Supporting Information

Löfke et al. 10.1073/pnas.1300107110

SI Materials and Methods

Quantitative Analysis of Root Gravitropism. Five-day-old seedlings were grown vertically, and gibberellic acid (GA_3; 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 gravistimulated 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 polyvinylpolypyrrolidone, 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 $\mu g \cdot mL^{-1}$ E64, 1 $\mu g \cdot mL^{-1}$ pepstatin, 1 $\mu g \cdot mL^{-1}$ leupeptin, 1 $\mu g \cdot mL^{-1}$ 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′-GTAAACGTAGGTGAGTCCA-3′). To monitor pharmacological treatments, we determined the *GA20ox1* expression with gene-specific GA20ox1 primers (GA20 sense 5′-CCGTAACTGTAGAAGACT-3′ and GA20 antisense 5′-TACTCTTGATACACCTTCCT-3′). AtGH3.1 cDNA was amplified by using the primers GH3.1 sense 5′-AACTTATGCCGACCATTAAAGAA-3′ and GH3.1 antisense 5′-TCTAGACCCGGCACATACAA-3′. The amplification mix consisted of 1× NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 100 μM dNTPs, 0.4 μM of primers, 0.25 U BIOTaq DNA polymerase (Bioline), 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).

1. Grabov A, et al. (2005) Morphometric analysis of root shape. *New Phytol* 165(2):641–651.
2. Abas L, et al. (2006) Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat Cell Biol* 8(3):249–256.
3. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_T$ method. *Methods* 25(4):402–408.

