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In the root, the transport of auxin from the tip to the elongation zone, referred to here as shootward, governs gravitropic bending. Shootward polar auxin transport, and hence gravitropism, depends on the polar deployment of the PIN-FORMED auxin efflux carrier PIN2. In *Arabidopsis thaliana*, PIN2 has the expected shootward localization in epidermis and lateral root cap; however, this carrier is localized toward the root tip (rootward) in cortical cells of the meristem, a deployment whose function is enigmatic. We use pharmacological and genetic tools to cause a shootward relocation of PIN2 in meristematic cortical cells without detectably altering PIN2 polarization in other cell types or PIN1 polarization. This relocation of cortical PIN2 was negatively regulated by the membrane trafficking factor GNOM and by the regulatory A1 subunit of type 2-A protein phosphatase (PP2AA1) but did not require the PINOID protein kinase. When GNOM was inhibited, PINOID abundance increased and PP2AA1 was partially immobilized, indicating both proteins are subject to GNOM-dependent regulation. Shootward PIN2 specifically in the cortex was accompanied by enhanced shootward polar auxin transport and by diminished gravitropism. These results demonstrate that auxin flow in the root cortex is important for optimal gravitropic response.

## INTRODUCTION

The plant hormone, auxin (indole-3-acetic acid [IAA]), regulates essentially all aspects of growth and development, including tropic responses. For gravitropism in roots, the response pathway has been separated into three sequential steps: gravity perception, signal transduction, and asymmetric growth leading to bending (Moullia and Fournier, 2009). The bending of the root is driven by formation of a differential auxin gradient between the upper and lower sides of the root and regulated by the polar transport of auxin (Muday and Rahman, 2008).

Auxin moves through plants by a unique, cell-to-cell polar transport mechanism (Benjamins et al., 2005; Kramer and Bennett, 2006). The mechanism depends on active uptake through an influx carrier, such as AUXIN INSENSITIVE1 (AUX1), and facilitated efflux through a carrier such as a member of the PIN-FORMED (PIN) family of proteins. Other proteins are involved in auxin transport, such as the ATP BINDING CASSETTE TYPE B TRANSPORTER4 class; however, the biochemical func-


tion of these proteins is less clear. To a large extent, the transport stream attains directionality by restricting the localization of the PIN-type efflux carriers; therefore, the deployment of these carriers plays a pivotal role in governing the spatial and temporal distribution of auxin (Grieneisen et al., 2007; Vieten et al., 2007; Petrášek and Friml, 2009). This is seen in the early embryo, where a shift in the localization of a PIN protein allows the embryo to establish its own independent polar axis and continues to be seen through development, as the placement of PIN proteins is vital for such diverse processes as phyllotaxis, vascular patterning, and lateral root emergence.


In the root, two auxin transport streams have been delineated. In one stream, shoot-derived IAA moves toward the root columella through the vasculature and central cylinder, a flow that is polarized by the auxin efflux carriers, PIN1 and PIN4 (Geldner et al., 2001; Friml et al., 2002). In the other stream, IAA moves away from the columella through the lateral root cap and epidermis, reaching the root's elongation zone but evidently not much farther. This second auxin transport stream is polarized by another member of the PIN family, PIN2 (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Utsuno et al., 1998). Any alteration of this second flow of auxin by chemical or genetic means affects the formation of an auxin asymmetry across the root and inhibits bending in response to gravity (Muday and Rahman, 2008).

Originally, the direction of auxin transport was described with respect to plant anatomy, so that transport toward the apex of

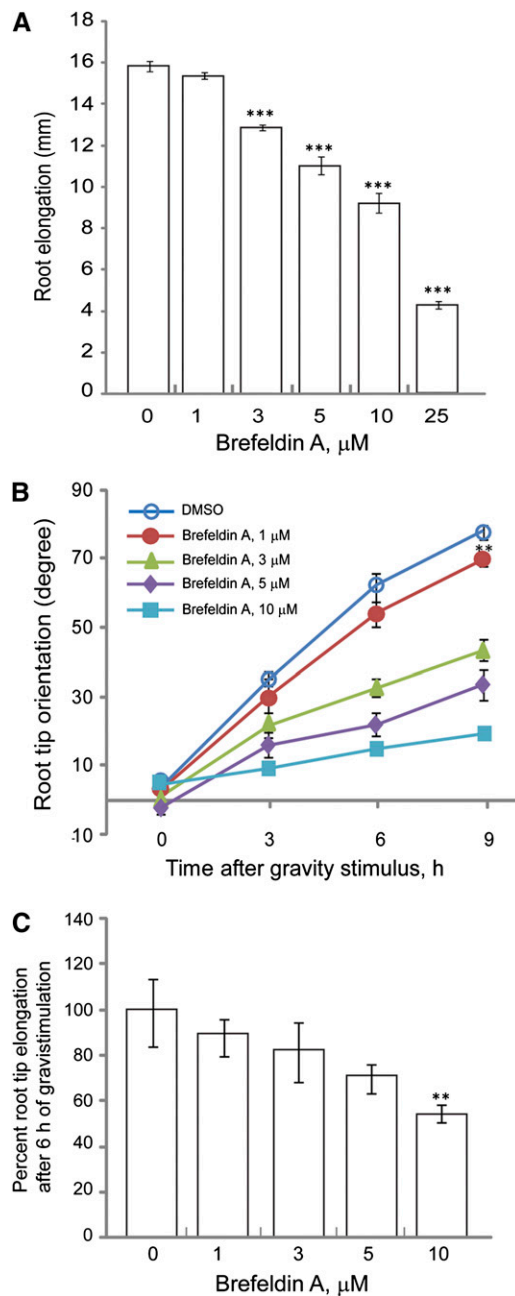
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**Figure 1.** Effect of Brefeldin on *Arabidopsis* Root Elongation and Gravitropism.

For assaying elongation (**A**), 5-d-old seedlings were transferred to plates containing brefeldin and incubated for 48 h. For assaying gravitropism (**B**) and (**C**), the seedlings were incubated on inhibitor plates for 18 h and then gravity stimulation applied by rotating the plate by 90°. Bars and symbols plot mean  $\pm$  SE from three independent experiments, with 10 to 12 seedlings per experiment. Asterisks represent the statistical significance between an experimental and control mean as judged by Student's *t* test: \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ . In (**B**), brefeldin treatments were significant ( $P < 0.001$ ) at all times past zero except for 1  $\mu\text{M}$ , which was significant at  $P < 0.01$  at 9 h and otherwise not significant.

(**A**) Root elongation.

either the shoot or the root was referred to as acropetal, and transport toward the base of either organ was referred to as basipetal (Baluška et al., 2005). With this anatomical reference, the apical domains of shoot and root cells face in opposite directions. Because cell polarity does not reverse across the shoot-root junction, many authors writing about cells now use apical as referring to the shoot apex exclusively and, likewise, basal as referring to the base of the shoot exclusively (Friml et al., 2006). While good reasons exist for each usage, because both are being used currently, apical and basal have contradictory meanings for the root. To avoid ambiguity, we will use the terms “rootward” and “shootward” to refer to the direction (or the domain) facing the shoot apex and root apex, respectively.

For both rootward and shootward auxin transport streams, the PIN proteins are localized as expected. In the vascular cylinder, where rootward flow occurs, PIN1 and PIN4 occupy a specific rootward domain on the plasma membrane; likewise, in lateral root cap and epidermis, in which shootward flow occurs, PIN2 is targeted to a specific, shootward plasma membrane domain. The importance for gravitropism of the shootward PIN2 localization in epidermis has been substantiated in experiments where that targeting is reversed to rootward and agravitropic roots result (Wiśniewska et al., 2006).

Given the interest in cell polarity and the importance of PIN targeting, the mechanism for polarizing PIN proteins is of keen interest. PIN proteins actively cycle between the plasma membrane and endosomes. This cycling was initially uncovered through sensitivity to the fungal toxin brefeldin A (brefeldin), which targets GNOM, a specific ARF-GEF required for that traffic (Geldner et al., 2001). When GNOM is inhibited, PIN proteins, among others, are recruited into conspicuous membranous aggregates (termed brefeldin bodies) presumably because internalization from the plasma membrane continues, but the restorative movement back to the plasma membrane is arrested. In fact, cargoes for GNOM-dependent recycling and hence recruitment into brefeldin bodies, are mainly, if not exclusively, proteins targeted to rootward domains, such as PIN1 in the stele and PIN2 in the cortex, whereas shootward domain proteins are targeted largely independent of GNOM (Kleine-Vehn et al., 2009).

Targeting PIN proteins also depends on phosphorylation, with antagonistic effects on targeting being found for the PINOID protein kinase and type 2A protein phosphatase (PP2A). Treatments that promote phosphorylation of PIN proteins tend to favor targeting to shootward domains. This has been shown, for example, by overexpressing the PINOID kinase (Friml et al., 2004) or by knocking down the regulatory A subunits of PP2A (Michniewicz et al., 2007). It has also been shown by phosphomimetic mutants of PIN proteins: proteins that mimic the phosphorylated state are insensitive to brefeldin and go to shootward domains, whereas mimics that cannot be phosphorylated are brefeldin sensitive and go to rootward ones (Kleine Vehn et al., 2009; Zhang et al., 2010). These studies establish the existence

(**B**) Curvature of root tips plotted against time after reorientation.

(**C**) Root elongation during gravitropism measured for the experiment in (**B**).

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of a rootward-to-shootward trafficking pathway, into which the likelihood of recruitment for PIN proteins appears to depend on their level of phosphorylation (Kleine-Vehn et al., 2009).

