of PIN1 in the root epidermis cells (6, 45). Immunolocalization studies of 35S::PIN1 roots showed a known ectopic presence of PIN1 in epidermis cells (Fig. 3F), whereas gaiΔ17 showed weaker, but overall normal pattern of PIN1 localization (Fig. 3H). The 35S::PIN1 × gaiΔ17 double mutant exhibited a strongly decreased ectopic presence of PIN1 in the epidermis (Fig. 3G), correlating with the rescue of normal gravitropic growth (Fig. 3C). This observation suggests that GA effects on root gravitropism are related to regulation of PIN abundance.
GA Increases PIN Protein Stability by Inhibiting PIN Vacuolar Trafficking.

High GA levels detected at the lower side of gravistimulated roots (Fig. 1) correlate with higher PIN2 abundance there (12, 35). In addition, the GA-deficient ga1-3 mutant has been shown to have lower PIN2 levels (26). GA does not change (DEX)-inducible expression of PIN2 genes (12) that allow dexamethasone (DEX)–inducible expression of PIN2 proteins. To test the effect of increased GA levels on PIN2 protein amounts independently of PIN transcription, we analyzed PIN2 turnover in TA::PIN2-GFP plants (12) that allow dexamethasone (DEX)–inducible expression of PIN2. After induction of PIN2 expression in a TA::PIN2-GFP line for 24 h, DEX was washed out and seedlings were treated with GA or with DMSO as a control. The GA treatment dramatically increased PIN2 stability compared with untreated controls (Fig. 4 A–C).

To investigate if this GA effect is specific for PIN proteins, we tested for differences in the PM amounts of the auxin influx carrier AUX1 (46), the auxin transporter P-glycoprotein19 (PGP19/ABC19) (47), and the aquaporin PLASMALEMMA INTRINSIC PROTEIN2 (PIP2) (48), and found that they were not influenced by GA or the GA biosynthesis inhibitor paclobutrazol (31) (Fig. S5 A–Q). In contrast, all Arabidopsis PM PIN proteins—PIN1–GFP (44), PIN2–GFP (12), PIN3–GFP (49), PIN4–GFP (50), and PIN7–GFP (10)—showed increased and decreased signals after GA and paclobutrazol treatments, respectively (Fig. S5 R–T). These data indicate that GA specifically influences the stability of PIN proteins.

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GFP-labeled intracellular compartments were identified as vacuoles by experiments that used the endocytic tracer FM4-64 (52). PIN2::PIN2-GFP plants were kept for 4 h in the dark and stained for 1 h with FM4-64 to visualize the tonoplast membranes. Overlay of GFP and FM4-64 fluorescence images showed GFP signal in the vacuolar lumen (Fig. S6A–C). In these experiments, GA treatment resulted in a dramatic decrease of the vacuolar signal, whereas the PIN2-GFP PM signal was notably increased (Fig. 4D, G, and H). Also, Western blot analysis showed an increase of PIN2 protein abundance in GA-treated roots (Fig. 4F). A complementary experiment in uniconazole-treated plants revealed that low GA levels increase the amount of the GFP signal in the lytic vacuoles (Fig. 4E, I, and J) consistent with previous reports (26). Together, these experiments show that GA increases stability specifically of PIN proteins by regulating the rate of PIN trafficking into the lytic vacuole, a process inhibited by high and promoted by low GA levels.

GA Targets PIN Vacular Trafficking Downstream of BFA-Sensitive Endosomes. The data so far raised the question at what particular PIN trafficking step GA acts to regulate PIN turnover. Up-take experiments with the endocytosis marker FM4-64 in ga1-3 and pentaple mutants and in seedlings treated with GA or uniconazole did not show any differences vs. untreated WT controls (Fig. S7A), not supporting a notion that GA acts on endocytosis.