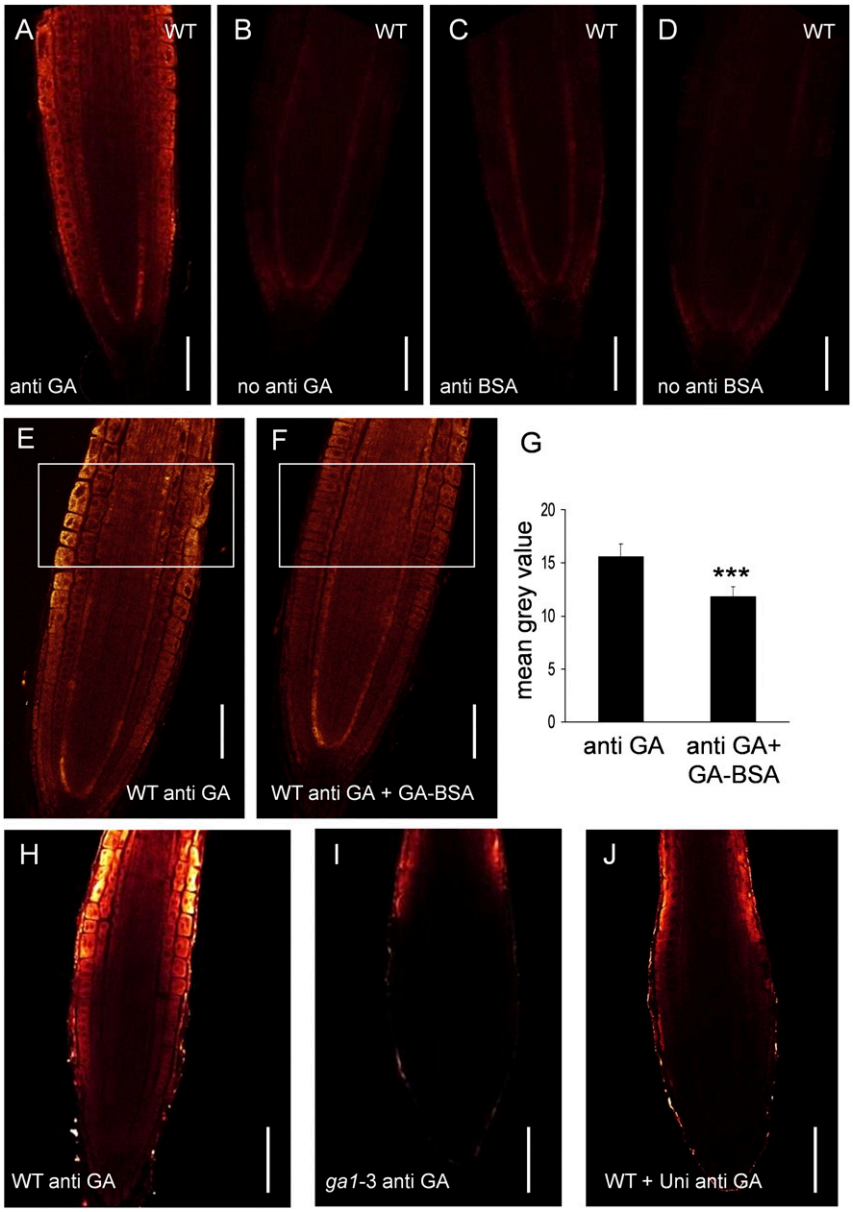

Fig. S1. The anti-GA antibody shows specificity to GA. Seedlings were grown vertically, and immunostaining was performed with a GA-specific antibody on vertically grown roots (*A*). In *B*, the primary antibody was omitted as control. (*C* and *D*) Immunostaining of roots was performed with antibodies 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*) Anti-GA serum was depleted of GA-specific antibody by incubation with GA-BSA conjugate before use for immunostaining of roots. (*E*) Immunostaining with nondepleted anti-GA serum. (*F*) immunostaining with GA-BSA conjugate depleted 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; $^{***}P \leq 0.001$). 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.)

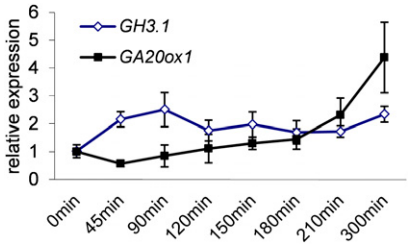

Fig. S2. Effect of gravistimulation on *GA20ox1* and *GH3.1* expression in the root. Real-time PCR analysis was performed with cDNA obtained from gravitropically induced roots. The graph shows a time course of *GA20ox1* and *GH3.1* expression after gravitropic induction ($n = 4$ pools of roots from 30–40 seedlings).

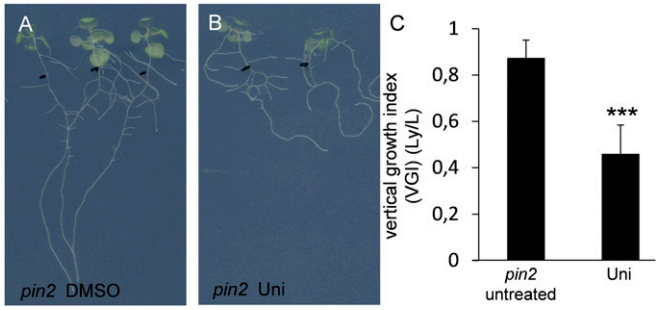

Fig. S3. Interfering with GA biosynthesis amplifies the *pin2* agravitropic defect. *pin2* was germinated on MS medium and then treated with DMSO (uniconazole control) (*A*) or 10 μM uniconazole for 10 d (*B*). The vertical growth index was determined according to the method described by (1) (*C*) (***$P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

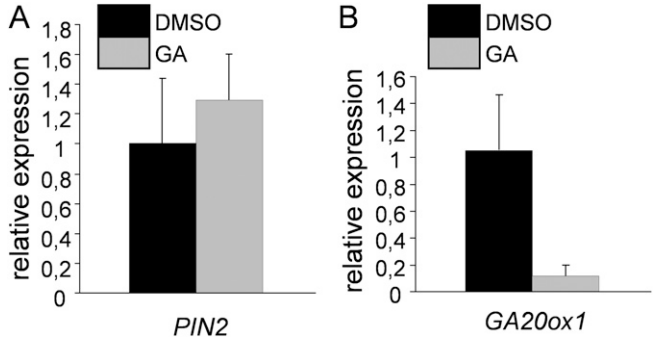

Fig. S4. Expression analysis of *PIN2* and *GA20ox1* by real-time PCR in GA-treated roots. Real-time PCR analysis was performed with cDNA obtained from control roots treated with DMSO and roots treated for 3 h with 50 μM GA ($n = 6$ pools of roots from 30–40 seedlings). (*A*) *PIN2* expression. (*B*) *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.