The functional relevance of the antagonism between PINOID kinase and PP2A has been demonstrated also with respect to gravitropism: for example, the weak gravitropism of a mutant with aberrant PP2A activity, *pp2aa1* (also known as *root curls on naphthylphthalamic acid1* [*rcn1*]) is largely rescued by concomitant loss of PINOID kinase function (Sukumar et al., 2009).

Taking results on cycling through endosomes together with those on phosphorylation, a model for PIN trafficking has been proposed based on a paradigm from animal epithelial cells (Kleine-Vehn and Friml, 2008; Robert and Offringa, 2008). These cells are highly polarized, with an apical domain that faces the lumen and a basolateral domain that faces away. Epithelial cells target many proteins by secreting them to both domains and selectively removing them from one domain followed by selective delivery to the other, a redeployment process called transcytosis. In transcytosis, selectivity occurs not through Golgi-based secretion but subsequently within endosomal compartments. For plant PIN proteins, the shootward targeting mechanism resembles transcytosis, but how deep the resemblance goes remains an open question.

While the recent elucidation of mechanisms responsible for PIN targeting represent an impressive advance in our understanding of plant polarity and auxin flow, important gaps remain. One of these concerns the status of PIN2 in the root cortex. The role of PIN2 in gravitropism outlined above reflects its localization in the lateral root cap and epidermis, where it transmits auxin from the perceiving cells of the root cap to the rapidly elongating cells of the growth zone. Nevertheless, PIN2 is also expressed in the root cortex, where its localization pattern is puzzling. Although in the elongation zone, cortical PIN2 has a shootward localization, in the meristem, the protein has a rootward locali-

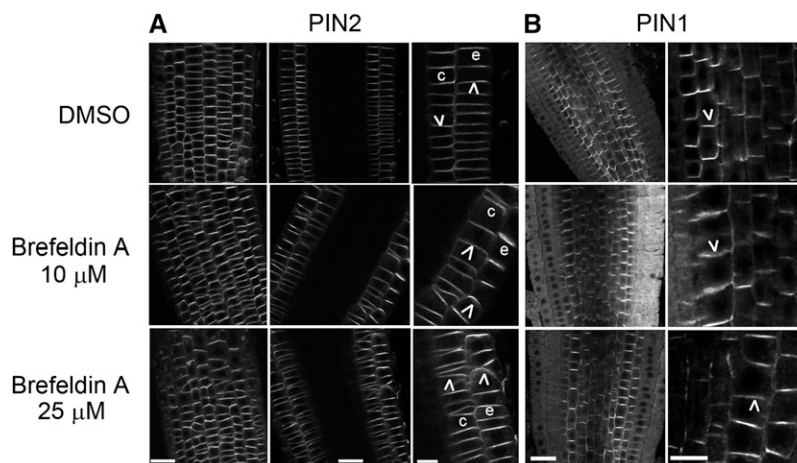
zation (Rahman et al., 2007). The functional significance of rootward localization of PIN2 in meristematic cortex cells remains enigmatic. For one thing, recent computational models predict little if any auxin flows in the cortex (Kramer, 2004; Laskowski et al., 2008). For another, were auxin to enter meristematic cortex cells, the polarization of PIN2 is such as to frustrate net flow toward the elongation zone.

Here, we take advantage of what is known about the mechanisms of PIN targeting to study the function of rootward cortical PIN2. We find that the rootward localization in the cortex is more susceptible to shootward relocation than is PIN1 in the stele. In all cases where cortical PIN2 changes polarity but PIN1 remains stable, gravitropism is impaired. We suggest that PIN2 deployment in the cortex represents a mechanism for fine-tuning the flow of auxin as required for optimal responses within the zone of elongation.

## RESULTS

### Brefeldin Affects Root Gravitropism and Elongation Differentially

Brefeldin is widely used to inhibit various protein trafficking pathways, including the trafficking of PIN proteins. Brefeldin induces relocation of rootward targeted PIN proteins to shootward domains (Kleine-Vehn et al., 2008a, 2008b, 2009). In most studies, brefeldin is used at 50  $\mu$ M and effects are assayed within a few hours. Although 50  $\mu$ M brefeldin has been frequently reported to disrupt targeting of PIN proteins and to alter auxin accumulation, to the best of our knowledge, no systematic investigation has been done to characterize the effects of brefeldin on root elongation and gravitropism. Therefore, we performed a dose-response assay with 5-d-old seedlings



**Figure 2.** Effect of Brefeldin on the Localization of PIN2 and PIN1.

Five-day-old *Arabidopsis* seedlings were treated for 48 h before being fixed and processed for immunofluorescence with anti-PIN2 and anti-PIN1 antibodies. The images are single confocal sections representative of three to four separate experiments, with four to six roots imaged per treatment for each experiment. Arrowheads illustrate polarity. For PIN2, the left-most column shows glancing views of the epidermis, and the central column shows roughly median planes, of which the right-most column shows magnified views. For PIN1, the left column represents the median views, and the right column shows magnified views of the left column. e, epidermis; c, cortex. Bars = 20  $\mu$ m for columns 1, 2, and 4 and 10  $\mu$ m for columns 3 and 5.

treated for 48 h. Gravitropism was more strongly inhibited by brefeldin than was elongation (Figure 1). For example, a 50% inhibition of elongation required  $>10 \mu\text{M}$  brefeldin, whereas a 50% inhibition of gravitropism required  $3 \mu\text{M}$  brefeldin, and at  $10 \mu\text{M}$ , the root was nearly agravitropic. Even  $1 \mu\text{M}$  brefeldin caused a small but significant delay in gravitropism (Figure 1B). Note that elongation of vertical roots (Figure 1A) as well as those bending in response to gravity (Figure 1C) responded similarly to the inhibitor. These results show that gravitropism is acutely sensitive to brefeldin.

### Shootward Relocation of PIN2 Can Be Induced Independently of That of PIN1

To understand how brefeldin inhibits gravitropism, we next investigated PIN targeting using both immunocytochemistry and live cell imaging. A 2-d incubation in  $10 \mu\text{M}$  brefeldin induced a rootward-to-shootward relocation of PIN2 in meristematic cortex cells, while the polarization of PIN2 in the epidermis and lateral root cap remained unaffected (Figure 2A). In contrast with PIN2, the rootward localization of PIN1 was not changed, although its expression level appeared to decrease (Figure 2B). We confirmed these immunostaining results with live imaging of PIN1 and PIN2 using the respective green fluorescent protein (GFP) fused transgenic lines (Benková et al., 2003; Xu and Scheres, 2005). In these lines, as with the wild type, a 2-d treatment with  $10 \mu\text{M}$  brefeldin induced rootward to shootward relocation in cortical PIN2 but not in PIN1 (see Supplemental Figure 1 online). These results differ from previously published results, insofar as 25 and  $50 \mu\text{M}$  brefeldin induce a rootward to shootward relocation of both PIN1 and cortical PIN2 (Kleine-Vehn et al., 2008a, 2008b, 2009).

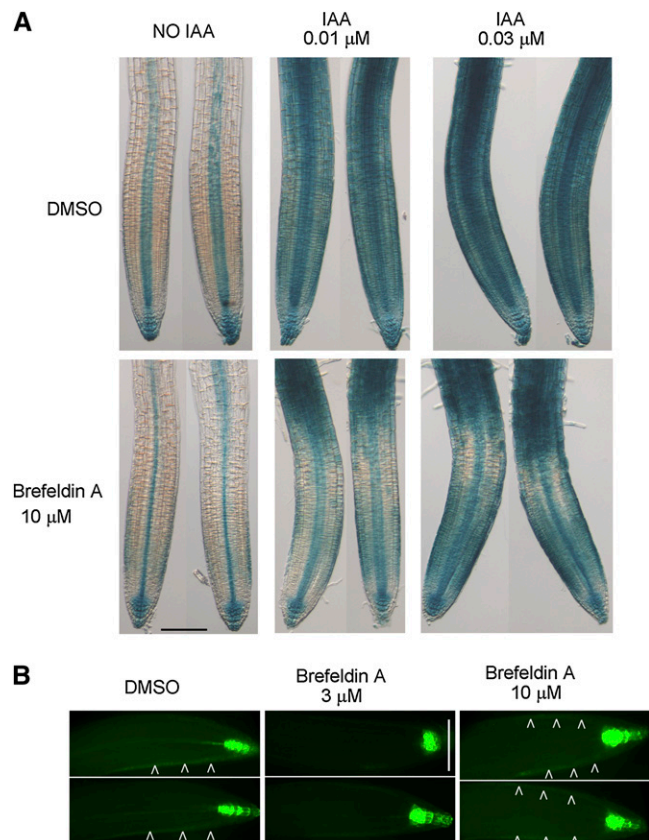
To examine this discrepancy, we investigated PIN1 and PIN2 localization in seedlings treated with  $25 \mu\text{M}$  brefeldin for 48 h. Consistent with previous reports, we found that the high concentration induced a shootward localization for both PIN1 and cortical PIN2 (Figure 2). We also investigated whether the differential sensitivity could be observed with shorter incubation. With as little as 2 h of treatment with  $10 \mu\text{M}$  brefeldin, cortical PIN2 underwent shootward relocation, whereas there was no detectable alteration in the targeting of PIN1 (see Supplemental Figures 2 and 3 online). As reported previously, brief incubation with  $50 \mu\text{M}$  brefeldin induced a pervasive shootward relocation of both PIN1 and cortical PIN2 (Kleine-Vehn et al., 2008a, 2008b, 2009). At high brefeldin, some cells had altered PIN polarity as early as 1 h, and by 2 h, altered polarity was observed in most cells (see Supplemental Figure 3 online). Interestingly, prolonged incubation in high brefeldin resulted in an increased cytoplasmic signal for PIN1 but not for cortical PIN2 (cf. Supplemental Figures 2 and 3 online).

