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Auxin Response in *Arabidopsis* under Cold Stress: Underlying Molecular Mechanisms [C][W]

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To understand the mechanistic basis of cold temperature stress and the role of the auxin response, we characterized root growth and gravity response of *Arabidopsis thaliana* after cold stress, finding that 8 to 12 h at 4°C inhibited root growth and gravity response by ~50%. The auxin-signaling mutants *axr1* and *tir1*, which show a reduced gravity response, responded to cold treatment like the wild type, suggesting that cold stress affects auxin transport rather than auxin signaling. Consistently, expression analyses of an auxin-responsive marker, *IAA2-GUS*, and a direct transport assay confirmed that cold inhibits root basipetal (shootward) auxin transport. Microscopy of living cells revealed that trafficking of the auxin efflux carrier PIN2, which acts in basipetal auxin transport, was dramatically reduced by cold. The lateral relocalization of PIN3, which has been suggested to mediate the early phase of root gravity response, was also inhibited by cold stress. Additionally, cold differentially affected various protein trafficking pathways. Furthermore, the inhibition of protein trafficking by cold is independent of cellular actin organization and membrane fluidity. Taken together, these results suggest that the effect of cold stress on auxin is linked to the inhibition of intracellular trafficking of auxin efflux carriers.

INTRODUCTION

The growth and development of plants are influenced by both external environmental factors, such as biotic and abiotic stresses, and by endogenous growth regulators, known as phytohormones. In many instances, plants respond to environmental stresses by changing their endogenous hormonal responses. For example, during abiotic stresses, plants produce increased amount of abscisic acid and ethylene. In addition, response pathways to abiotic stress have also been suggested to involve jasmonic acid and salicylic acid (for review, see Xiong et al., 2002).

Among various abiotic stresses, cold is a major stress that limits plant growth and development. The phytohormone abscisic acid is well known to play an important role in facilitating the adaptation process during cold stress (for review, see Chinnusamy et al., 2004; Mahajan and Tuteja, 2005). Although auxin (indole-3-acetic acid [IAA]), another phytohormone, essentially plays a role in virtually every aspect of growth and development of plants, including the tropic responses (Davies, 1995), little is known about the response of this hormone under cold stress.

Potentially linking cold stress to auxin are reports that cold stress inhibits the inflorescence gravity response in *Arabidopsis thaliana* (Fukaki et al., 1996; Wyatt et al., 2002). Gravity response critically depends on auxin. The gravity response pathway has been separated into three sequential steps: gravity perception, signal transduction, and asymmetric growth response (for review, see Sack, 1991). Although perception and signal transduction, which involve the sedimentation of amyloplasts in specialized cell types, such as root columella or shoot endodermis (Sack, 1991; Kiss, 2000; Weise et al., 2000), might be auxin independent, auxin is intimately involved in bending. Gravitropic curvature is driven by an asymmetric distribution of auxin that induces differential growth and bending (for review, see Muday and Rahman, 2008). Furthermore, mutations in auxin transport or signaling components commonly result in agravitropic or reduced gravitropic phenotypes in *Arabidopsis* roots (Maher and Martindale, 1980; Estelle and Somerville, 1987; Wilson et al., 1990; Leyser et al., 1996; Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Utsuno et al., 1998).

During cold treatment at 4°C, the *Arabidopsis* inflorescence does not respond to the gravity vector; however, when gravistimulated in the cold and then returned to vertical at room temperature, the inflorescence bends in response to the previous horizontal gravistimulation. This demonstrates the existence of a gravity-persistent signal and indicates that cold stress acts after gravity perception (Fukaki et al., 1996; Wyatt et al., 2002). Furthermore, basipetal auxin transport in the inflorescence was abolished at 4°C but was restored to the wild-type level soon after the plant was returned to room temperature (Wyatt et al., 2002; Nadella et al., 2006). To identify components that separate the perception events from the response, Wyatt et al. (2002) screened for mutants in which the gravity-persistent signal was aberrant. Although these *gps* mutants respond abnormally to the gravity stimulus, the amyloplast sedimentation is apparently normal, suggesting that the aberrant response is caused by an event (or events) that links gravity perception to auxin transport

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(Wyatt et al., 2002). Additional studies of *gps* mutants revealed that these mutants fail to establish the proper auxin gradient in the inflorescence after gravistimulation and also show altered polar and lateral auxin transport (Nadella et al., 2006). In an earlier report, Morris (1979) demonstrated that temperature affects the velocity of exogenous auxin transport in a number of species. Collectively, these results establish a link between cold stress and auxin response in planta, but the molecular mechanism that regulates the response under cold stress remains unclear.

To provide a mechanistic explanation to the effect of cold stress on auxin response, we used gravitropism of *Arabidopsis* roots as a model system and developed an assay, wherein seedlings grown at 23°C were transiently treated at 4°C and the effects of the cold stress were monitored subsequently at 23°C. Our results reveal that cold stress blocks the gravity response reversibly. Using molecular and cellular approaches, we also show that cold stress primarily targets the auxin transport system rather than auxin signaling and inhibits basipetal auxin transport by blocking the intracellular trafficking of the auxin efflux carrier, PIN2. Finally, we show that the inhibition of protein trafficking by cold stress is selective and independent of cellular actin organization and membrane fluidity.

RESULTS

Effect of Cold Stress on *Arabidopsis* Root Growth and Gravity Response

To understand the effect of cold stress on auxin response, we developed a new assay system using the *Arabidopsis* root. In this system, the vertically oriented seedlings were cold stressed at 4°C for various time lengths. After the cold incubation, the seedlings were returned to 23°C and then gravistimulated by rotating the plate 90°. Growth and bending of the roots were followed for the next 9 h using root tip reorientation assay as described earlier (Lincoln et al., 1990; Rahman et al., 2001). The time-course analysis revealed that cold treatment for 24 h blocked gravitropism and elongation completely over the 9-h assay window (Figure 1). However, shorter durations of cold slowed gravitropism and reduced elongation, roughly in proportion to the cold duration. After 6 h at 23°C, compared with controls, the 12-h cold treatment reduced gravitropism by 40% and elongation by 60%. Hence, we selected 12-h incubation at 4°C as an optimal treatment for analyzing the effects of cold stress. The delayed gravitropism and slower growth suggests that cold stress affects the auxin response.

In cold-stressed seedlings, the root tip orientation and elongation showed differential responses during the recovery period (Figures 1A and 1B). After 9 h of 23°C incubation, while the gravity response of cold-stressed roots recovered to 80% of control, the recovery of root elongation was only 50%, indicating that root gravity response and elongation are controlled by partially distinct response pathways. Interestingly, during the recovery process, in contrast with root elongation, the kinetics of root gravitropism seem to follow parallel lines independent of duration of cold treatment (Figure 1A), raising a possibility that most of the differences at gravitropic kinetics may occur at an early time point of recovery. To explore this scenario, we compared the early kinetics of gravity response and root elongation between control and cold-stressed seedlings (see Supplemental Figure 1 online). For root tip orientation, we observed a response lag of 120 min, and for root elongation it was 90 min (see Supplemental Figure 1 online). These results indicate that root elongation responds to recovery earlier than gravity response. However, during the 9-h recovery window, we found that the root tip of cold-stressed roots responded to gravity much faster than did the elongation process (Figure 1). Taken together, these results indicate that the differential responses of root gravity and elongation take place during the later part of the recovery process instead of the early part and also reinforce the idea that root gravity responses and elongation pathways are regulated by partially distinct pathways.

Cold Stress Affects Auxin Transport Rather Than Auxin Signaling

Both the auxin transport and signaling have been implicated in regulating the root gravity response in *Arabidopsis*, as evidenced in part by mutant phenotypes. For example, the auxin transport mutants *aux1* and *eir1* are completely agravitropic, and the auxin signaling mutants exhibit both agravitropic and reduced gravitropic phenotypes (Maher and Martindale, 1980; Estelle and Somerville, 1987; Wilson et al., 1990; Leyser et al., 1996; Luschnig et al., 1998). To understand the molecular pathway by which cold stress inhibits the auxin response, we analyzed root gravity response and elongation of two auxin signaling mutants *axr1-3* and *tir1-1* (Figure 2). AXR1 and TIR1 are components of the ubiquitin proteasome pathway, which plays a central role in regulating cellular auxin signaling (Leyser et al., 1993; Ruegger et al., 1998). In addition, TIR1 also serves as an auxin receptor (Dharmasiri et al., 2005; Tan et al., 2007). Mutations in these proteins result in reduced, but not abolished root gravitropism. We therefore examined these mutants to understand the role of auxin signaling in cold stress. If cold stress inhibits the auxin response through auxin signaling, then one would expect a weaker response in these mutants compared with control. However, both gravitropism and elongation were inhibited in *axr1* and *tir1* roots to roughly the same extent as in the wild type (Figure 2). Therefore, it appears that an intact auxin signaling system is not responsible for the observed responses to cold stress.