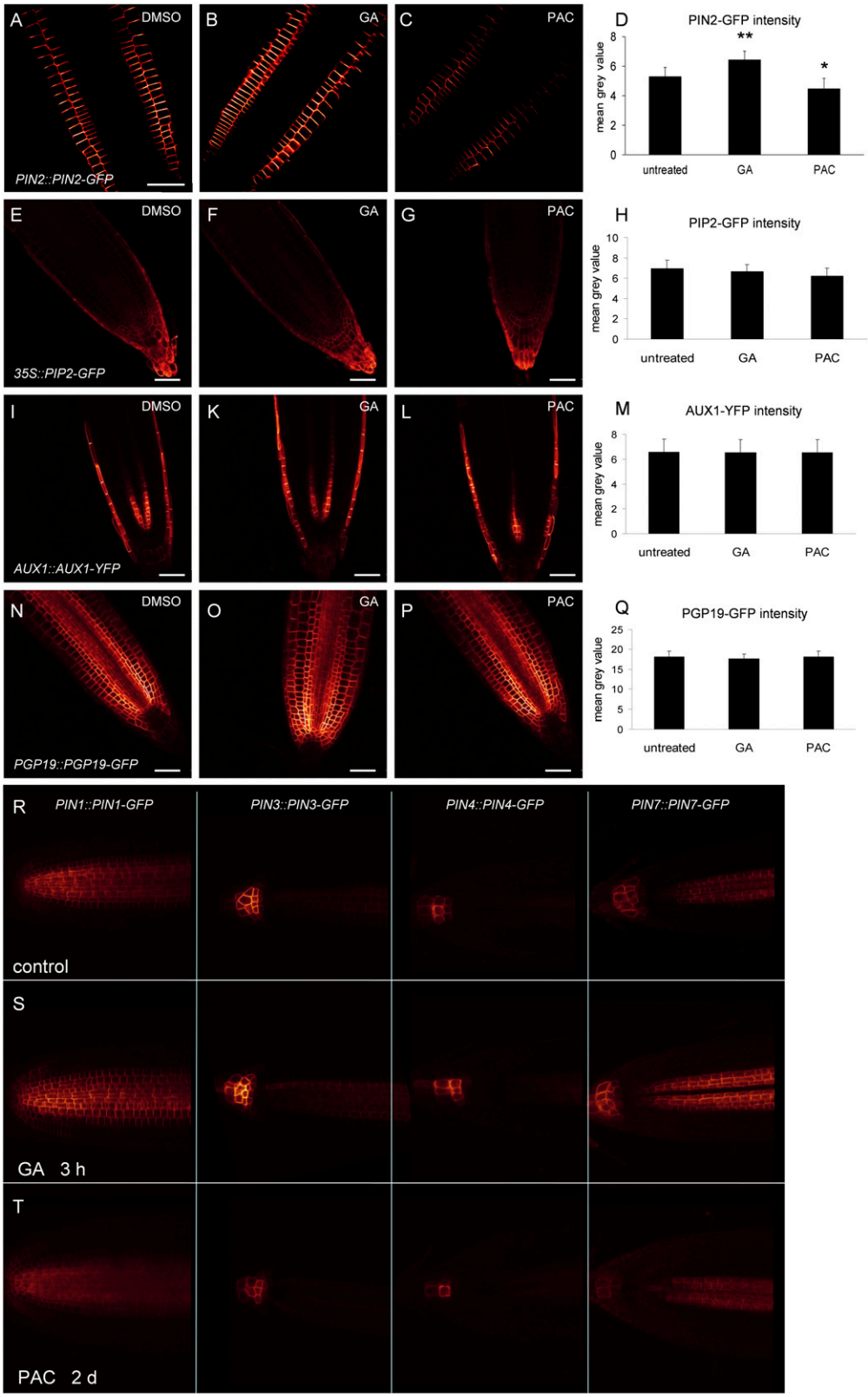

Fig. S5. Effect of altered GA levels on plasma membrane proteins is PIN-specific. *PIN2::PIN2-GFP* (*A–C*), *35S::PIP2-GFP* (*E–G*), *AUX1::AUX1-YFP* (*I–L*), and *PGP19::PGP19-GFP* (*N–P*) were treated with DMSO (GA and paclobutrazol solvent) for 3 h (*A*, *E*, *I*, and *N*), 50 μM GA for 3 h (*B*, *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.)

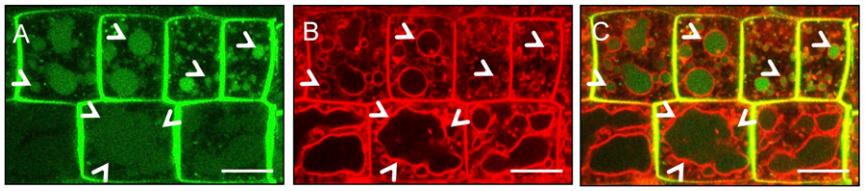

Fig. S6. Identification of GFP-labeled vacuoles by FM4-64 staining. *PIN2::PIN2-GFP* plants were kept for 4 h in the dark (*A*) and stained for 1 h with FM4-64 followed by 1 h washout (*B*). (*C*) Overlay of *A* and *B*. Selected vacuoles are marked by arrowheads to allow comparison between GFP and FM4-64 label. (Scale bars: 10 μm.)