The difference between low and high concentrations of brefeldin raises the possibility that rootward targeting of PIN2 and PIN1 has differential requirements for GNOM activity. Alternatively, vascular PIN1 might be less accessible to brefeldin compared with cortical PIN2. To examine access of brefeldin to the root, we used a line expressing LOW TEMPERATURE-INDUCED protein 6b fused to GFP (GFP-LTI6b) in all root tissues (Kurup et al., 2005). Incubating GFP-LTI6b in  $10 \mu\text{M}$  brefeldin for 48 h

gave rise to prominent, GFP-positive aggregates (see Supplemental Figure 4 online). It was difficult to image the aggregates in the stele within the meristem, but in the elongation zone, these aggregates formed similarly in epidermis, cortex, endodermis, and pericycle. These results confirm our suggestion that cortical PIN2 and PIN1 have differential sensitivity toward brefeldin-induced shootward relocation.

### Effect of Shootward PIN2 in Meristematic Cortex Cells on Auxin Transport

Our finding that rootward-localized cortical PIN2 in the meristem specifically can be shifted to a shootward plasma membrane domain allows us to test the function of cortical PIN2 localization



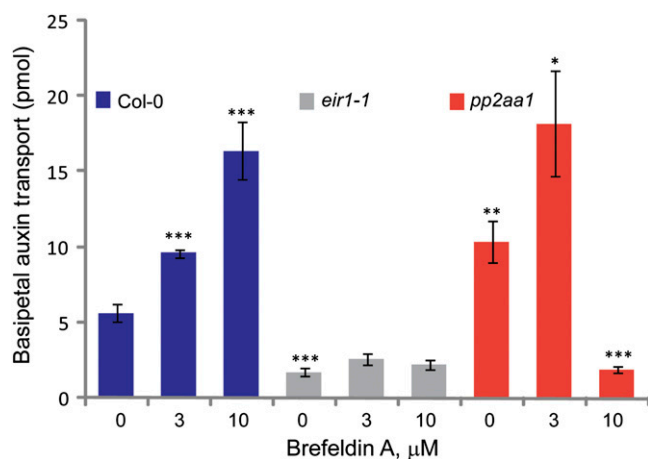
**Figure 3.** Brefeldin Alters Auxin Responsiveness.

**(A)** Five-day-old *IAA2-GUS* transgenic seedlings were treated for 48 h, stained in a buffer containing 1 mM X-gluc for 1 h at  $37^{\circ}\text{C}$ , and cleared for photography. For IAA treatment, seedlings were transferred to plates containing IAA and incubated for 4 h prior to staining. Images are representative of 20 to 30 seedlings, stained in two to three separate experiments. Bar =  $100 \mu\text{m}$ .

**(B)** Five-day-old *DR5-mGFP* transgenic seedlings were treated for 18 h in the vertical position and then rotated by  $90^{\circ}$  for 3 h prior to imaging. Two representative images are shown out of 20 seedlings imaged in two separate experiments. Arrowheads indicate *DR5-GFP* expression in the peripheral layers, which was generally asymmetric in controls, weak or absent on  $3 \mu\text{M}$ , and symmetric on  $10 \mu\text{M}$  brefeldin. Bar =  $100 \mu\text{m}$ .

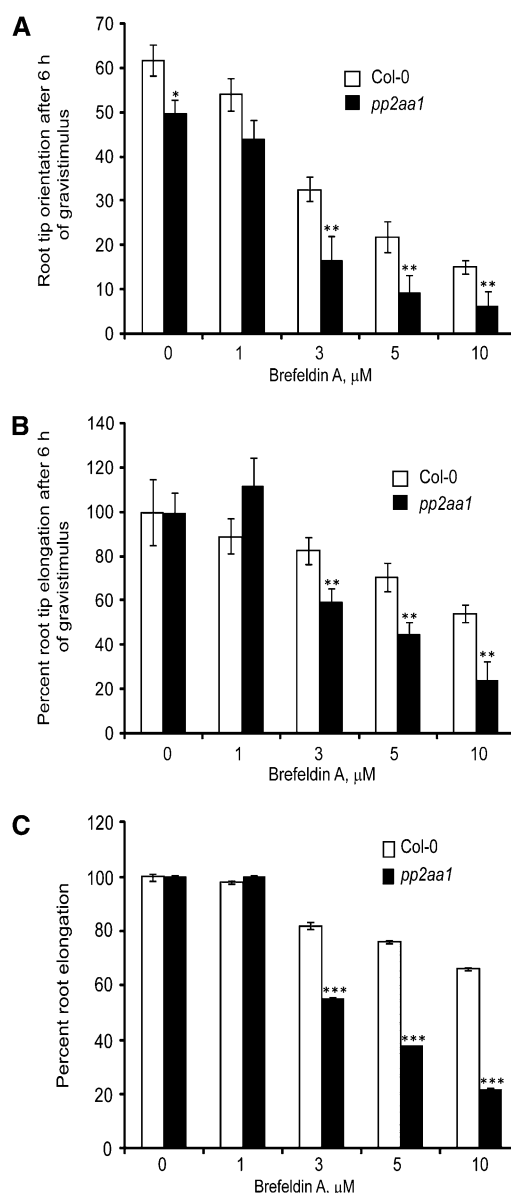
without interference from concomitant PIN1 relocation. To determine whether the polarity of PIN2 in the cortex impacts auxin transport, we first imaged  $\beta$ -glucuronidase (GUS) staining of the auxin-responsive marker *IAA2-GUS*, whose activity has been correlated with endogenous auxin in a variety of settings (Casimiro et al., 2001; Benková et al., 2003; Swarup et al., 2005; Grieneisen et al., 2007) and responds more sensitively to IAA than does *DR5* (Okamoto et al., 2008; Shibasaki et al., 2009). Untreated *IAA2-GUS* roots have GUS activity confined to columella and to stele cells, where auxin is believed to be present at high concentration, with little if any staining present in cortex and epidermis (Figure 3A). When the intracellular level of auxin was increased by application of IAA for 4 h, GUS staining was observed throughout the root and the staining was stronger as the concentration of IAA increased. In contrast with roots not treated with brefeldin, roots treated with 10  $\mu$ M brefeldin for 2 d had reduced GUS staining in response to the IAA, particularly in the peripheral layers. The reduced staining in the periphery is consistent with enhanced transport based on the shootward polarity shift of PIN2.

To examine auxin transport specifically with respect to gravitropism, we used the *DR5* promoter fused to a modified GFP (*mGFP*). Consistent with previous reports (Ottenschläger et al., 2003; Paciorek et al., 2005), control roots expressed *DR5-GFP* asymmetrically, as evident from the enhanced expression in the outermost cell layer on the lower side of the root meristem (Figure 3B). By contrast, in seedlings treated with 3  $\mu$ M brefeldin for 2 d and then rotated, expression in the outermost layer was weak in 70% of the roots (i.e., 14/20). Interestingly, in 10  $\mu$ M brefeldin, where roots barely responded to gravity (Figure 1B), *DR5-GFP* was expressed more strongly in the outermost layer but more or less symmetrically in 80% of roots (16/20) (Figure 3B). That brefeldin reduced both *IAA2-GUS* staining in meriste-



**Figure 4.** Brefeldin Enhances Shootward Auxin Transport in the Root.

Five-day-old seedlings were treated for 48 h, and transport of tritiated IAA over 1 h was measured as described in Methods. Bars plot mean  $\pm$  SE of three replicate experiments, each done in triplicate. Asterisks represent the statistical significance between control and brefeldin-treated means, as judged by Student's *t* test: \*  $P < 0.02$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ . [See online article for color version of this figure.]



**Figure 5.** Roots of *pp2aa1* Respond to Brefeldin More Strongly Than Those of the Wild Type.

Five-day-old seedlings were transferred to treatment plates and either incubated for 18 h and then rotated by 90° (**A**) and (**B**) or incubated for 48 h (**C**). Bars plot mean  $\pm$  SE from three to five independent experiments, with 10 to 12 seedlings per experiment. Asterisks represent the statistical significance between the means for each genotype, as judged by Student's *t* test: \*  $P < 0.02$ , \*\*  $P < 0.01$ , and \*\*\*  $P < 0.001$ .

(**A**) Root tip orientation after 6 h of stimulation.

(**B**) Root elongation of the same plants shown in (**A**).

(**C**) Root elongation of vertical plants.

matic cortical cells exposed to IAA as well as the *DR5-GFP* signal during gravitropism led us to hypothesize that the shootward translocation of PIN2 in the cortex stimulates the rate of shootward auxin transport and thus depletes auxin from the peripheral layers of the root.



To test the above hypothesis, we measured shootward auxin transport directly. As described in Methods, the net amount of IAA transported toward the shoot in a 5-mm tip segment of the root was assayed. Consistent with the hypothesis, a 2-d brefeldin treatment stimulated auxin transport, with 10  $\mu$ M tripling the amount (Figure 4). Further consistent with the hypothesis, brefeldin had no effect on auxin transport in the PIN2 null mutant, *eir1-1* (Luschnig et al., 1998). Taken together, these results suggest that the rootward localization of PIN2 in meristematic cortical cells exerts a negative regulatory effect on auxin transport, a regulation that is important for optimal gravitropism.