Cold Stress Inhibits the Root Basipetal Auxin Transport

Because mutants defective in root basipetal (shootward) auxin transport are completely agravitropic, we assessed the status of auxin transport in response to cold stress in two ways, indirectly and directly. First, we imaged the β-glucuronidase (GUS) staining pattern of the auxin-responsive marker *IAA2-GUS* (Luschnig et al., 1998; Swarup et al., 2001), whose activity has been suggested to correlate with endogenous auxin (Casimiro et al., 2001; Benkova et al., 2003, Swarup et al., 2005; Grieneisen et al., 2007; Okamoto et al., 2008). Untreated roots showed a typical staining pattern, with GUS activity confined to the columella and stele (Figure 3A). By contrast, roots cold stressed for 12 h had

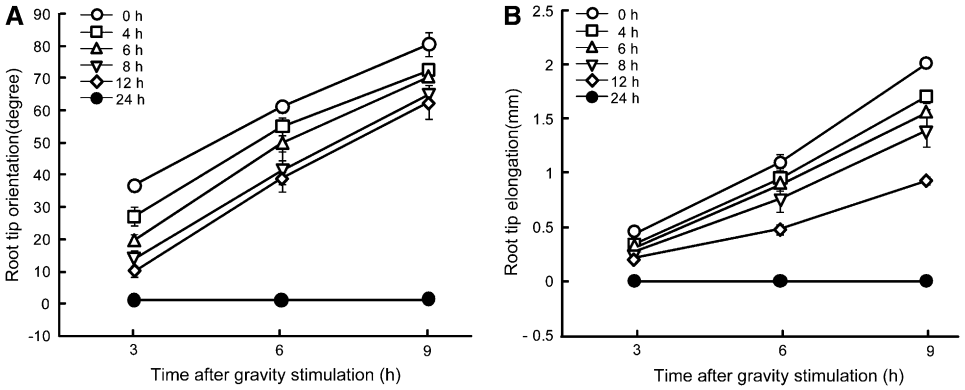

Figure 1. Effect of Cold Stress on *Arabidopsis* Root Gravity Response and Elongation.

Five-day-old seedlings were transferred to new agar plates and subjected to cold treatment (4°C) for various time lengths. After the cold treatment, gravity stimulation was provided to the roots by rotating the plates 90° at 23°C. Data for root tip orientation and elongation were collected for 3, 6, and 9 h. Vertical bars represent mean ± SE. Data are from three independent experiments with 10 to 12 seedlings per experiment.

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

(B) Root elongation.

additional GUS activity in the outer cell layers, such as epidermis and cortex. The appearance of staining in the peripheral cells of cold-stressed roots could be explained by the accumulation of auxin in these tissues. To confirm this auxin accumulation, we incubated the plants in 1 μM IAA for 30 min. The exogenous IAA strongly increased GUS staining without changing its pattern (Figure 3B). In the untreated roots, there was greater staining in columella and stele without signal in peripheral regions, whereas in the cold-stressed roots, staining increased in the periphery as well as in columella and stele. These results offer additional evidence that cold does not alter signaling in response to auxin and imply that auxin accumulates in the cortex and epidermis of the cold-stressed root. The latter is consistent with a block in auxin transport. However, we do not rule out the possibility of changed auxin metabolism or synthesis in root meristem under cold stress.

Next, we monitored the asymmetric distribution of auxin during gravity response using the same marker line (Figure 3). In control roots, after 3 h of gravistimulation, we observed the formation of a GUS gradient extending basipetally from the lower side of the root tip to the meristematic region (Figure 3C, left panel). This gradient was faint by 6 h of gravistimulation and completely absent after 9 h (Figure 3C, left panel). By contrast, in cold-treated roots, GUS activity was observed on both sides of the meristem after 3 h of gravity stimulation; after 6 h of gravity stimulation, the asymmetric auxin gradient formed on the lower side of the root; and by 9 h, the GUS signal had faded (Figure 3C, right panel). These results are consistent with the physiological

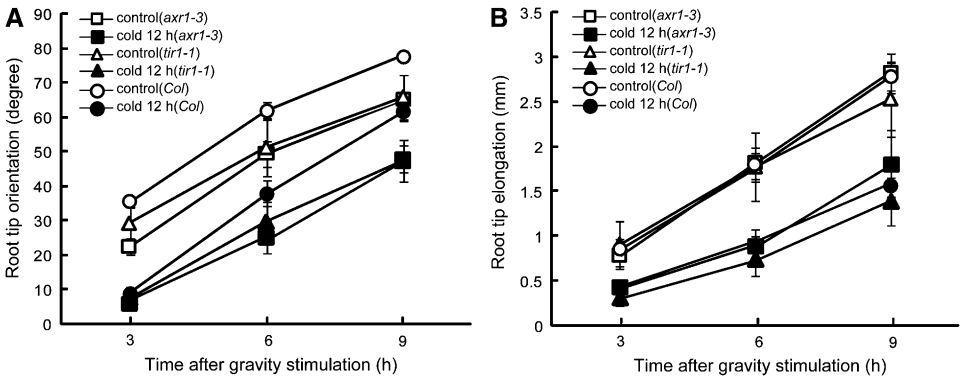

Figure 2. Effect of Cold Stress on Auxin Signaling Mutants.

Root gravity response and elongation of Columbia-0, *axr1-3*, and *tir1-1* were compared after 12 h of 4°C treatment. Gravity response and root elongation were measured as described in Figure 1. Vertical bars represent mean ± SE. Data are from three independent experiments with 10 to 12 seedlings per experiment.

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

(B) Root elongation.

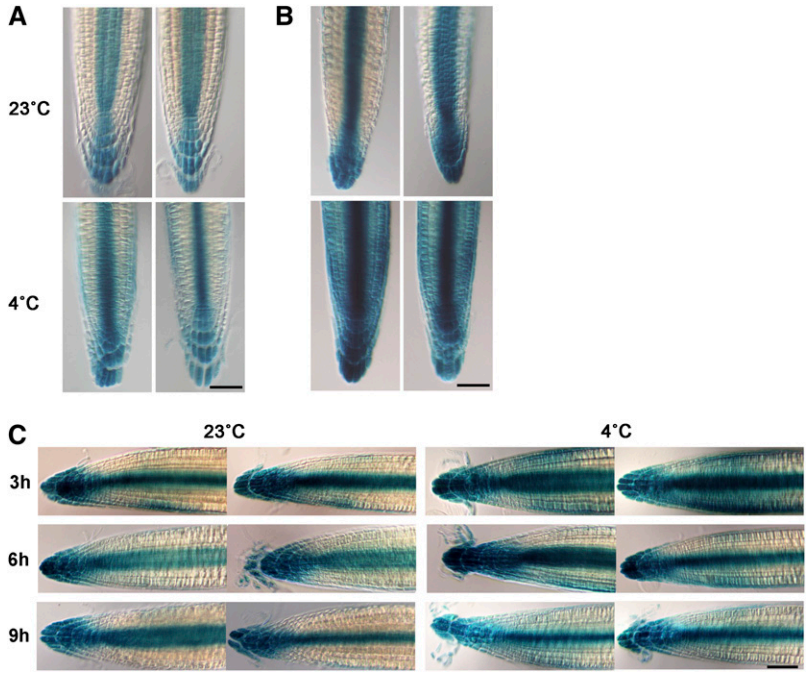

Figure 3. Cold Stress Alters the Auxin Response in *Arabidopsis* Roots.

Five-day-old *IAA2-GUS* seedlings were treated at 4°C for 12 h. Seedlings were stained in a buffer containing 1 mM X-gluc for 1 h at 37°C and cleared for photography. These are representative images of 30 seedlings for **(A)** and **(B)** and 25 seedlings for **(C)** stained in at least three separate experiments. Bars = 50 μm.