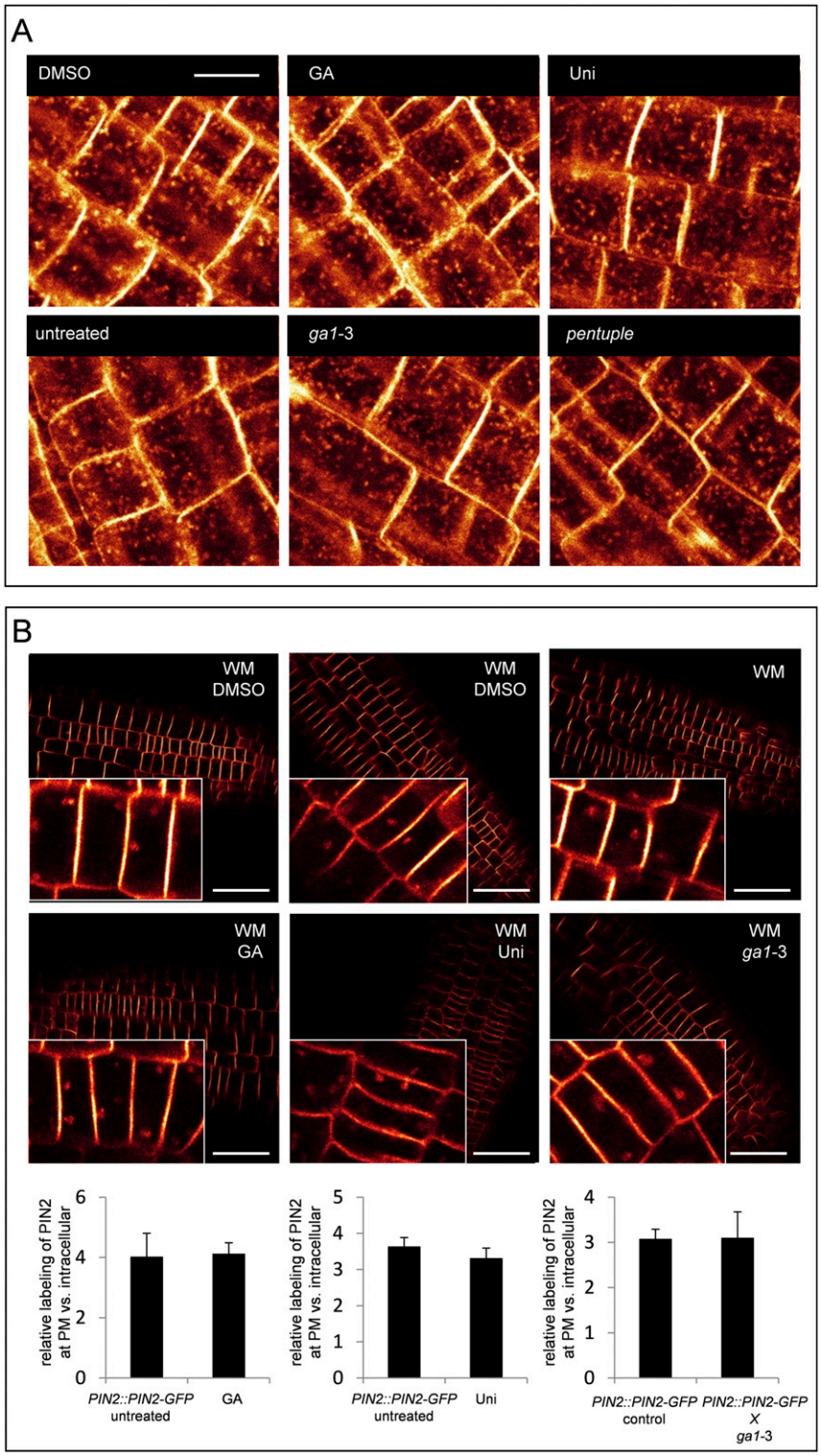

Fig. S7. No GA effect on FM4-64 uptake and wortmannin-induced intracellular aggregation of PIN2-GFP. (*A*) Uptake of the endocytosis marker FM4-64 in WT roots treated with DMSO (GA and uniconazole control), 50 μM GA for 3 h, or uniconazole for 2 d, and uptake in roots of the *ga1*-3 and *pentuple* mutants. (*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.)