### Loss of PP2AA1 Enhances Responses to Brefeldin

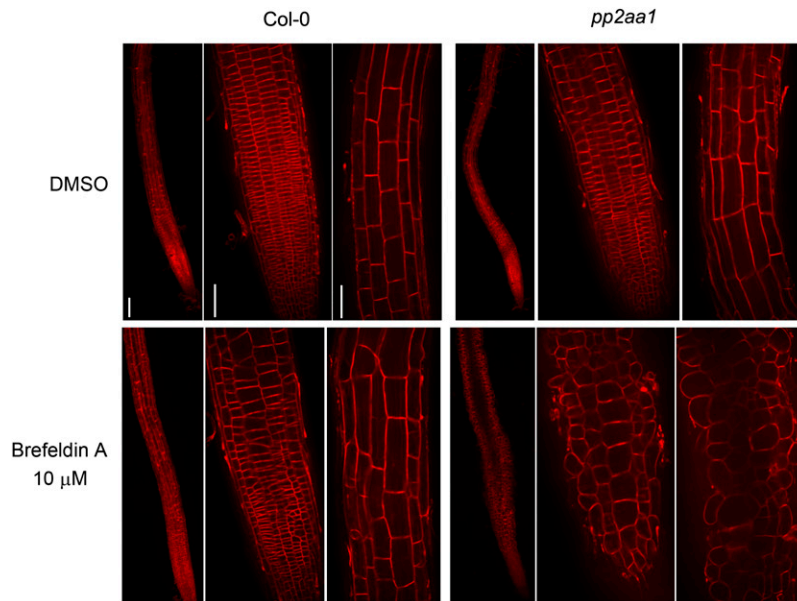
Recent investigations into the targeting mechanisms for PIN proteins have revealed a pivotal role for phosphorylation, with PINOID implicated as the protein kinase and PP2A as the phosphatase (Kleine-Vehn and Friml, 2008). Because in *Arabidopsis thaliana*, regulatory subunits of this protein phosphatase are encoded by three genes (PP2AA1, 2, and 3), most work has involved double or triple mutants or the use of RNA interference, in which shootward translocation of both PIN1 and cortical PIN2 typically occur (Michniewicz et al., 2007). Since PP2AA1 has been implicated specifically in regulating gravitropism (Sukumar et al., 2009), we used the loss-of-function mutant *pp2aa1* (also known as *rcn1*) to investigate cortical PIN2 targeting further.

First, we assayed gravitropism in response to low concentrations of brefeldin given for 2 d (Figure 5). Both gravitropism and elongation in *pp2aa1* were hyperresponsive to brefeldin: inhibi-

tions observed at 10  $\mu$ M in the wild type occurred at 3  $\mu$ M in the mutant. Interestingly, *pp2aa1* roots on 10  $\mu$ M brefeldin were morphologically abnormal (Figure 6). Even though untreated wild-type and *pp2aa1* roots were morphologically alike, and the inhibitor did not induce notable alterations in the wild type, nevertheless, 10  $\mu$ M brefeldin severely affected cell patterning in *pp2aa1*, causing histology to become irregular and cells to swell.

Because we hypothesize above that brefeldin inhibits gravitropism through increasing shootward polar transport of auxin (Figures 1 and 4), we next investigated whether shootward auxin transport in *pp2aa1* responds more strongly to brefeldin. Consistent with previous results (Rashotte et al., 2001), untreated *pp2aa1* had nearly double the auxin transport of the wild type (Figure 4). Brefeldin effectively stimulated shootward auxin transport in the mutant, with 3  $\mu$ M inducing a similar amount of transport as typically caused by 10  $\mu$ M in the wild type. Surprisingly, 10  $\mu$ M brefeldin reduced shootward auxin transport in *pp2aa1* to the level of *eir1-1*, implying that this transport stream was in fact eliminated. Along with the morphological phenotype, it appears that 10  $\mu$ M brefeldin is able to strongly enhance defects in *pp2aa1* associated with auxin transport, initiating a phenotype possibly related to the root collapse induced by stronger reduction of PP2A activity (Michniewicz et al., 2007).

To link brefeldin responsiveness in *pp2aa1* to the targeting of cortical PIN2, we investigated the effect of brefeldin on PIN localization, again using a 2-d treatment. In *pp2aa1*, cortical PIN2 attained a completely shootward localization on 3  $\mu$ M brefeldin, whereas the wild type required 10  $\mu$ M for a similarly complete conversion (Figure 7). Similar to the wild type, the shootward



**Figure 6.** Brefeldin Alters the Morphology of *pp2aa1* Roots.

Five-day-old seedlings were transferred to 10  $\mu$ M brefeldin plates for 48 h and then stained in 2  $\mu$ M FM 4-64 dye for 15 min. Left columns show the whole root, center columns the meristem, and right columns the transition zone. Images represent single confocal sections of surface views, obtained from three separate experiments, with five roots imaged per treatment for each experiment. Bars = 100  $\mu$ m for left columns and 50  $\mu$ m for middle and right columns.

[See online article for color version of this figure.]

localization of PIN2 in *pp2aa1* was altered neither in epidermis nor in lateral root cap, even on 10  $\mu$ M brefeldin. Furthermore, as observed in the wild type, in the *pp2aa1* background, neither 3 nor 10  $\mu$ M brefeldin induced any shootward relocation of PIN1 (Figure 8). Collectively, these results indicate that the rootward localization of PIN2 in meristematic cortical cells is required for optimal shootward auxin polar transport and hence for gravitropism.

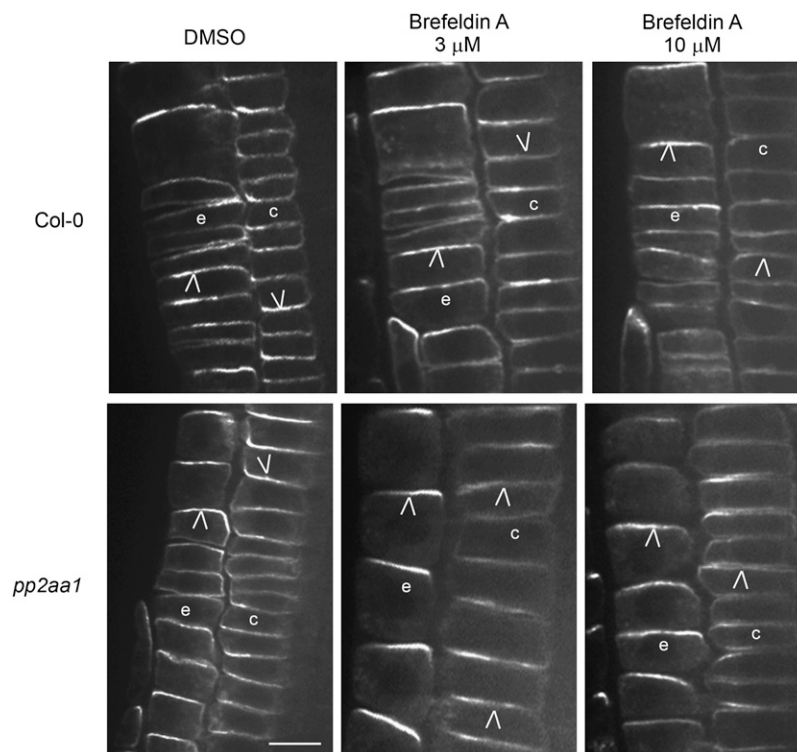
### Brefeldin Affects the Localization of PP2A Regulatory Subunit A1

We next sought to investigate PP2A activity by means of localization of PP2AA1-GFP (Michniewicz et al., 2007). Although it has been previously shown that PP2AA1 is localized cytosolically but cofractionates to an appreciable extent with plasma membrane proteins (Michniewicz et al., 2007; Blakeslee et al., 2008), to our knowledge, the effect of brefeldin on this localization has not yet been reported. PP2AA1-GFP permeated the cytoplasm; however, following 2-d treatment with as low as 3  $\mu$ M brefeldin, appreciable PP2AA1-GFP moved into large central bodies (Figure 9) identified as nuclei by staining with a DNA-specific dye (see Supplemental Figure 5 online). While the significance of PP2AA1-GFP moving into the nucleus is not known, it implies that brefeldin treatment changes the deployment of this protein phosphatase regulatory subunit.

To investigate trafficking quantitatively, we used fluorescence recovery after photobleaching (Figure 10). Control PP2AA1-GFP seedlings recovered virtually 100% of their prebleach intensity by 30 s, whereas seedlings treated with brefeldin (10  $\mu$ M for 2 d) recovered <80%. These results imply that brefeldin immobilized  $\sim$ 20% of PP2AA1. There is insufficient resolution to say whether the immobilized fraction was at the plasma membrane, within endosomes, or in some other compartment. Insofar as the immobilized PP2AA1 is inactive, this result is consistent with low concentration of brefeldin leading to an increased phosphorylation of PIN2 and hence to shootward polarization.