(A) Control roots (top panel) and cold-treated roots (bottom panel).

(B) Seedlings treated with 1 μM IAA for 30 min, control roots (top panel), and cold-treated roots (bottom panel).

(C) Monitoring the formation of asymmetric auxin gradient in gravity stimulated roots. Left panels represent control roots, and right panels represent cold stressed roots.

responses shown in Figure 1. The *IAA2-GUS* marker analyses collectively suggest that cold stress inhibits the gravity response by enhancing the accumulation of endogenous auxin in root meristem, possibly by inhibiting the basipetal auxin transport.

To confirm the above hypothesis, we measured the root basipetal auxin transport directly. Figure 4 represents the net amount of basipetally transported IAA in the 5-mm apical segment. Cold treatment decreased the amount of auxin transported basipetally by >50%. When the cold-treated seedlings were returned to 23°C for 9 h, basipetal transport actually exceeded the control level, suggesting compensatory recovery. To explore the possibility that cold treatment may reduce the membrane uptake capacity, we performed an uptake assay with the structurally related compound, benzoic acid. There was no significant difference in the amount of benzoic acid taken up between untreated and cold-stressed seedlings (see Supplemental Figure 2 online). Taken together, these results suggest that the primary target for cold stress is auxin transport.

Cold Stress Inhibits the Intracellular Trafficking of Auxin Efflux Carrier PIN2

Our physiological and molecular data along with the auxin transport data indicate that cold stress inhibits gravitropism by blocking auxin transport. To understand the mechanistic basis of this pathway, we next investigated the cellular localization and intracellular trafficking of PIN2, which belongs to a major family of auxin efflux carriers, the PIN proteins, and plays an important role in regulating the root gravity response (for review, see Muday and Rahman, 2008). It has been suggested that both the polar deployment and the intracellular trafficking of PIN proteins are required for functionality and optimal auxin transport (for review, see Vieten et al., 2007). Hence, the cold stress–induced inhibition of basipetal transport and gravity response could be linked to either polar targeting or the intracellular trafficking of PIN2.

To investigate these possibilities, we followed the localization and intracellular trafficking of PIN2 in living cells using PIN2-green fluorescent protein (GFP) transgenic seedlings (Xu and Scheres, 2005). Our analyses revealed that cold stress for 12 h did not appear to alter the asymmetric localization of PIN2 but did appear to suppress trafficking (Figures 5A to 5D). Control roots had numerous PIN2- positive small bodies in the cytosol, consistent with the idea that PIN2 cargo is continuously cycled between plasma membrane and endosomal compartments. When the PIN2 localization and trafficking were observed in the seedlings immediately after cold treatment, the cold-stressed roots showed an asymmetric localization of PIN2 as observed in control roots, but no PIN2 endosomal bodies were observed

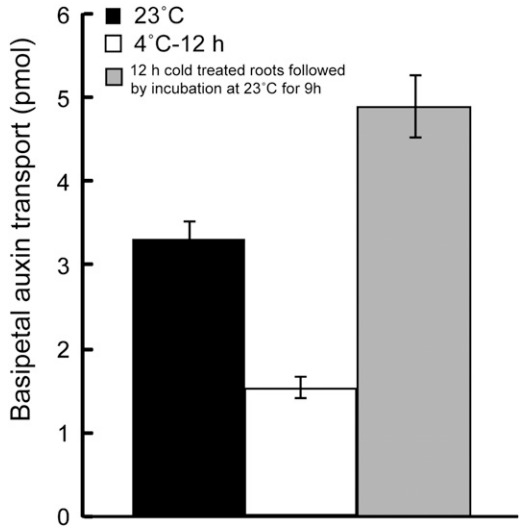

Figure 4. Effect of Cold Stress on Root Basipetal Auxin Transport.

Five-day-old Columbia seedlings were subjected to cold stress at 4°C for 12 h, and root basipetal transport of auxin was measured for 1 h at room temperature immediately after the cold treatment. The same transport assay was performed for the 12 h cold-treated roots followed by incubation at 23°C for 9 h. The experiments were done in triplicate and repeated at least three times (control $n = 96$; 12 h cold treatment $n = 150$; 12 h cold treatment followed by incubation at 23°C for 9 h = 50). Vertical bars represent mean ± SE.

(Figures 5A to 5D), indicating that the cold treatment suppressed the endosomal cycling of PIN2.

To confirm that cold stress indeed inhibits PIN2 trafficking, we monitored cycling of PIN2 in the presence of a widely used protein trafficking inhibitor, brefeldin A (BFA). Previously, BFA treatment has been shown to result in the accumulation of PIN1, PIN2, PIN3, and PIN4 proteins in so-called BFA bodies (Geldner et al., 2001; Paciorek et al., 2005). Based on our results, we hypothesize that if cold stress inhibits the intracellular cycling of PIN2, then cold-stressed roots would form fewer BFA bodies compared with roots grown at optimum temperature. Consistent with this hypothesis, in BFA-treated roots at 23°C, PIN2 accumulated in numerous large bodies (BFA bodies) in both meristem and elongation zones; whereas in cold-stressed roots, few if any BFA bodies were formed (Figures 5E to 5H). To ensure the reproducibility of these observations, we quantified the number of BFA bodies, which confirmed their strong suppression in the cold and also showed that sensitivity was fully restored after a 9 h recovery at 23°C (see Supplemental Figure 3 online). Collectively, these results confirm our hypothesis that cold stress inhibits the basipetal transport of auxin by interfering with the trafficking of PIN2. However, this effect is a transient one, as the PIN2 cycling is restored after seedlings are returned to a nonstressful temperature.

Cold Stress Blocks the Early Phase of Gravity Response by Inhibiting the Lateral Relocalization of PIN3

PIN3, a member of the PIN family transmembrane efflux carriers, has been suggested to serve as a link between the early phase of root gravity response and asymmetric auxin redistribution (Friml et al., 2002b; Harrison and Masson, 2008). In vertically oriented roots, PIN3 exhibits a symmetrical localization at the plasma membrane of root statocytes, while upon gravistimulation, it readily relocalizes laterally to the bottom membrane and facilitates the establishment of a lateral auxin gradient across the root tip (for review, see Harrison et al., 2008). In addition, like PIN2, PIN3 has also been shown to cycle rapidly between the plasma membrane and undefined endosomal compartments in a BFA-sensitive manner (Friml et al., 2002b). To understand the possible involvement of PIN3 in cold stress–induced inhibition of gravity response, we compared the intracellular localization of PIN3 in control and cold-treated seedlings using a PIN3-GFP transgenic line (Laskowski et al., 2008). Consistent with previous results of Harrison and Masson (2008), we also found that upon gravistimulation, in ~50% of control roots, PIN3 relocalized to the bottom part of root columella cells (Figures 6A and 6B, Table 1). By contrast, in cold-treated seedlings, PIN3 relocalization was not observed in 80% of roots (Figures 6C and 6D, Table 1). Since the relocalization process has been suggested to be mediated by the rapid cycling of PIN3 (Friml et al., 2002b), we next investigated the effect of BFA on PIN3 trafficking in control and cold-stressed roots (Figures 6E and 6F). In contrast with PIN2, BFA treatment even at a higher concentration, failed to induce any BFA bodies in the control PIN3-GFP transgenic roots (cf. Figures 5E and 5F with Figure 6E). Instead, we observed a diffuse cytoplasmic signal of PIN3 in ~85% of BFA-treated control roots (Figure 6E, Table 2). On the other hand, in cold-treated seedlings, in 75% of roots, BFA treatment did not induce any diffused cytoplasmic signal (Figure 6F, Table 2), suggesting that cold stress possibly inhibits the rapid cycling of PIN3. Taken together, these results indicate that cold stress affects PIN3 relocalization by interfering its intracellular trafficking and thereby inhibits the early phase of gravity response in *Arabidopsis* roots.