Following endocytosis, PIN proteins are sorted in the endosomes for recycling back to the PM or for further trafficking toward the vacuole (14). The recycling and, to a lesser extent, vacuolar trafficking are sensitive to the vesicle trafficking inhibitor brefeldin A (BFA) (13, 35). BFA treatment aggregates different types of endosomes and thus allows better visualization of the endosomal and vacuolar trafficking cargoes, including PIN proteins (35, 53). GA treatment preceding BFA application increased (Fig. 5A–C)—whereas low GA levels in uniconazole-treated roots or in the ga1-3 mutant decreased—the PIN2-GFP signal in BFA-induced aggregates compared with controls (Fig. 5D–F). This suggests that high GA promotes and low GA inhibits PIN retrograde trafficking somewhere downstream of the BFA-sensitive endosomes. A contrasting outcome was seen in similar experiments with another trafficking inhibitor, wortmannin (WM), that leads to swelling and aggregation of prevacuolar compartments (13, 54). We did not observe any change of the PIN2-GFP accumulation in WM-induced intracellular aggregations with increased (i.e., GA treatment) or decreased (i.e., uniconazole treatment, ga1-3 mutant) GA levels (Fig. S7B).

In summary, these experiments with the use of different markers and trafficking inhibitors confirmed that GA interferes with vacuolar trafficking of PIN proteins. GA targets presumably a trafficking step downstream of BFA-sensitive endosomes toward the vacuole.

Conclusion
PM proteins targeted for degradation are endocytosed and subsequently hydrolyzed by proteases in the lytic vacuole after a passage through the early and late endosomes. During gravitropism, the PIN2 auxin transporter is degraded at the upper side of the root and stabilized at the lower root side, thus contributing to the asymmetric auxin flow that mediates gravitropic bending (12, 13, 35). The PIN2 destabilization at the upper side is mediated by the low auxin levels and signaling (35), and the initial increase of PIN2 levels at the lower side is presumably caused by auxin-mediated inhibition of PIN2 endocytosis (18). Here, we propose an additional or alternative mechanism for PIN stabilization at the lower side. We detected asymmetry of another plant signaling molecule, GA, in gravistimulated roots with high GA levels at the lower side. GA also stabilizes specifically PIN proteins by inhibiting their trafficking to the lytic vacuole, providing the possibility that GA contributes to the stabilization of PIN2 at the lower side of the root and, thus, promotes asymmetric auxin flow and distribution for gravitropic bending. In line with this hypothesis, interference with GA levels or signaling modifies the gravity-induced asymmetric auxin response and gravitropic bending. On the contrary, GA asymmetry appears to develop only after the initial gravity-induced auxin asymmetry, suggesting mutual feed-back regulation of these two pathways. Based on these different kinetics, auxin may control the early

**Fig. 4.** GA effects on PIN stability and vacuolar trafficking. (A and B) PIN2 expression was induced in TA::PIN2-GFP seedlings by treatment with 30 μM DEX for 24 h. After depletion of DEX by washing, roots were mounted with or without GA and PIN2-GFP signal intensities were recorded after the indicated time points. Roots were treated with DMSO as control (A) or with 50 μM GA (B). To rule out that the DEX-inducible promoter responds to GA, TA::PIN2-GFP seedlings were treated for 24 h with GA without DEX, but did not show induction of PIN2-GFP expression. To have a strong PIN2-GFP signal for quantification, root columella cells were imaged. (C) Total PIN2-GFP signal intensity at time point 1 h DMSO and GA, respectively, was set to 100%, and relative signal intensity of subsequent time points was calculated as percentage of total signal intensity at time point 1 h. (D and E) PIN2-GFP signal at the PM relative to intracellular signal after GA treatment (D) and after uniconazole treatment (E). A small box of the size of the PM signal was used to define the region of interest for the PM quantification; the intracellular signal was quantified within a rectangle region of interest covering the cell volume. The ratio of PM vs. internal signal was measured in seven cells in a row (n = 8 individual roots). **P ≤ 0.01. (F) Western Blot analysis of roots treated for 3 h with DMSO or 50 μM GA. (G–J) Vacular targeting of PIN2 was analyzed after treatment [GA and uniconazole (Uni)] of seedlings in the light and then transferred to the dark for 6 h (G and H) or 3 h (I and J). PIN2::PIN2-GFP seedlings were pretreated with DMSO (GA solvent) for 3 h (G), 50 μM GA for 3 h (H), DMSO (uniconazole solvent) for 2 d (I), or 10 μM uniconazole for 2 d (J). (Scale bars: A and B, 50 μm; G–J, 25 μm.)
response of the root to a gravitropic stimulus and GA subsequently maintains gravitropic curvature by stabilizing the PIN-dependent auxin stream along the lower side of the root. The emerging picture involves GA as a part of the complex network consolidating asymmetric auxin action during gravitropic responses and, possibly, also other auxin-mediated processes, as consolidating asymmetric auxin action during gravitropic re-