### PINOID Kinase Is Not Required for the Responses to Low Concentrations of Brefeldin

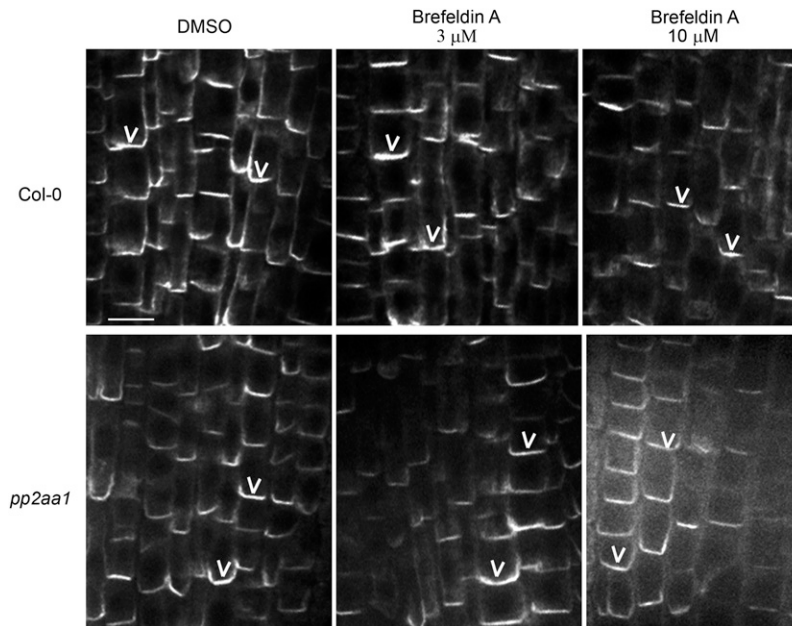
Recent studies on the targeting of PIN proteins have shown that phosphatase 2A is antagonized by PINOID kinase (Michniewicz et al., 2007; Kleine-Vehn et al., 2009; Sukumar et al., 2009). In principle, brefeldin could alter PP2A activity, increase PINOID kinase activity, or both; since brefeldin interferes with PP2AA1, we next investigated whether brefeldin treatment increases PINOID activity. Because there is no accepted activity assay, we examined the amount of the protein using a PINOID-yellow fluorescent protein (YFP) transgenic line (Michniewicz et al., 2007). A 2-d treatment with 10  $\mu$ M brefeldin increased fluorescence levels detected through confocal microscopy, and the



**Figure 7.** Cortical PIN2 in *pp2aa1* Is Hypersensitive to Brefeldin-Induced Relocation.

Five-day-old seedlings were treated for 48 h before being fixed and processed for immunostaining with an anti-PIN2 antibody. Images represent single confocal sections of median views, obtained from two separate experiments, with 8 to 10 roots imaged per treatment for each experiment. Arrowheads illustrate PIN2 polarity. c, cortex; e, epidermis. Bar = 10  $\mu$ m.





**Figure 8.** PIN1 in *pp2aa1* Is Not Translocated on Low Concentrations of Brefeldin.

Five-day-old seedlings treated for 48 h before being fixed and processed for immunostaining with an anti-PIN1 antibody. Images are single confocal sections, representative of two separate experiments, with eight to ten roots imaged per treatment in each experiment. Arrowheads illustrate PIN1 polarity. Bar = 10  $\mu$ m.

effect of 25  $\mu$ M brefeldin appeared to be stronger (Figure 11A). PINOID expression was also increased in 4-h treatments, by either 10 or 50  $\mu$ M brefeldin (see Supplemental Figure 6 online). As shown previously (Kleine-Vehn et al., 2009) and confirmed here, PINOID is largely excluded from brefeldin bodies (Figure 11A; see Supplemental Figure 6 online). That brefeldin treatment increases the expression of native PINOID was found from an immunoblot of samples treated for 4 h with 50  $\mu$ M brefeldin (Figure 11B). These results are consistent with brefeldin enhancing shootward polarization by increasing the activity of PINOID and, hence, of the phosphorylation of PIN2. Therefore, low concentrations of brefeldin both increase the abundance of PINOID and interfere with the PP2AA1 regulatory subunit of PP2A, implying that GNOM is poised to exert cooperative control on the partitioning of PIN2 in the cortex between rootward and shootward domains.

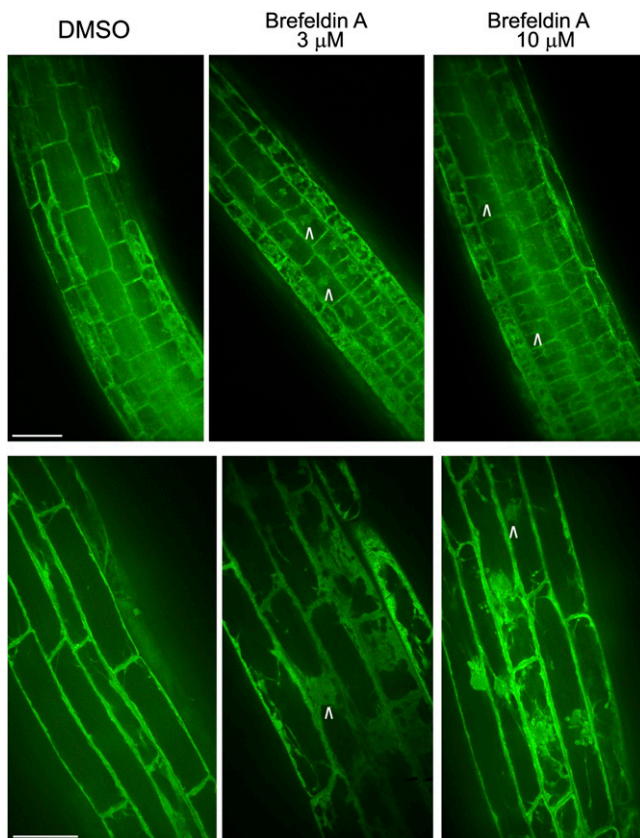
Because, as shown above, the loss of PP2AA1 enhanced the response to brefeldin, we hypothesized that the loss of PINOID kinase would make the root less responsive. To test this hypothesis, we assayed the effect of brefeldin on *pid-9*, which contains a loss-of-function allele of PINOID (Bennett et al., 1995) and has modestly delayed gravitropism (Sukumar et al., 2009). The *pid-9* mutant responded to brefeldin similarly to the wild type, both in terms of gravitropism (Figure 12A) and elongation (Figure 12B). In addition, despite the loss of PINOID, 10  $\mu$ M brefeldin drove shootward relocation of cortical PIN2, as effectively as in the wild type (Figure 12C). These results refute the above hypothesis and indicate that a PINOID-independent pathway for shootward PIN2 polarization exists in meristematic cortex cells. Furthermore, insofar as loss of PINOID kinase restores gravitropic bending in

the *pp2aa1* background (Sukumar et al., 2009), the fact that the loss of PINOID does not likewise restore gravitropism on low concentrations of brefeldin provides evidence that the partial loss of GNOM function is not solely affecting PP2A function.

Considering that PINOID belongs to a small family of protein kinases (Galván-Ampudia and Offringa, 2007), one or more members of the clade might function redundantly. To explore this possibility, we investigated the effect of brefeldin on a quadruple mutant in which all four members of the *PINOID* clade (*PID*, *PID2*, *WAG1*, and *WAG2*) are knocked out (Cheng et al., 2008). The quadruple mutant roots grow slowly and respond to gravity sluggishly; therefore, we assayed gravitropism 24 h after a 90° rotation. The quadruple mutant was resistant to brefeldin, for both root growth and gravitropism (Figure 13). Although the extreme dwarf phenotype of the mutant is a potential complication, these results support the idea that at least one other member of the clade can substitute for PINOID in conditioning a shootward localization of cortical PIN2 and hence for regulating gravitropism.

## DISCUSSION

In the ever fluctuating environment, gravity provides a uniquely constant signal. To respond to gravity, plants have evolved a complex sensory system, sharing construction principles with systems such as those for vision or hearing that evolved in animals. The stimulus is sensed by a group of cells adapted for this purpose, which then transmit information to adjust the organism's behavior. In the plant root, gravity is sensed by



**Figure 9.** Brefeldin Alters the Localization of PP2AA1-GFP.

Five-day-old PP2AA1-GFP transgenic seedlings were treated for 48 h and imaged with confocal fluorescence microscopy. Images were captured using the same confocal settings and are representative of 40 roots obtained from at least five independent experiments. Arrowheads indicate nuclei. Top panel shows the transition zone; bottom panel shows the elongation zone. Bars = 50  $\mu$ m.

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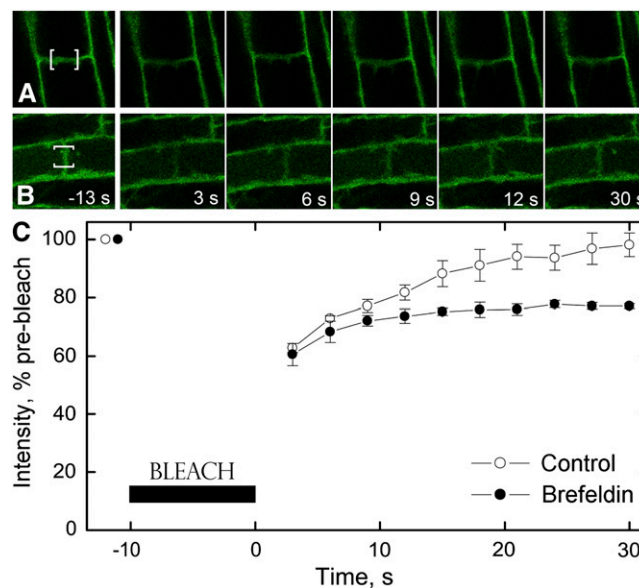
statocytes, specialized cells within the root cap columella, and a signal is then transmitted to the meristem and elongation zone. Without the equivalent of nerves, cells whose function is transmitting signals, plants transmit messages extensively by means of the hormone auxin. The plant moves auxin in specific directions and at speeds commensurate with the growth it regulates.