Cold Stress–Induced Inhibition of Protein Trafficking Is Selective

To elucidate the specificity of cold stress–induced inhibition of PIN trafficking, we next investigated its effect on other protein trafficking pathways using three independent markers and the general endocytic tracer FM 4-64. For tracking endosomal motility, we used GFP-ARA7, which is a homolog of Rab5 GTPase and a widely used plant endosomal marker (Ueda et al., 2004); for Golgi trafficking, *N*-acetylglucosaminyl transferase fused to GFP (NAG-GFP) (Essl et al., 1999); and for membrane trafficking, low temperature inducible protein 6b fused to GFP (GFP-LTI6b; Kurup et al., 2005), which has been shown to cycle between the plasma membrane and endosome (Grebe et al., 2003; Jaillais et al., 2008). Like PIN2, these markers are known to be recruited to BFA bodies (Grebe et al., 2003). As expected, both endosomes (see Supplemental Movie 1 online; Figure 7, top panel) and Golgi (see Supplemental Movie 3 and Supplemental Figure 4 online) were highly dynamic at 23°C, which was captured in movies and visualized through superimposition of two frames, separated by 30 s. Cold stress considerably slowed endosomal movement (see Supplemental Movies 2 and 4 online; Figure 7, second panel) and to a lesser extent the

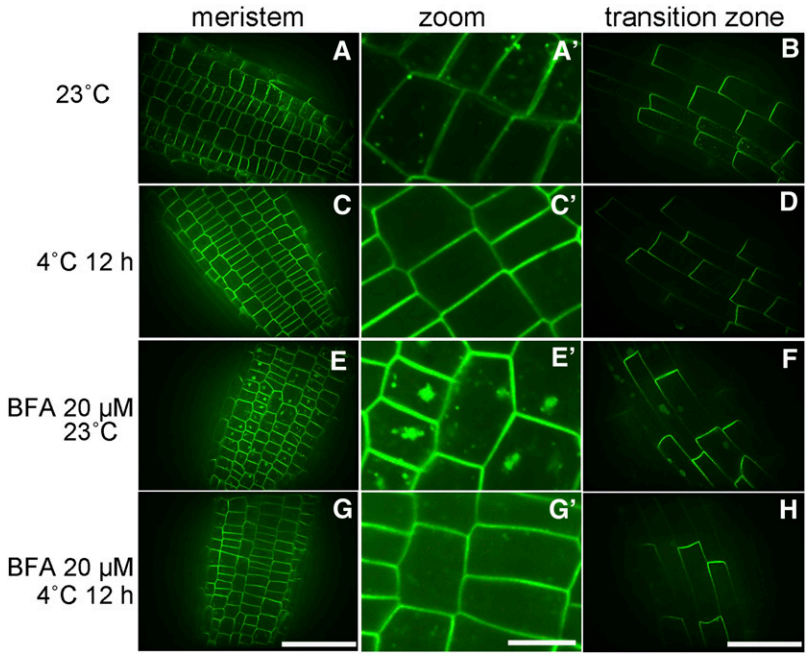

Figure 5. Cold Stress Affects the Intracellular Dynamic Cycling of PIN2 but Not Its Polar Localization.

Five-day-old PIN2:PIN2–GFP transgenic seedlings were treated at 4°C for 12 h. Seedlings grown at 23°C (**[A]**, **[B]**, **[E]**, and **[F]**) and cold-treated seedlings (**[C]**, **[D]**, **[G]**, and **[H]**). Roots treated with 20 μM BFA for 30 min (**[E]** to **[H]**). The images were captured using the same confocal setting and are representative of 40 roots obtained from at least five independent experiments. Middle panels (**[A']** to **[G']**) represent zoomed images of the left panels. Bars = 50 μm in **(A)** to **(H)** and 10 μm in zoomed images.
[See online article for color version of this figure.]

Golgi movements. Furthermore, during cold stress, the endosomal marker was recruited to BFA bodies only to a limited extent (Figures 7G to 7L; see Supplemental Figure 4 online). As with PIN2, endosomal and Golgi trafficking were restored almost to the normal level after 9 h of recovery at 23°C, as evidenced by the restoration of formation of BFA bodies (see Supplemental Figures 3 and 4 online). However, to our surprise, cold stress did not suppress the plasma membrane to endosomal trafficking of another plasma membrane protein LTI6b (Figure 8). Consistently, cold stress also failed to inhibit the recruitment of LTI6b

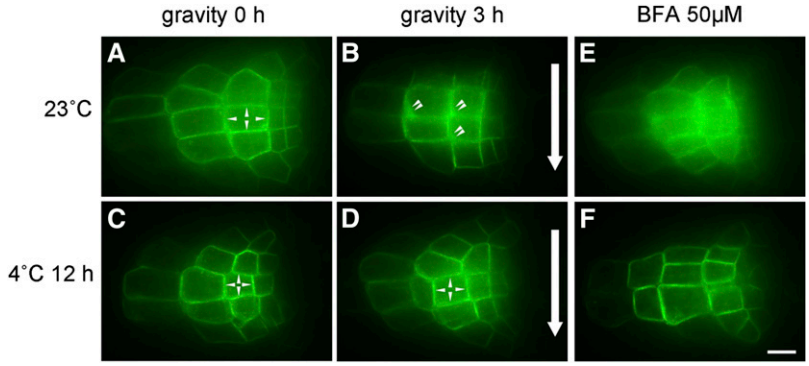

Figure 6. Cold Stress Inhibits the Lateral Relocalization and Intracellular Cycling of PIN3.

Five-day-old PIN3:PIN3–GFP transgenic seedlings were treated at 4°C for 12 h. Seedlings grown at 23°C (**[A]**, **[B]**, and **[E]**) and cold-treated seedlings (**[C]**, **[D]**, and **[F]**). After the cold treatment, gravity stimulation was provided to the roots by rotating the plates 90° at 23°C. The large arrows in the middle panel show the direction of gravity. PIN3 relocalization was imaged after 3 h of gravistimulation (**[B]** and **[D]**). Arrowheads indicate symmetric (**[A]**, **[C]**, and **[D]**) and lateral localizations **(B)** of PIN3. To observe the intracellular cycling of PIN3, control or cold-treated roots were treated with 50 μM BFA for 30 min (**[E]** and **[F]**). The images were captured using the same confocal setting and are representative of 20 roots obtained from three independent experiments. Bars = 10 μm.
[See online article for color version of this figure.]

Table 1. PIN3 Relocalization upon Gravistimulation in Control and Cold-Stressed Root Columella Cells

Temperature	Position	Number of Seedlings	Percentage Value
23°C	Lower[a]	14	53.8%
	No bias[b]	12	46.2%
4°C	Lower[a]	6	26.1%
	No bias[b]	17	73.9%

[a]PIN3 relocalized to the bottom membrane of columella cells.
[b]PIN3 showed no bias toward lower or upper sides of the columella cells.

into BFA bodies. Although this result is interesting and significant in proving the selective nature of cold stress–induced inhibition of protein trafficking, there is a potential problem in interpreting this experimental data as the GFP-LTI6b marker is expressed under a 35S constitutive promoter, as opposed to PIN2 and PIN3, which are expressed under their own promoters (Kurup et al., 2005; Xu and Scheres, 2005; Laskowski et al., 2008). Hence, the observed differences between PINs and LTI6b trafficking under cold stress could be attributed to differences in their construct promoters and basal expression levels. To address this concern, we investigated PIN2 trafficking under cold stress using a molecularly complemented transgenic line, *eir1-4* 35S:PIN2-GFP, where PIN2 is expressed under a 35S promoter (Abas et al., 2006). Consistent with the results observed for PIN2-GFP (Figure 5), cold stress also inhibited the PIN2 trafficking in the roots of *eir1-4* 35S:PIN2-GFP, as evidenced by the lack of formation of BFA bodies in presence of BFA (see Supplemental Figure 5 online). This result confirms that the differential trafficking responses observed under cold stress for PIN2 and LTI6b are not due to promoter artifact or increased basal expression.

In addition, we used a general endocytic tracer FM 4-64 (Bolte et al., 2004) to see the effect of cold stress on general endocytosis. Compared with control roots, we observed fewer endosomal bodies stained by FM 4-64 in cold-stressed roots (see Supplemental Figure 6 online). Consistently, in presence of BFA, a reduced number of BFA bodies was observed in cold-treated roots (see Supplemental Figure 6 online). Furthermore, we also identified a distinct difference in the size of the BFA bodies. In control roots, BFA treatment resulted in larger BFA bodies, but in cold-stressed roots, the BFA bodies were smaller in diameter (see Supplemental Figure 6 online). The apparent presence of general endocytic trafficking in cold-treated seedlings indicates that cold stress does not completely shut down all protein trafficking pathways. Collectively, these results indicate that immobilization of PIN2 or PIN3 during cold stress is not a global slowdown of trafficking but instead represents a selective process to regulate the activity of specific proteins.