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*Pharmacological Treatments and Experimental Conditions.* Chemicals were applied in solid or liquid MS medium or by spraying 7-d-old seedlings with BFA (50 mM stock in ethanol; final concentration (f.c.) 50 μM), DEX (30 mM stock in DMSO; f.c. 30 μM), FM4-64 (2 mM stock in DMSO; f.c. 2 μM), GA3 (100 mM stock in DMSO; f.c. 50 μM), uniconazole (10 mM stock in DMSO; f.c. 10 μM), paclobutrazol (10 mM stock in DMSO; f.c. 10 μM), or WM (10 mM stock in DMSO; f.c. 30 μM). Mock treatments were done by using equal amounts of solvent (DMSO), and double treatments were carried out within 1 h-interval. All independent experiments were performed at least in triplicate, with a minimum of eight individual plants. BFA, WM, and FM4-64 were applied in liquid MS medium for 2 h (BFA), 4.5 h (WM), and 1 h (FM4-64).

Dark treatments were done as described previously (13). To compare protein concentrations in the lytic vacuole, epidermal cells at the interface of the meristematic and elongation zone were recorded. For live cell GFP imaging, a Leica DM6000 CS, TCS SPS AOBBS confocal scanning microscope was used, and fluorescent signals were quantified by using the integrated Leica quantification module (LAS AF 2.1.0). Fluorescent measurements were done on the original Leica image files. Representative images are shown. Statistics were evaluated with GraphPad QuickCalc test (http://graphpad.com/quickcalc/index.cfm).

**Immunodetection of GA in Roots and Competition Assays.** Whole-mount immunolocalization was done as described previously (58) after a prefixation step using a freshly prepared 3% W-ethyl-N-(3-dimethylaminopropyl) carbodiimidine-hydrochloride (Sigma) solution in methanol for 1 h (44). Antibodies were diluted as follows: 1:100 BSA (Agrisera), 1:200 DyLight 488 (Agrisera), 1:100 anti-GA1 antibody and anti-rat/goat conjugate obtained from Agrisera. Anti-GA antibody and GA3-BSA conjugate were used in a 1:10 ratio. The setup for antibody depletion was designed with competition calculator from Agrisera. Antibody and GA3-BSA conjugate were incubated in 1x PBS solution (24 h, 4 °C, rotator) in a total volume of 150 μL and then used as a primary antibody for immunodetection of GA in parallel preparations. In parallel, microscopes were used to differentiate between GA-specific and non-specific signals in the immunolocalization experiments. To distinguish between the upper and lower side after gravistimulation, we coexpressed DRS5rev::GFP or used the already established bending of the root for orientation. The immunodetected of GA was done in independent triplicates repeated with eight individual plants. The competition assay with the GA3-BSA conjugate was performed twice with eight individual plants.

**Immunodetection of PIN Proteins in Roots.** Automated whole mount protein immunolocalization was done as described previously (32). The anti-PIN1 rabbit antibody (18) was used at a dilution of 1:500. The whole-mount protein immunolocalization was repeated three times.