The polarity of auxin transport is strictly regulated by the polarity of the PIN proteins (Kleine-Vehn and Friml, 2008; Robert and Offringa, 2008). For gravitropism in the root, PIN2 deployment plays a pivotal role in transmitting auxin shootward through the peripheral layers of the root meristem and elongation zone (Muday and Rahman, 2008). However, PIN2 is targeted to shootward domains in epidermis and lateral root cap but to rootward domains in meristematic cortical cells; in other words, PIN2 is polarized appropriately in epidermis and lateral root cap to handle a shootward flow of auxin, but in meristematic cortical cells, PIN2 is polarized inappropriately. The reason for this wrong-way polarity remains unclear. Using a combination of molecular physiology and cell biology, here we show that (1)

rootward PIN2 and PIN1 are differentially sensitive to shootward relocation, (2) shootward relocation of PIN2 requires a PINOID family protein kinase but not necessarily PINOID itself, and (3) rootward PIN2 in cortical cells plays an important role in gravitropism.

### Cortical PIN2 Targeting Mechanisms

Our results indicate that rootward PIN2 in the cortex is more susceptible to brefeldin-induced shootward relocation than is rootward PIN1. In previous work, this relocation process was discovered in experiments with higher concentrations of brefeldin (usually 50  $\mu$ M). At this concentration, both rootward domain proteins, PIN1 and PIN2, are relocated shootward (Kleine-Vehn et al., 2008a). On 50  $\mu$ M brefeldin, PIN proteins appear first in brefeldin bodies and subsequently in shootward domains. In our experiments with 10  $\mu$ M brefeldin, in both long-term (2 d) and short-term (few hour) treatments, shootward relocation of PIN2 occurs in the apparent absence of brefeldin bodies. Insofar as brefeldin bodies represent a fairly drastic disruption to membrane trafficking, that relocation can be observed in their absence supports the idea that the shootward delivery is always operative but revealed when brefeldin blocks traffic to rootward domains. The nature of the shootward delivery has



**Figure 10.** Brefeldin Immobilizes a Fraction of PP2AA1-GFP.

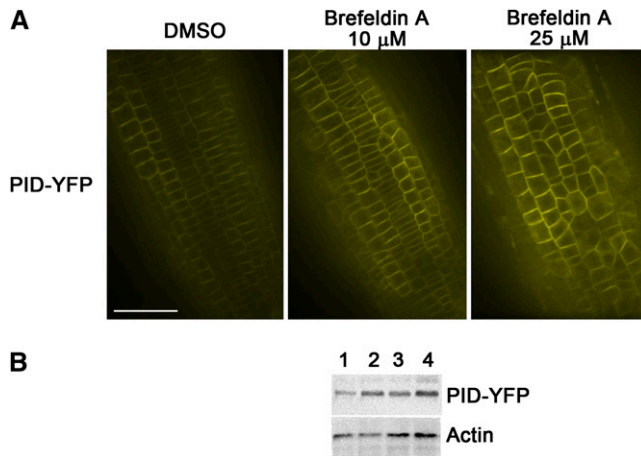
Five-day-old PP2AA1-GFP transgenic seedlings were treated for 48 h before being imaged with confocal fluorescence microscopy and subjected to photobleaching. Symbols plot mean  $\pm$  SE of three separate experiments, with three or four roots imaged per treatment for each experiment. In each root, three to five cells were photobleached. In (A) and (B), white brackets show the approximate region used for bleaching and for measurement of fluorescence intensity.

(A) Control.

(B) Brefeldin (10  $\mu$ M).

(C) Quantification of recovery.

[See online article for color version of this figure.]



**Figure 11.** Brefeldin Increases the Abundance of PINOID Kinase.

Five-day-old PID-YFP transgenic seedlings were treated for 48 h and imaged with confocal fluorescence microscopy. Images are single confocal sections of surface views, representative of 30 seedlings obtained from four independent experiments, and were captured using the same confocal settings. For immunoblotting, 5-d-old seedlings were treated for 4 h with 50  $\mu$ M brefeldin. Total proteins were extracted, resolved by SDS-PAGE, and probed with a monoclonal antibody against YFP. The blot is representative of three independent experiments. Bar = 50  $\mu$ m.

(A) PID-YFP localization in the meristem.

(B) Immunoblot. Lanes 1 and 3 are control; lanes 2 and 4 are brefeldin treated. Lanes 1 and 2 were loaded with 10  $\mu$ g total protein, and lanes 3 and 4 were loaded with 20  $\mu$ g.

[See online article for color version of this figure.]

been postulated to reflect a process of transcytosis, where material is moved from the rootward domain to the shootward domain via endosomal intermediates (Kleine-Vehn and Friml, 2008). However, in phenotypically wild-type plants and in the absence of brefeldin, direct evidence for such a transcytosis has been demonstrated only for newly divided cells (Kleine-Vehn et al., 2008a); therefore, to what extent shootward delivery occurs by transcytosis or by means of other polarized targeting mechanisms remains an open question.

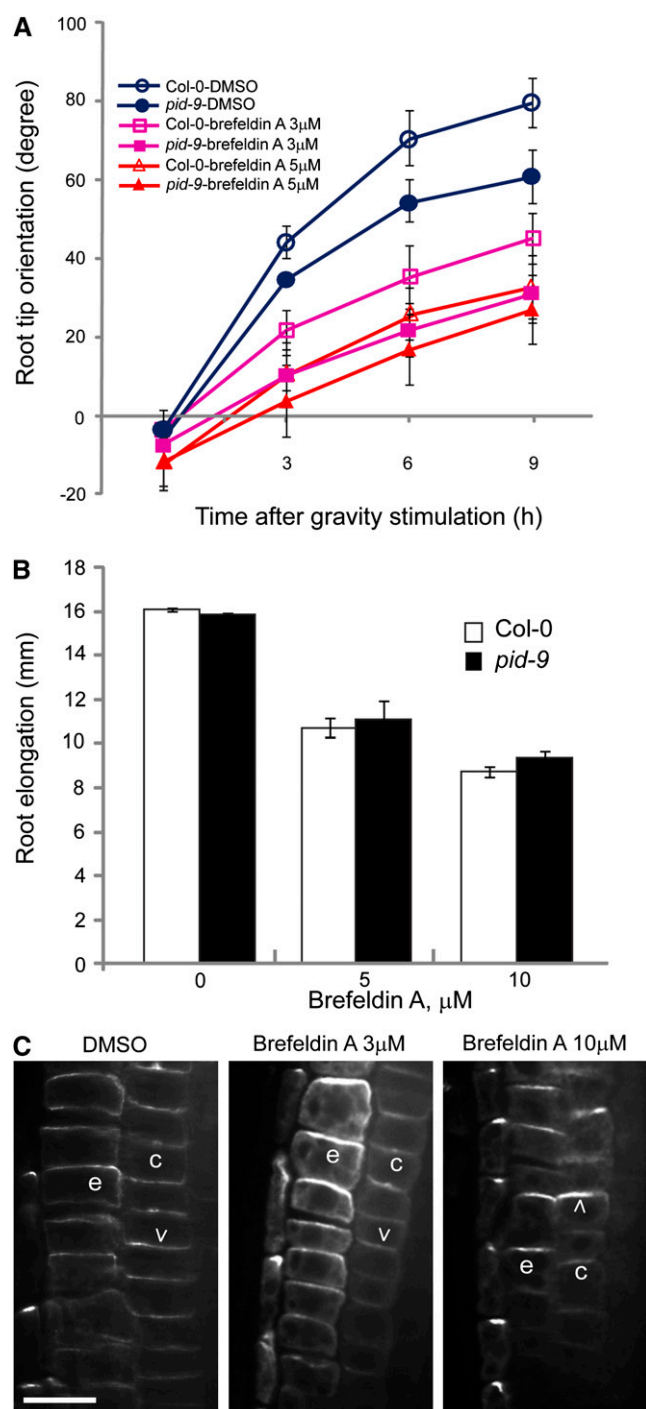
Along with sensitivity to brefeldin, our results show that cortical PIN2 trafficking is also more sensitive to the status of the type 2A phosphatase than is PIN1. The regulatory *pp2aa1* mutant, although able to target cortical PIN2 to rootward domains, is hyperresponsive to brefeldin, with 3  $\mu$ M inducing shootward transcytosis compared with 10  $\mu$ M in the wild type. PP2A is regulated by the A subunit, encoded in the *Arabidopsis* genome by three genes with partially overlapping function (Zhou et al., 2004). Previous work has reported shootward relocation of both PIN1 and PIN2 with stronger misregulation of PP2A, either from double or triple mutant combinations of the regulatory A subunit genes or from RNA interference knockdown of all three genes together (Michniewicz et al., 2007; Kleine-Vehn et al., 2009). That a single mutant, *pp2aa1*, increases the sensitivity of PIN2 to brefeldin-induced relocation implies that PIN2 trafficking is regulated acutely by PP2A status, at least as compared with PIN1.

In general, shootward PIN targeting appears to be promoted by phosphorylating PIN proteins, and the sole protein kinase implicated to date is PINOID. Both PIN1 and PIN2 move to shootward domains when PINOID kinase is overexpressed (Friml et al., 2004). Overexpressing PINOID also appears to exacerbate the effects of misregulated PP2A function, in terms of targeting, auxin transport, and related phenotypes, while the loss of function of PINOID tends to weaken such phenotypes (Michniewicz et al., 2007; Kleine-Vehn et al., 2009; Sukumar et al., 2009). However, we show here that PINOID is not required for the brefeldin-induced shootward relocation of cortical PIN2. The *pid-9* mutant responded to brefeldin as did the wild type in elongation, gravitropism, and PIN targeting assays. Previously, shootward targeting independent of PINOID had been proposed because the shootward relocation of PIN1 and PIN2 induced by 50  $\mu$ M brefeldin is delayed in *pid-9* but not prevented (Kleine-Vehn et al., 2009). Consistently, we find that a mutant lacking all four members of the *PINOID* clade is resistant to brefeldin, as expected if activity from this protein kinase family is required for PIN2 to traffic to a shootward domain in the root cortex. Apparently functional redundancy exists within the *PINOID* clade.