Effect of Cold Stress on the Cytoskeleton

To assess the consequences of cold stress on the cytoskeleton, we investigated the organization of actin and microtubules in *Arabidopsis* root. To monitor actin, we first imaged living root cells expressing a GFP-tagged actin binding domain from fimbrin (GFP-ABD2-GFP; Wang et al., 2008). The cold stress did not induce any notable change in actin organization: long filamentous actin cables were observed both in the roots grown in optimum and cold-stressed conditions (Figure 9). This observation was complemented by imaging actin through chemical fixation followed by staining with an anti-actin antibody (see Supplemental Figure 7 online). The similar results observed by two independent approaches confirm that 12 h of cold treatment does not alter the root cellular actin organization.

We also investigated the effect of cold stress on microtubule organization, the other component of the cytoskeleton, by live cell imaging using a tubulin-GFP transgenic line. In contrast with actin, 12 h of cold stress severely disrupted cortical microtubules (Figure 10).

Considering the current proposed role of actin in plant endocytosis and PIN trafficking (Muday and Murphy, 2002; Grebe et al., 2003; Dhonukshe et al., 2008) and since cold stress slowed down the trafficking of several proteins, including PIN2, our observation that cold stress does not affect the actin organization is surprising. Recently, Dhonukshe et al. (2008) showed that the chemicals [such as 2,3,5 triiodobenzoic acid (TIBA) and 2-(1-pyrenoyl) benzoic acid (PBA)] that stabilize actin by inducing its bundling also slow down the endosomal and vesicle motility. Therefore, we compared the effect of TIBA and cold stress on endosomal motility. Consistent with Dhonukshe et al. (2008), we found that the movement of endosomal marker ARA7 was considerably slowed in TIBA-treated roots (see Supplemental Figure 8 and Supplemental Movies 5 and 6 online). The effect of 5 μM TIBA on endosomal movement was comparable to 12 h of cold stress (cf. Supplemental Movies 1, 2, and 5 online), and at 25 μM TIBA, there was no trace of endosomal movement (see Supplemental Figure 8 and Supplemental Movie 6 online). However, when we imaged actin, we could not reproduce the bundling effect of TIBA as reported earlier (Dhonukshe et al., 2008). In both live cell imaging and immunostaining methods, actin organization remained apparently unaffected (Figure 9; see Supplemental Figure 7 online).

Role of Membrane Rigidity in Root Gravity Response and PIN2 Trafficking

One of the earliest effects of an alteration in temperature on cells is a change in fluidity of cellular membranes (Levitt, 1980). A decrease in temperature increases the membrane rigidity

Table 2. Effect of BFA on Intracellular Cycling of PIN3 in Control and Cold-Stressed Roots

Temperature	Signal Type	Number of Seedlings	Percentage Value
23°C	Membrane localized[a]	3	15.8%
	Cytoplasmic[b]	16	84.2%
4°C	Membrane localized[a]	11	73.3%
	Cytoplasmic[b]	4	26.7%

[a]PIN3 localization is in the membrane of columella cells.
[b]PIN3 localization is in the cytoplasm.

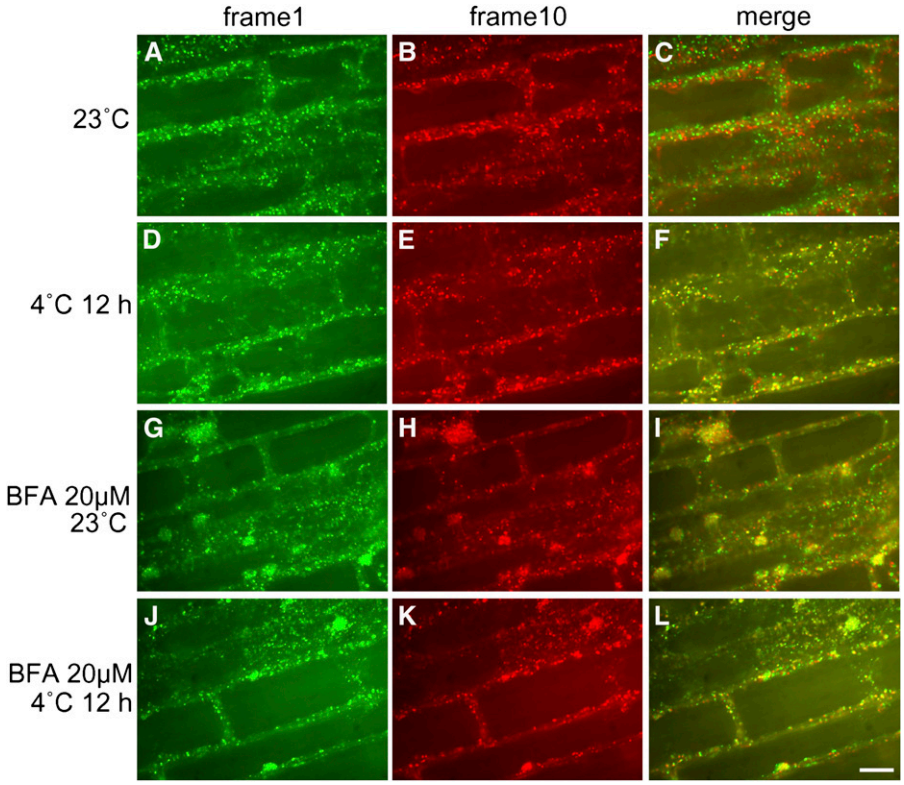

Figure 7. Effect of Cold Stress on Endosomal Movement.

Five-day-old GFP-ARA7 transgenic plants were cold stressed at 4°C for 12 h and subjected to real-time imaging. **(A)** to **(C)** show the trafficking in control plants at 23°C; **(D)** to **(F)** show the trafficking under cold stress. Effect of BFA on endosomal movement (**[G]** to **[I]**) grown at 23°C and (**[J]** to **[L]**) under cold stress. The roots were treated with 20 μM BFA for 30 min. Movies were captured for 30 s with 3-s time lapse between frames. Merged image is composed by superimposing frame 1 and frame 10. Color codes are green for frame 1 and red for frame 10. Images are representative of 20 roots obtained from three independent experiments. Bar = 10 μm.

(Alonso et al., 1997). To understand the significance of membrane rigidity in auxin response, we used the widely accepted membrane rigidifier DMSO (Lyman et al., 1976; Orvar et al., 2000). By analyzing the polarization index of alfalfa (*Medicago sativa*) protoplast cells, Orvar et al. (2000) showed that at 25°C, 3% DMSO effectively reduces the membrane fluidity. Therefore, we treated the *Arabidopsis* seedlings with 3% DMSO for 12 h at 23°C and then assayed root gravitropism and PIN2 trafficking, as described earlier. Although 3% DMSO treatment inhibited root elongation, it did not affect the root gravity response (Figures 11A and 11B). Consistently, it affected neither the intracellular PIN2 trafficking nor the PIN2-BFA body formation (Figure 11C). Furthermore, the preincubation in 3% DMSO for 12 h largely enhanced the inhibitory effect of cold pretreatment on root elongation, while this combination of treatments only slightly affected the root gravity response (see Supplemental Figure 9 online). These observations confirm the idea that root growth and gravity responses use partially distinct regulatory mechanisms, and a change in membrane fluidity preferentially targets one of these pathways. Collectively, these results suggest that the root gravity response and trafficking of PIN2 are not appreciably affected by membrane rigidification.