**DEX Depletion Assay.** TA::PIN2-GFP seedlings were grown on solid one-half MS for 6 d and transferred to 30 μM DEX-supplemented solid one-half MS medium for 24 h. After washing three times for 20 min in liquid one-half MS without sucrose, the roots were mounted in 3% glycerine with 50 μM GA3 or equal amount of GA solvent, and PIN2-GFP signal was recorded after 2, 4, 6, 7, and 24 h. To estimate the possible induction of the inducible DEX promoter by GA, the TA::PIN2-GFP line was transferred to only GA (50 μM) supplemented solid one-half MS medium for 24 h and recorded. Half MS media for 24 h and recorded. Half MS media for 24 h and recorded. The experiment was performed in triplicate with a minimum of five individual plants.

**ACNOWLEDGMENTS.** We thank Volker Lipka for providing the infrastructure of his department; Alexandra Matei, Charlotte Roth, Mena Erkenschwick, and Stefan Jakobs for providing the Atto647N conjugated anti-rat/goat antibodies were diluted as follows: 1:100 BSA (Agrisera), 1:200 DyLight 488 (Agrisera), 1:100 anti-GA1 antibody and anti-rat/goat conjugate obtained from Agrisera. Antibody and GA3-BSA conjugate were used in a 1:10 ratio. The setup for antibody depletion was designed with competition calculator from Agrisera. Antibody and GA3-BSA conjugate were incubated in 1x PBS solution (24 h, 4 °C, rotator) in a total volume of 150 μL and then used as a primary antibody for immunodetection of GA in parallel preparations. In parallel, microscopes were used to differentiate between GA-specific and non-specific signals in the immunolocalization experiments. To distinguish between the upper and lower side after gravistimulation, we coexpressed DRS5rev::GFP or used the already established bending of the root for orientation. The immunodetected of GA was done in independent triplicates repeated with eight individual plants. The competition assay with the GA3-BSA conjugate was performed twice with eight individual plants.

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Supporting Information

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SI Materials and Methods

Quantitative Analysis of Root Gravitropism. Five-day-old seedlings were grown vertically, and gibberelic acid (GA3; Duchefa) or uniconazole (Wako Pure Chemical) was applied for 3 h and 2 d, respectively, before plates were turned through 135° for an additional 12-h gravity stimulation in the dark. After turning for 135°, roots that showed a normal gravitropic response grew in the diagonal of the Petri dish and deviations of the response could be monitored easily. All gravitropically stimulated roots were assigned to one of the eight 45° sectors on a gravitropism diagram. The length of the bars in the diagram represents the percentage of seedlings assigned to the respective sector. As a control, the ecotype Landsberg erecta was used. For time course experiments, 7-d-old seedlings were gravstimulated in the light by a 90° rotation, and the angle the root tips were deviating from the vertical plane was recorded after 2.5, 4, 6, 8, and 24 h by using ImageJ software (National Institutes of Health). The independent experiments were carried out at least in triplicate with eight individual plants. The vertical growth index (Fig. S3) was determined as described previously (1).