Nevertheless, brefeldin treatment did increase the abundance of PINOID kinase. We detected this within 4 h following treatment with 50  $\mu$ M brefeldin on an immunoblot; also, the increase in PID-YFP signal was clear even with 10  $\mu$ M treatment. While PINOID appears not to be required for cortical PIN2 shootward localization, it might nevertheless be involved. This highlights the complexity of PIN targeting mechanisms. Currently, experiments with brefeldin are interpreted based on the drug's trapping PIN proteins in the endosome; however, at least some other proteins also are likely to be trapped (Grebe et al., 2003; Shibasaki et al., 2009) and brefeldin also affects endoplasmic reticulum to Golgi traffic (Nebenführ et al., 2002). These disruptions allow for brefeldin to alter PIN status indirectly. We find that brefeldin increases PINOID expression and partially immobilizes PP2AA1-GFP, which is arguably consistent with a misregulation of PP2A activity. Given the pronounced effects of both PINOID kinase and PP2A on PIN targeting, we hypothesize that, in addition to direct effects on PIN cycling, brefeldin affects PIN polarity indirectly, based on its ability to affect the trafficking of regulatory kinases and phosphatases.

### Cortical PIN2 and Gravitropism

Our results indicate that the rootward targeting of PIN2 in the cortex plays a major role in regulating gravitropism. We initially observed that gravitropism is quite sensitive to brefeldin, with 3  $\mu$ M inhibiting the response by 50%. On 10  $\mu$ M brefeldin, PIN2 in the cortex is relocated to a shootward domain, while PIN1 is not, and the rate of shootward auxin transport is tripled. Subsequently, we found that the responsiveness to brefeldin is heightened in *pp2aa1*, with 3  $\mu$ M sufficient to cause specific shootward relocation of PIN2 and to inhibit gravitropism and stimulate auxin transport to the same extent as caused by 10  $\mu$ M brefeldin in the wild type. We interpret these results by hypothesizing that PIN2 in the cortex is functional and therefore increases the shootward flux of auxin when present in the shootward domain and decreases that flux when present in the rootward domain. In this



**Figure 12.** Gravitropism, Root Elongation, and Cortical PIN2 Translocation in *pid-9* Show a Wild-Type Sensitivity to Brefeldin.

For gravitropism, seedlings were treated vertically for 18 h and then rotated by 90°. Symbols plot mean  $\pm$  SE from three independent experiments with 10 to 12 seedlings per experiment. For root elongation, 5-d-old seedlings were treated vertically for 48 h. For PIN2 localization, 5-d-old seedlings were treated for 48 h before being fixed and processed for immunostaining with an anti-PIN2 antibody. Images represent single confocal sections of median views, obtained from two separate experi-

view, the wild-type rootward targeting of PIN2 in the cortex acts to limit the flow of auxin into the elongation zone.

In previous work, relocation of cortical PIN2 was induced along with PIN1, for example, by 50 μM brefeldin (Kleine-Vehn et al., 2008a, 2008b), which prevented assessing the role of PIN2 in the cortex specifically. Interestingly, one exception is an experiment where a phosphomimetic PIN1 was expressed in the *pin2* (null) background, driven by the PIN2 promoter: while the native PIN1 goes shootward in epidermis and rootward in the cortical meristem and restores gravitropism fully, the phosphomimetic protein is targeted to shootward domains in both cortex and epidermis and restores gravitropic bending by only ~50% (Zhang et al., 2010), a responsiveness not unlike what we show here for 3 μM brefeldin treatment. These results support the relevance for gravitropism of PIN2 polarization in the meristematic cortex.

It might seem paradoxical that increasing the flow of auxin would hinder gravitropism. Nevertheless, this conclusion was recently reached by Sukumar et al. (2009), who used a combination of genetic and pharmacological methods to show that enhanced shootward auxin transport is invariably associated with reduced gravitropism. Our results strengthen the association by adding treatment with low concentrations of brefeldin to the conditions known to increase auxin transport and at the same time decrease gravitropism. Although the mechanistic basis remains to be elucidated, it appears that sustaining a gradient of auxin across the root is sufficient to drive differential growth and hence bending requires an optimal flux of auxin, with either too much or too little diminishing the response (Muday and Rahman, 2008).

Our hypothesis assumes PIN2 is functional whether it be in rootward or shootward domains. This assumption is consistent with recent work that has induced the relocation of both PIN1 and PIN2 and found a profound depletion of auxin within the root meristem (Friml et al., 2004; Michniewicz et al., 2007; Kleine-Vehn et al., 2008a). Although the depletion might be attributed to the failure of rootward transport to deliver auxin to the root tip, this attribution accounts neither for the auxin synthesized in the root cap itself (Bhalerao et al., 2002), which would be expected to accumulate in the absence of shootward transport, nor for the measured enhanced rates of shootward auxin transport. Further evidence for PIN proteins functioning in whichever domain they are delivered comes from experiments with PIN1. When this protein is expressed under the PIN2 promoter in a *pin2* (null) background, the transgenic PIN1 adopts the localization of native PIN2 and fully restores gravitropism, even though endogenous PIN1 is targeted primarily to rootward domains (Wiśniewska et al., 2006; Zhang et al., 2010).

ments, with 8 to 10 roots imaged per treatment for each experiment. Arrows illustrate PIN2 polarity. In (B), no significant difference between means for the two genotypes was found. c, cortex; e, epidermis. Bar = 20 μm.

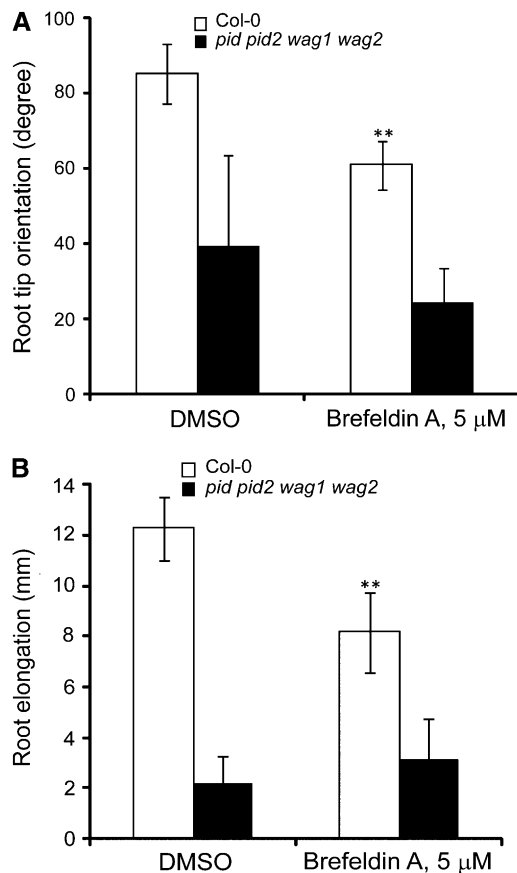
(A) Gravitropism.

(B) Root elongation.

(C) PIN2 localization.

[See online article for color version of this figure.]





**Figure 13.** The Quadruple Mutant (*pid pid2 wag1 wag2*) Is Resistant to Brefeldin-Induced Inhibition of Root Elongation and Gravitropism.

For gravitropism, 5-d-old wild-type and 7-d-old quadruple mutant seedlings were treated vertically for 24 h and then rotated by 90°. The gravity response was measured after 24 h of gravity stimulation. For root elongation, elongation rate of the same seedlings was measured. Symbols plot mean  $\pm$  SD from two independent experiments with seven seedlings per experiment for *pid pid2 wag1 wag2* and 10 seedlings for the wild type. Asterisks represent the statistical significance between brefeldin and DMSO-treated seedlings, as judged by Student's *t* test: \*\*  $P < 0.01$ . For the quadruple mutant, the effect of brefeldin was not significant in either assay.

**(A)** Gravitropism after 24 h of gravity stimulation.

**(B)** Root elongation after 48 h of brefeldin treatment.

Our hypothesis further assumes that auxin enters cortical cells. The most well characterized auxin influx carrier, AUX1, is not expressed in cortex cells (Swarup et al., 2001), but other carriers might be able to permeate auxin. In the apoplast, some auxin is protonated; therefore, a sufficient quantity might be able to diffuse across the membrane directly. A recent computational model has emphasized the necessity of having an influx carrier to sustain a canalized flux of auxin (Kramer, 2004), but it is precisely the wrong-way polarization of cortical PIN2 that suggests the influx must be limited for a net shootward flux to occur. We predict that expressing AUX1 in the cortex would diminish, if not eliminate, shootward polar auxin transport as well as gravitropism, a prediction that could be tested by means of tissue-specific promoters.

Nevertheless, brefeldin stimulates shootward auxin transport and delays gravitropism at lower concentrations than it induces shootward relocation of cortical PIN2. Similarly, untreated *pp2aa1* has stimulated auxin transport and delayed gravitropism but expresses cortical PIN2 in the usual rootward domain. Brefeldin might influence auxin transport and PIN targeting via independent mechanisms, but if so then misregulation of PP2A must affect both of them similarly. Alternatively, we hypothesize that the amount of PIN2 reaching the shootward domain in the cortex increases in proportion to the loss of GNOM activity and, when PP2A is misregulated, the increase is steeper and that the amount of PIN2 reaching the shootward domain increases auxin transport (and hence reduces gravitropism) proportionately. By contrast, the bulk relocation of cortical PIN2 happens only after a certain threshold has been exceeded, perhaps reflecting a cooperative interaction between the PINOID kinases, PP2A, and GNOM-dependent targeting. We propose that being able to subtly modulate the deployment of PIN2 allows the root to better negotiate the competing demands of obstacles, nutrients, and water as it grows through the soil.