DISCUSSION

Auxin response plays a crucial role in regulating growth phases, ranging from embryo development to tropic responses during plant development (Davies, 1995; Benkova et al., 2003; Friml, 2003). However, comparatively little insight has been gained so far about the response of this hormone under temperature stress. Previous studies have shown that high temperature alters the plant's auxin response by altering its biosynthesis (Gray et al., 1998), whereas cold temperature affects the polar and lateral transport of auxin (Morris, 1979; Wyatt et al., 2002; Nadella et al., 2006). Although these studies demonstrate a link between auxin and temperature stress, the molecular and cellular mechanisms that regulate the auxin response under temperature stress, particularly for cold, remain elusive. By developing a new assay system and using *Arabidopsis* root gravity response as a model for auxin response, here we show that (1) cold stress affects the auxin response pathway in a reversible manner and primarily targets the auxin transport pathway instead of a signaling pathway, (2) the inhibition of auxin transport by cold stress is linked to the inhibition of intracellular trafficking of a subset of proteins, including auxin efflux carriers, and (3) membrane fluidity and

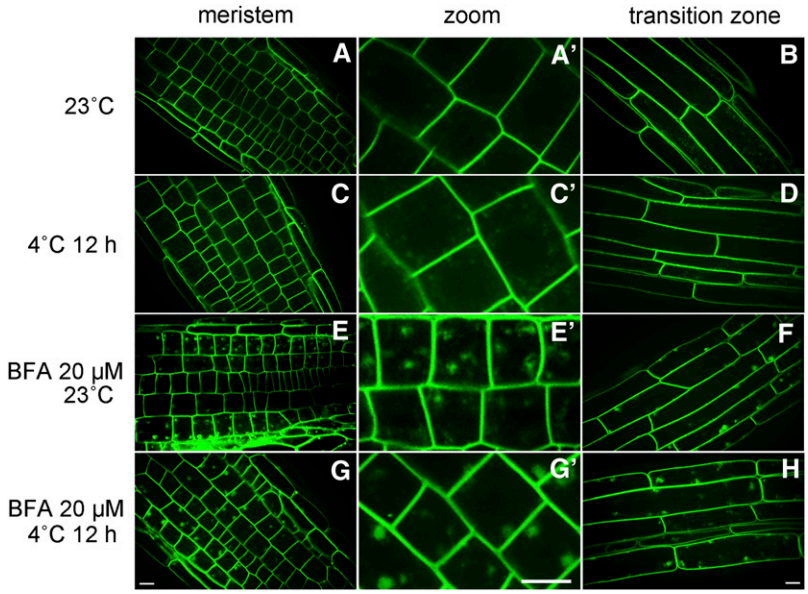

Figure 8. Effect of Cold Stress on the Intracellular Trafficking of PM Protein LTI6b.

Five-day-old EGFP-LTI6b seedlings were treated at 4°C for 12 h. **(A)**, **(B)**, **(E)**, and **(F)** show seedlings grown at 23°C, and **(C)**, **(D)**, **(G)**, and **(H)** show cold-treated seedlings. **(E)** to **(H)** show roots treated with 20 μM BFA for 30 min. Middle panels (**[A']**, **[C']**, **[E']**, and **[G']**) represent zoomed images of left panels. The images were captured using same confocal setting and are representative of 30 roots obtained from five independent experiments. Bars = 10 μm.

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actin play limited roles in mediating the cold- induced inhibition of protein trafficking.

Root growth and gravity response are tightly regulated by intracellular auxin response. For instance, accumulation of IAA in the root inhibits elongation by affecting the residence time of the cells in the elongation zone (Rahman et al., 2007). Similarly, a failure to make a proper auxin gradient across the meristem in response to gravity alters the root gravity response (Rashotte et al., 2001; Sukumar et al., 2009; this work). Physiological and molecular analyses revealed that our assay condition (12 h at 4°C) induced the ectopic accumulation of auxin in the root meristem (Figures 1 and 3). The transport assay further confirmed that this accumulation of auxin in the meristem is due to the inhibition of basipetal polar transport of auxin (Figure 4). This increased concentration of auxin inhibited both growth and gravity responses. The idea that the ectopic auxin concentration alters growth and gravitropism is further substantiated by the fact that both processes tended to return to normal with the restoration of auxin efflux (Figures 3 and 4). In addition, the wild-type response of auxin signaling mutants *axr1-3* and *tir1-1* to cold stress confirmed that cold stress does not primarily target auxin signaling. These results are consistent with previous findings showing that the *Arabidopsis* inflorescence failed to respond to gravity during cold treatment but responded normally to gravity when returned to room temperature, albeit with a lag period (Fukaki et al., 1996; Wyatt et al., 2002).

Auxin moves through plants by a unique cell-to-cell polar transport mechanism from the shoot meristem and young leaves down the length of the stem eventually reaching to the tip of the root (for review, see Muday and Rahman, 2008). The polar transport of auxin is regulated by a family of proteins that facilitate cell-to-cell efflux of auxin (for review, see Benjamins et al., 2005; Vieten et al., 2007). Compared with inflorescence, the auxin transport in root is more complex as two distinct transport pathways are functional. Shoot-derived IAA moves acropetally (toward the root apex) through the central cylinder and is regulated by auxin efflux carriers, such as PIN1 and PIN4 (Geldner et al., 2001; Friml et al., 2002a) Shoot-derived IAA also moves basipetally (from the apex toward the base) through the outer layers of root cells and is regulated by another efflux carrier PIN2 (Tsurumi and Ohwaki, 1978; Luschnig et al., 1998; Muller et al., 1998; Chen et al., 1998). This basipetal transport of auxin is particularly important for root gravitropism as any alteration of this flow, either by genetic or chemical means, affects gravitropism (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Rashotte et al., 2000). In addition to basipetal transport of auxin, formation of an asymmetric auxin gradient across the root meristem is also important for root gravity response (for review, see Muday and Rahman, 2008).

A current hypothesis suggests that the PIN3, a member of auxin efflux carrier family functions as a molecular link between early gravity perception and auxin redistribution in roots by relocalizing itself to the bottom membrane of root statocytes upon gravistimulation (Friml et al., 2002b). However, recent observations suggest that this may be partially true (Harrison and Masson, 2008; this work). In these studies, PIN3 relocalization was observed only in 50% of roots after gravistimulation, indicating that PIN3 may function as a minor player in redirecting

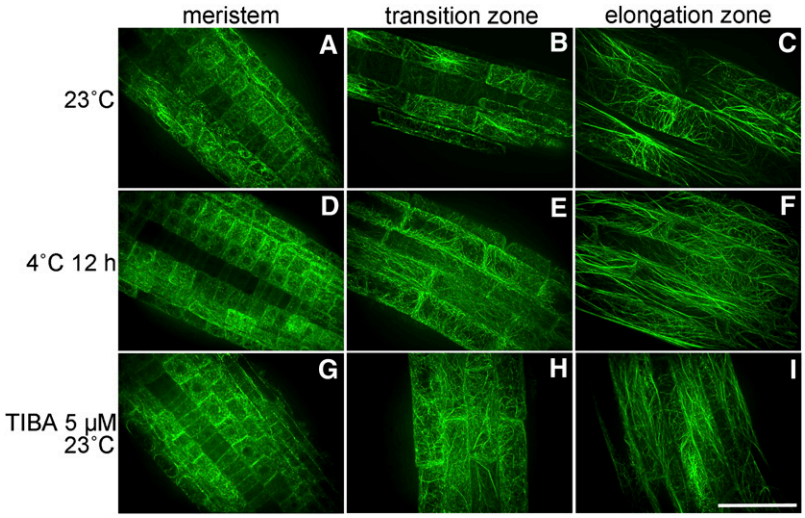

Figure 9. Effect of Cold Stress on Actin Cytoskeleton Structure.

Five-day-old GFP-ABD2-GFP transgenic seedlings were subjected to cold treatment at 4°C for 12 h. Seedlings grown at 23°C (**[A]** to **[C]**), cold-treated seedlings (**[D]** to **[F]**), and seedlings treated with 5 μM TIBA for 30 min at 23°C (**[G]** to **[I]**). The images were captured using same confocal setting and are representative of 30 roots obtained from at least four independent experiments. Images are projections of 10 to 12 optical sections. Bar = 50 μm.
[See online article for color version of this figure.]

auxin. In addition, the weak gravitropic phenotype of *PIN3* null alleles (Harrison and Masson, 2008) suggests that auxin redistribution in the root meristem may be mediated by proteins in addition to PIN3. In fact, ARL2 and ARG1 have been suggested to act in concert with PIN3 to link the gravity sensation in the root statocytes to auxin redistribution through root cap (Harrison and Masson, 2008). Nevertheless, we found that cold stress blocked the relocalization of PIN3 in approximately half of the roots where relocalization exists, indicating that PIN3 relocalization in statocytes is susceptible to cold stress (Figure 6, Table 1).