Membrane Protein Extraction and SDS/PAGE Analysis. Roots of seedlings (100 mg; 5–6 d after germination) were homogenized and resuspended in extraction buffer [50 mM Tris, pH 6.8, 25% (vol/vol) D-sorbitol, 1.5% (wt/vol) insoluble polyvinylpyrrolidone, 10 mM Na-EDTA, 10 mM Na-EGTA, 1 mM 1,4-dithioerythritol, 50 mM NaF, 10 mM potassium phosphate buffer (pH 7.8), 40 mM β-glycerophosphate, 0.2% (wt/vol) casein, and protease inhibitors as follows: 1 mM benzamidine, 1 mM PMSF, 3.5 μg·mL−1 EDTA, 1 μg·mL−1 pepstatin, 1 μg·mL−1 aprotinin, and one Roche complete mini protease inhibitor tablet per 10 mL]. Three steps of extraction were followed by centrifugation in a standard benchtop centrifuge (470 × g for 2 min, 4 °C). The collected supernatant was combined, mixed by vortexing, and centrifuged (18,800 × g for 90 min, 4 °C). Pellets were resuspended in 50 mM Tris (pH 7.5), 20% glycerol, 2 mM EGTA, 2 mM EDTA, 50 to 500 μM 1,4-dithioerythritol, and protease inhibitors as described above. Equal amounts of protein were separated by 10% SDS–urea PAGE and probed with affinity-purified anti-PIN2 (1:20) (2), followed by HRP-conjugated donkey anti-rabbit IgG (1:10,000; ECL Western Blotting Detection Reagents; GE Healthcare).

RT-PCR. RNA extraction of 50 mg root material was performed according to the manufacturer’s instruction (innuPREP Plant RNA-Kit; Analytik Jena). DNA was digested by using the TURBO DNA-free kit from Ambion/Applied Biosystems. cDNA was synthesized with 1.5 μg of total RNA and 20 pmol of oligo(dT) (18 dT) oligonucleotides as described in the manual of the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas).

The iCycler system (Bio-Rad) was used for amplification and quantification of cDNA by using QuantiTect primers from Qiagen for PIN2 (QT00843850) and as reference UBQ5 (UBQ5 sense 5′-GACGCTTCATCTCGTCC-3′ and UBQ5 antisense 5′-GTTAAACGTAGGTGAGTCCA-3′). To monitor pharmacological treatments, we determined the GA20ox1 expression with gene-specific GA20ox1 primers (GA20 sense 5′-CCGTAACTGTTAGAGCT-3′ and GA20 antisense 5′-TACTCTTGATACACCTTCT-3′). AtGH3.1 cDNA was amplified by using the primers GH3.1 sense 5′-AACCATTGCGACCATTAAAGAAG-3′ and GH3.1 antisense 5′-TCTAGACCCCCGACATACA-3′. The amplification mix consisted of 1× NH4 reaction buffer (Bio-lime), 2 mM MgCl2, 100 μM dNTPs, 0.4 μM of primers, 0.25 U BIOTaq DNA polymerase (Bio-lime), 10 nM fluorescein (Bio-Rad), 1:100,000 diluted SYBR Green I solution (Cambrex), 1 μL of a 1:10 dilution of cDNA as template, and double-distilled water to a total volume of 25 μL. The PCR regime consisted of an initial 90-s denaturation step at 95 °C followed by 40 cycles of 20 s at 95 °C, 20 s at 55 °C, and 40 s at 72 °C. Calculations were done according to the 2−ΔΔCT method (3).

Fig. S1. The anti-GA antibody shows specificity to GA. Seedlings were grown vertically, and immunostaining was performed with a GA-specific antibody on vertically grown roots (A). In B, the primary antibody was omitted as control. (C and D) Immunostaining of roots was performed with antibodies directed against BSA. To verify that the antibody against BSA-conjugated GA would not detect plant proteins related to BSA, an anti-BSA antibody was used for control experiments. (D) Primary anti-BSA antibody was omitted as control. In contrast to the anti-GA antibody, the anti-BSA antibody did not cross-react with plant proteins in the Arabidopsis root. To confirm specificity of the anti-GA antibody, additional experiments were carried out. First, the serum containing the antibody directed against GA was depleted of GA-specific antibodies by incubating the serum with BSA-coupled GA before immunostaining experiments. (E–G) Immunostaining with GA-BSA conjugate depleted anti-GA serum. (F) Immunostaining with nondepleted anti-GA serum. (G) Quantification of immunosignal in the areas indicated in E and F. Data represent means ± SD (n = 8 seedlings per treatment, experiments repeated two times, representative data shown; ***P ≤ 0.001). This treatment drastically decreased the immune signal in the root epidermis and, therefore, showed that the antibody specifically detected GA in the epidermal cell layer of roots (E–G). (H–J) To show the detection of GA by the anti-GA antibody, immunostaining was performed on WT roots (H), ga1-3 roots with low GA content (I) and WT roots after 10 μM uniconazole treatment for 2 d (J). (A–F) DyLight 488 (Agrisera) was used as secondary antibody. (H–J) Atto647N-conjugated anti-rat/goat secondary antibody was used. (Scale bar: 50 μm.)
**Fig. S2.** Effect of gravistimulation on GA20ox1 and GH3.1 expression in the root. Real-time PCR analysis was performed with cDNA obtained from gravitropically induced roots. The graph shows a time course of GA20ox1 and GH3.1 expression after gravitropic induction (n = 4 pools of roots from 30–40 seedlings).