## METHODS

### Plant Materials and Growth Conditions

All lines were in the Columbia (Col-0) background of *Arabidopsis thaliana*. The transgenic *DR5-mGFP* was a gift of E.B. Blancaflor (Samuel Roberts Noble Foundation, Ardmore, OK), the PIN2-GFP line (Xu and Scheres 2005) was a gift of B. Scheres (University of Utrecht, The Netherlands), PP2AA1-GFP, PID-YFP, and *pp2aa1* (Michniewicz et al., 2007) were gifts of J. Friml (Flanders Institute for Biotechnology, VIB, Gent, Belgium), *IAA2-GUS* (Swarup et al., 2001) was a gift of M. Bennett (University of Nottingham, Sutton Bonington, UK), and *pid pid2 wag1 wag2* was a gift of Yunde Zhao (University of California, San Diego, CA). Quadruple mutants were selected from a segregating population based on a cotyledon phenotype (Cheng et al., 2008). *pid-9* (Christensen et al., 2000) was provided by G. Muday (Wake Forest University, Winston-Salem, NC), and homozygous *pid-9* seedlings were selected from a segregating family using the tricot phenotype, as described earlier (Sukumar et al., 2009). Columbia and PIN1-GFP were obtained from the ABRC (Columbus, OH).

Surface-sterilized seeds were placed in round, 9-cm Petri plates on modified Hoagland medium (Baskin and Wilson, 1997) containing 1% (w/v) sucrose and 1% (w/v) agar (Difco Bacto agar; BD laboratories). Two days after stratification at 4°C in the dark, plates were transferred to a growth chamber (NK system, LH-70CCFL-CT) at 23°C under continuous white light at an irradiance of  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The seedlings were grown vertically for 5 d. For both long and short-term inhibitor treatments, 5-d-old seedlings were transferred to new plates containing the inhibitors and incubated as indicated under the same conditions.

### Chemicals

Brefeldin A, IAA, and 4',6-diamidino-2-phenylindole were purchased from Sigma-Aldrich. FM 4-64 was purchased from Invitrogen. [ $^3\text{H}$ ] IAA (20 Ci mmol $^{-1}$ ) was purchased from American Radiolabeled Chemicals. Other chemicals were from Wako Pure Chemical Industries.

### Gravitropism Assay

Root tip reorientation was assayed as described earlier (Rahman et al., 2001). Briefly, 5-d-old vertically grown seedlings were transferred to

square plates containing brefeldin and allowed to remain vertical for 18 h before being rotated by 90°. To measure the bending of roots and elongation, photographs of plates were taken at specific time points after reorientation using a digital camera (Canon Power Shot A 640) and analyzed with Image J (<http://rsbweb.nih.gov/ij/>). In the same experiments, elongation rate of vertical roots was obtained for seedlings handled in the same way but not rotated.

### GUS Staining, Immunostaining, and Live Cell Imaging

GUS staining was performed as described earlier (Okamoto et al., 2008). In brief, 5-d-old seedlings were transferred to plates containing the desired concentration of inhibitor and grown vertically for 48 h in the growth chamber. At that time, to expose seedlings to IAA, they were transferred to plates containing the same concentration of inhibitor as well as IAA for 4 h. Seedlings were transferred to GUS staining buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100) containing 1 mM X-gluc and incubated at 37°C in the dark for 1 h. Roots were cleared as described earlier (Malamy and Benfey, 1997) with minor modifications. In brief, roots were incubated at 57°C in a solution containing 0.24 N HCl in 20% methanol for 15 min. This solution was replaced with 7% NaOH in 60% ethanol and incubated for 15 min at room temperature. The roots were rehydrated stepwise through 40, 20, and 10% ethanol for 5 min each, and finally vacuum-infiltrated for 15 min in 5% ethanol and 25% glycerol and mounted in the same solution. The roots were imaged with a light microscope (Nikon Diaphot) equipped with a digital camera control unit (Digital Sight, DS-L2; Nikon).

For immunostaining, we used the protocol described earlier by Rahman et al. (2007). Briefly, seedlings were fixed in PIPES buffer (50 mM PIPES, pH 7, 4% paraformaldehyde, and 0.5 mM  $\text{CaCl}_2$ ), permeabilized in PME buffer (50 mM PIPES, pH 7, 5 mM EGTA, and 2 mM  $\text{MgSO}_4$ ) containing glycerol (10%) and triton (0.2%), and then in ice-cold methanol. Following rehydration in PBS, seedlings were subjected to brief cell wall digestion with pectinase (0.01% [w/v]) and pectolyase (0.001% [w/v]) made up in PBS, and then incubated in 10% (v/v) DMSO and 3% (v/v) Nonidet P-40 in PME for 1 h. After incubation, seedlings were rinsed with PME (3 × 5 min) and blocked in 1% (w/v) goat serum in PBA (1% BSA and 0.01% sodium azide in PBS). After 1 h, the blocking solution was replaced carefully with primary antibody. The primary antibodies were anti-PIN2, a gift of P. Mason (University of Wisconsin, Madison, WI), or anti-PIN1, a gift of J. Friml (Flanders Institute for Biotechnology, VIB, Gent, Belgium) diluted 1:100 in PBA. The secondary was Cy-3-goat anti-rabbit IgG (1:200 in PBA; Jackson ImmunoResearch).

To image GFP, seedlings were mounted in liquid growth medium on a slide for observation on an Olympus BX-61 upright microscope, equipped with a Yokogawa confocal attachment, and imaged with a ×60 oil immersion objective. To observe *DR5-GFP* during gravitropism, seedlings were mounted 3 h following rotation of the plate and imaged with a ×20 objective. For photobleaching, seedlings were mounted under a piece of agar saturated with growth medium and imaged on a Nikon inverted microscope (TE2000-S) interfaced to a confocal attachment (Eclipse-C1) with a ×60 water immersion objective. A region of interest (ROI) was defined as a thin rectangle that spanned a transverse cell wall and surrounding cytoplasm, and the ROI was irradiated at 100% power of the 488-nm laser for 10 s. Recovery images were collected automatically every 3 s, with approximately a 0.2-s frame acquisition. The fluorescence intensity within the ROI was analyzed using Image J.

For nuclear staining, brefeldin-treated PP2A1-GFP seedlings were transferred to liquid growth media containing 5  $\mu\text{g}/\text{mL}$  DAPI and incubated for 20 to 30 min at room temperature. After incubation, seedlings were placed on slides and imaged with a spinning disc fluorescence microscope (Olympus BX-61 equipped with Bx-DSU).

### Auxin Transport Assay

Five-day-old vertically grown seedlings were transferred to treatment plates and grown vertically for 48 h. For measuring shootward transport, a 2-mm-wide strip of Parafilm was placed on the surface of plates containing assay medium (2% agar, 5 mM MOPS, 5 mM  $\text{KNO}_3$ , 2 mM  $\text{CaNO}_3$ , 2 mM  $\text{MgSO}_4$ , and 1 mM  $\text{KH}_2\text{PO}_4$ , pH 6.7), and seedlings were transferred with the root tip near the middle of the Parafilm strip. Donor agar was prepared, containing 5  $\mu\text{M}$  [ $^3\text{H}$ ] IAA (3.7 MBq  $\text{mL}^{-1}$ ), 1.5% agar, 5 mM MES, 5 mM  $\text{KNO}_3$ , 2 mM  $\text{CaNO}_3$ , 2 mM  $\text{MgSO}_4$ , and 1 mM  $\text{KH}_2\text{PO}_4$ , pH 5.7, cut into 1-mm<sup>3</sup> cubes, and placed on the edge of the Parafilm strip, so that the agar block just touched the root tip. Plates were then incubated vertically under nearly saturating humidity for 1 h at room temperature. Subsequently, the apical 2 mm of the root was cut and discarded, and the subsequent 5-mm segment was cut and soaked overnight in 5 mL of liquid scintillation fluid (ACSII; Amersham Biosciences). The radioactivity was measured with a scintillation counter (model LS6500; Beckman ACSII). Eight root segments were used per datum.

### Immunoblotting

Total protein extracts were obtained from the apical 5-mm segment of 5-d-old PID-YFP root tips. The root tips were homogenized in SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibodies against anti-Living Colors-YFP (Clontech) and anti-actin (C4; Chemicon). Proteins were detected with a luminoimage analyzer (AE-6972C; ATTO) using chemiluminescence reagents, as described previously (Nakayama et al., 2007).

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PIN2 (At5g57090), PIN1 (At1g73590), IAA2 (At3g23030), PID (At2g34650), PP2AA1 (At1g25490), and LTI6b (At3g05890).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Effect of Long-Term Brefeldin Treatment on the Localization of PIN2 and PIN1 Assessed in Living Cells.

**Supplemental Figure 2.** Effect of Short-Term Brefeldin Treatment on the Localization of PIN2.

**Supplemental Figure 3.** Effect of Short-Term Brefeldin Treatment on the Localization of PIN1.

**Supplemental Figure 4.** Effect of Brefeldin on the Localization of GFP-LTI6b.

**Supplemental Figure 5.** Partial Nuclear Localization of PP2A1-GFP with Long-Term Brefeldin Treatment.

**Supplemental Figure 6.** Effect of Short-Term Brefeldin Treatment on the Localization of PINOID Kinase (PID-YFP).

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