For basipetal transport, recent molecular and cellular findings suggest that the polar deployment of PIN2 and the constitutive cycling of this protein from membrane to endosomes are required for the proper functionality (Paciorek et al., 2005; Sukumar et al., 2009). The constitutive cycling of PIN2 has been inferred by its sensitivity to the protein trafficking inhibitor, BFA, which captures the PIN2 in an unknown intracellular compartment, popularly called the BFA compartment (Geldner et al., 2001; Paciorek et al., 2005). Our observations that cold stress inhibits the basipetal transport of auxin and the formation of an asymmetric auxin gradient (Figures 3 and 4) could in principle be explained by its effect on either or both of these processes. The live cell imaging of PIN2 and comparison of formation of BFA bodies between stressed and control roots suggest that cold stress does not alter the polarity of PIN2 proteins but inhibits their cycling. Based on these observations, we hypothesize that the loss of dynamic cycling of PIN2 results in a reduced basipetal transport of auxin and diminishes the root's capability to form an auxin gradient.

This hypothesis is consistent with recent findings showing that reduced basipetal transport of auxin and slower gravitropism occur in *pid-9* mutants and in wild-type plants treated with the protein kinase inhibitor, staurosporine, along with a stimulation of the rate of PIN2 cycling (Sukumar et al., 2009). The role of this dynamic cycling in regulating PIN activity may be multiple. PIN cycling may facilitate the rapid change in PIN polarity, such as relocalization of PIN3 after gravity stimulation (Friml et al., 2002b), or it may provide a mechanism by which it can control its occurrence in PM and regulates its own efflux (Paciorek et al., 2005). Our results suggest that severe reduction in intracellular cycling reduces auxin efflux, even though more proteins are

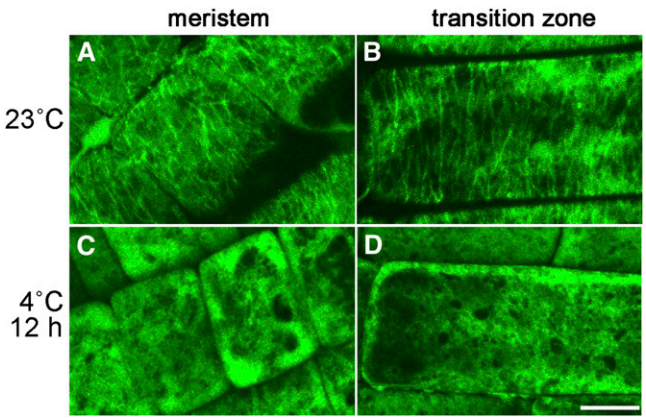

Figure 10. Effect of Cold Stress on Microtubule Cytoskeleton Structure.

Five-day-old TUB6-GFP transgenic seedlings were incubated at 23°C or cold stressed at 4°C for 12 h and imaged using the same confocal setting. The images are representative of 30 seedlings obtained from three independent experiments. Seedlings grown at 23°C (**[A]** and **[B]**) and cold-treated seedlings (**[C]** and **[D]**). Images are projections of 10 to 12 optical sections. Bar = 10 μm.
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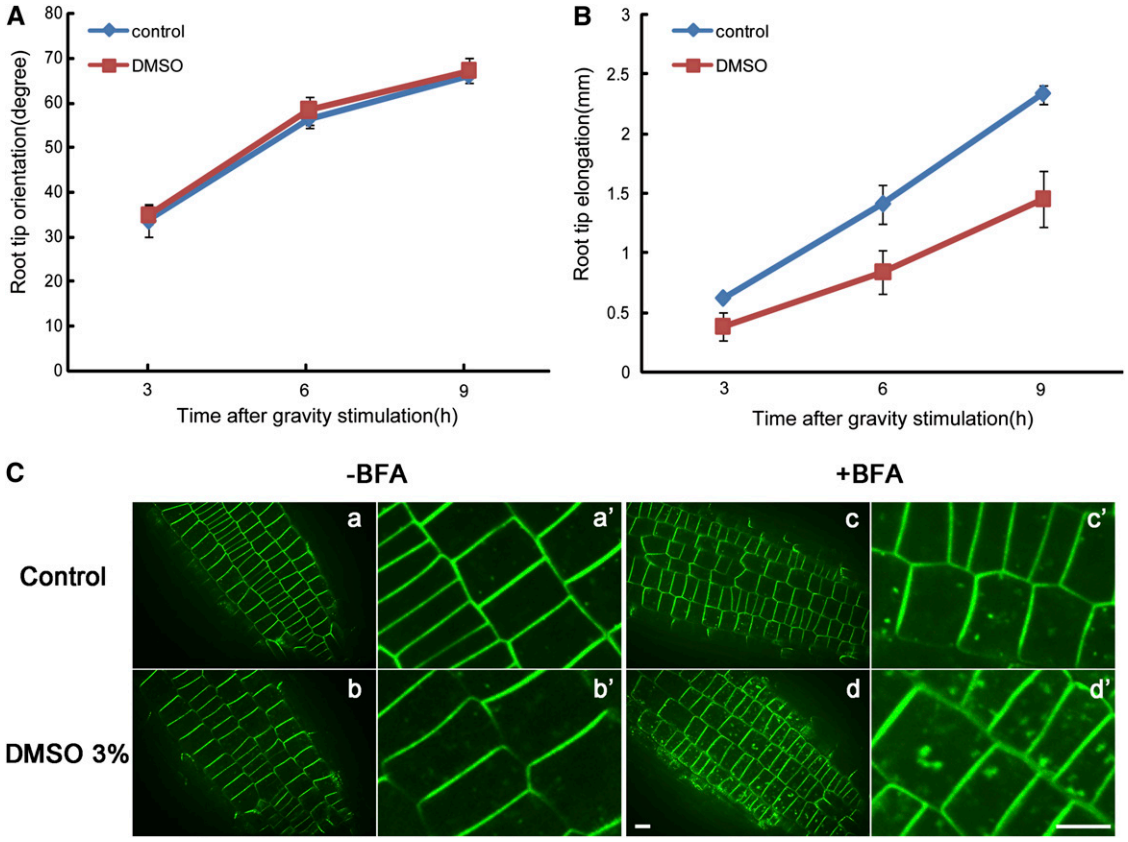

Figure 11. Effect of Membrane Rigidification on Root Gravity Response and PIN2 Cycling.

Five-day-old Columbia-0 seedlings were transferred to new agar plates containing 3% DMSO for 12 h at 23°C. After the DMSO treatment, seedlings were transferred to new agar plates without DMSO, and gravity response and root elongation were measured as described in Figure 1 **[A]** and **[B]**. Five-day-old PIN2:PIN2-GFP seedlings were treated with or without 3% DMSO for 12 h (**[a]** and **[b]**). Control and 12-h 3% DMSO–treated seedlings were treated with 20 μM BFA for 30 min (**[c]** and **[d]**). The images were captured using same confocal setting and are representative of 40 roots obtained from at least five independent experiments. Right panel (**[a']** to **[d']**) represents zoomed images of left panel. Bars = 10 μm.

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

(B) Root elongation. Vertical bars represent mean ± SE. Data are from three independent experiments with 20 seedlings per experiment.

(C) The intracellular trafficking of PIN2 protein in DMSO treatment was investigated using PIN2:PIN2–GFP transgenic plants.

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present in the membrane. Similarly, an increase in cycling also diminished efflux activity (Sukumar et al., 2009), possibly because some time is required for PIN2 to form regulatory associations with accessory proteins required for function. Taken together, these results suggest that proper intracellular cycling of PIN2 is essential for its optimal functionality.

Since low temperature typically inhibits enzymatic activity, the question remains open whether the effect of cold stress on auxin efflux carriers reflects a nonspecific metabolic slowdown. Although cold stress inhibited endosomal movement to a great extent and Golgi trafficking to a lesser extent, it exerted no effect on the trafficking of a low temperature–induced membrane protein, LTI6b (Figures 7 and 8). In addition, although less compared with control, general endocytosis process seemed to be active under cold stress (see Supplemental Figure 6 online). The inability of the cold stress to inhibit the trafficking of LTI6b or completely shut down the general trafficking pathways make it unlikely that the observed loss of PIN2 cycling is linked to generalized loss of enzymatic activity.