**Fig. S3.** Interfering with GA biosynthesis amplifies the pin2 agravitropic defect. pin2 was germinated on MS medium and then treated with DMSO (uniconazole control) (A) or 10 μM uniconazole for 10 d (B). The vertical growth index was determined according to the method described by (1) (C) (**P ≤ 0.001).

**Fig. S4.** Expression analysis of PIN2 and GA20ox1 by real-time PCR in GA-treated roots. Real-time PCR analysis was performed with cDNA obtained from control roots treated with DMSO and roots treated for 3 h with 50 μM GA (n = 6 pools of roots from 30–40 seedlings). (A) PIN2 expression. (B) GA20ox1 expression. Real-time PCR analyses of the GA-treated seedlings revealed no significant effects of GA on PIN2 transcription, whereas the significant GA-induced decrease of GA20ox1 expression showed that the gene expression in the root was generally GA-responsive.
Fig. S5. Effect of altered GA levels on plasma membrane proteins is PIN-specific. PIN2::PIN2-GFP (A–C), 35S::PIP2-GFP (E–G), AUX1::AUX1-YFP (I–L), and PGP19::PGP19-GFP (N–P) were treated with DMSO (GA and paclobutrazol solvent) for 3 h (A, E, I, and N), 50 μM GA for 3 h (B, F, K, and O), or 10 μM paclobutrazol for 2 d (C, G, L, and P). Quantification of expression was done by image analysis (D, H, M, and Q). Data represent means ± SD (n = 8 seedlings). Arabidopsis lines expressing PIN1, PIN2, PIN3, PIN4, and PIN7 as GFP fusion under control of the native promoters were treated with (R) DMSO, (S) 50 μM GA for 3 h, or (T) 10 μM paclobutrazol for 2 d. (Scale bar: 50 μm.)
Fig. S6. Identification of GFP-labeled vacuoles by FM4-64 staining. *PIN2::PIN2-GFP* plants were kept for 4 h in the dark (A) and stained for 1 h with FM4-64 followed by 1 h washout (B). (C) Overlay of A and B. Selected vacuoles are marked by arrowheads to allow comparison between GFP and FM4-64 label. (Scale bars: 10 μm.)
Fig. S7. No GA effect on FM4-64 uptake and wortmannin-induced intracellular aggregation of PIN2-GFP. (A) Uptake of the endocytosis marker FM4-64 in WT roots treated with DMSO (GA and uniconazole control), 50 μM GA for 3 h, or uniconazole for 2 d, and uptake in roots of the ga1-3 and pentuple mutants. (B) PIN2-GFP signal in wortmannin compartments of PIN2::PIN2-GFP seedlings after treatment with DMSO for 3 h (GA control), 50 μM GA for 3 h, DMSO for 2 d (uniconazole control), 10 μM uniconazole for 2 d, and PIN2-GFP signal in roots of the ga1-3 mutant. PIN2-GFP signal at the plasma membrane was quantified relative to intracellular signals. (Scale bars: A, 25 μm; B, 50 μm.)