Another factor that might influence the cycling of proteins is membrane fluidity. It has been shown that a decrease in temperature lowers membrane fluidity and an increase enhances it (Mejia et al., 1995; Alonso et al., 1997). The rigidification of membranes could result in less trafficking and less BFA body formation by reducing the ability to form vesicles and allow their fusion. We provide several lines of evidence against this scenario. First, the contrasting effects of cold stress on the trafficking of a group of plasma membrane proteins, PIN2, PIN3, and LTI6b. Like PINs, LTI6b rapidly cycles between plasma membrane and endosomal compartments in a BFA-sensitive manner (Grebe et al., 2003). However, cold stress selectively inhibited the cycling of PIN2/PIN3 but not that of LTI6b (Figures 5, 6, and 8). Second, the uptake capacity of the membrane was not altered by cold stress, as evidenced by the unaffected uptake of auxin

structural analog BA (see Supplemental Figure 2 online). Finally, the membrane rigidifier DMSO did not affect the PIN2 cycling or gravity response, although it inhibited root elongation (Figure 11). DMSO treatment has been shown to rigidifiy membranes and mimics the effects of cold on gene expression, calcium influx, and cold acclimatization (Orvar et al., 2000). Taken together, we suggest selective inhibition of protein trafficking by cold stress is not linked to membrane rigidification.

The dynamic cycling of PIN proteins as well as their polarized localizations have been widely speculated to be mediated by actin (Muday and Murphy, 2002; Blakeslee et al., 2005). This speculation came from several observations, including the ability of cytochalasin to prevent BFA-induced mislocalization of PIN1 (Geldner et al., 2001), and the actin-sensitive relocalization of PIN3 during tropisms (Friml et al., 2002b). In a recent article, Dhonukshe et al. (2008) hypothesized a role for actin-based vesicle trafficking in the mechanism of polar auxin transport, which they supported by showing that some auxin transport inhibitors, such as TIBA and PBA, inhibit endosomal movement and cause actin filaments to bundle in plant, yeast, and animal cells. Increases in bundle length and thickness suggest that actin is stabilized through reactions favoring polymerization and lateral association over depolymerization and dissociation. This change in actin dynamicity was hypothesized to inhibit vesicle movement, including PIN cycling. If this hypothesis is correct, then the simplest model to explain the effect of cold stress on subcellular trafficking would be through its effect on actin dynamicity.

To test this hypothesis, we imaged actin by both live cell imaging and immunostaining (Figure 9; see Supplemental Figure 7 online) and found no indication of enhanced bundling under cold stress, although endosomal movement, PIN dynamics, and microtubule structure were all severely disrupted (Figures 5, 6, 8, and 9). Likewise, although TIBA inhibited the endosomal movement (see Supplemental Figure 7 and Supplemental Movies 5 and 6 online; Dhonukshe et al., 2008), it did not induce actin bundling even at the highest concentration we tested (see Supplemental Figure 7 online). The actin stabilization is directly linked to bundle thickness and bundle dynamicity. Although we did not observe any significant change in actin bundle thickness under cold stress or short-term TIBA treatment, we cannot rule out the possibility that the dynamicity of the actin bundle might have changed. Nevertheless, our results suggest that actin plays a limited role in suppressing the trafficking of PIN2 and ARA7 in the cold-stressed *Arabidopsis* root.

The role of actin in directing the PIN proteins is also questionable. Classic experiments have shown that cytochalasin at a concentration sufficient to stop streaming had no effect on the rate of polar transport in either grass coleoptiles or pea (*Pisum sativum*) epicotyls, although it caused a lag in uptake (Cande et al., 1973). More recently, it was shown for the *Arabidopsis* root meristem that even in the absence of actin, PIN2 retained its apical (shootward) and basal (rootward) localization in epidermal and cortical cells (Rahman et al., 2007); and Dhonukshe et al. (2008) showed that the stabilization of actin by TIBA did not affect the polar localization of PIN2. Consistently, cold stress, although inhibiting the dynamic cycling of PIN2, neither affected the polar localization of PIN2 nor actin structure. Taken together, these results suggest that polar deployment of PIN2 is independent of actin.

Information concerning low-temperature effects on actin organization is scarce and contradictory. For instance, in a single-cell system, cold stress disrupts actin organization (Egierszdorff and Kacperska, 2001; Pokorna et al., 2004), whereas chilling temperatures were found to have no effect on the arrangement of actin filament bundles in onion bulb cells (Quader et al., 1989). In addition, it has also been shown that cold acclimatization greatly enhances the resistance of winter oilseed rape (*Brassica napus*) actin filaments to cold-induced degradation (Egierszdorff and Kacperska, 2001). In this work, we did not observe any significant change in root actin organization after 12 h of cold treatment. This could be due to a species effect or might be related to the length of the treatment. Prolonged cold incubation might disrupt actin structure in the *Arabidopsis* root. On the other hand, consistent with previous reports (Sakiyama and Shibaoka, 1990; Baluska and Barlow, 1993; Lazareva et al., 2008), we found that cold stress severely disrupts the microtubule structure (Figure 10). Given these results, it is interesting to wonder whether microtubules play a role in PIN trafficking. In fact, a recent report indicated that intact microtubules are required for polar trafficking in plant cells (Kleine-Vehn et al., 2008).

Although in yeast, both mutational and cell biological approaches have provided direct evidence that regulated assembly of actin filament network is crucial for endocytic process (for review, see Galletta and Cooper, 2009), such evidence is lacking for plants. Our current understanding about the involvement of actin in plant protein trafficking largely comes from studies where inhibitors are used at high concentrations, potentially eliciting nonspecific effects. For example, Kleine-Vehn et al. (2006) reported that latrunculin mislocalized PIN1 in *Arabidopsis* roots at 30 μM but not at 20 μM; nevertheless, in this material, 1 μM latrunculin is all but saturating for degrading the actin filaments (Rahman et al., 2007). Hence, the effect of high concentration of latrunculin on PIN localization could be due to simple toxicity, and these data should be interpreted with additional caution. In this regard, our newly developed assay system with cold stress can be used as an alternative approach to study the cellular mechanism of protein trafficking as it selectively inhibits the intracellular trafficking, represents a natural environmental stress, and completely avoids the use of chemical inhibitors.

In conclusion, our observations provide new insights into the auxin response pathway under cold stress. We have shown that cold stress affects the polar transport of auxin by selectively inhibiting the intracellular trafficking of a pool of proteins that includes the auxin efflux carriers. We also provide evidence that membrane fluidity and the actin cell cytoskeleton play limited roles in cold induced inhibition of protein trafficking.

METHODS

Plant Materials and Growth Conditions

All lines, except EGFP-LTI6b (C24 background), are in the Columbia background of *Arabidopsis thaliana*. The transgenic GFP-ABD2–GFP (Wang et al., 2008) was the gift of E.B. Blancaflor (Samuel Roberts Noble Foundation, Ardmore, OK), the PIN2-GFP line (Xu and Scheres, 2005), was the gift of B. Scheres (University of Utrecht, The Netherlands), GFP-ARA7 (Ueda et al., 2004) was a gift of T. Ueda (University of Tokyo, Japan),

eir1-4 35S-PIN2-GFP (Abas et al., 2006) was a gift of C. Luschnig (University of Natural Resources and Applied Life Sciences, BOKU, Wien, Austria), and NAG-GFP (Essl et al., 1999) was a gift from C. Hawes (Oxford Brookes University, Oxford, UK). EGFP-LTI6b (Kurup et al., 2005), and PIN3-GFP (Laskowski et al., 2008) were provided by G. Muday (Wake Forest University, Winston-Salem, NC), and the β-tubulin6-GFP (Bannigan et al., 2006) was provided by T.I. Baskin (University of Massachusetts, Amherst, MA). Columbia-0, *axr1-3*, and *tir1-1* 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 ~100 μmol m^{-2} s^{-1}. The seedlings were grown vertically for 5 d. For cold treatment, 5-d-old seedlings were transferred to a 4°C growth chamber (NK System; LH-1-120.S) and incubated for various time lengths under continuous white light at an irradiance of ~100 μmol m^{-2} s^{-1}.

Chemicals

IAA and BFA were purchased from Sigma-Aldrich Chemical Co. FM 4-64 was purchased from Invitrogen. [^{3}H] IAA (20 Ci $mmol^{-1}$) was purchased from American Radiolabeled Chemicals. The [ring-^{14}C] Benzoic acid (60 mCi $mmol^{-1)}$ was purchased from Moravek Biochemicals. Other chemicals were from Wako Pure Chemical Industries.

Root Tip Reorientation Assay

Root tip reorientation assay was performed as described earlier (Rahman et al., 2001). Briefly, 5-d-old vertically grown seedlings were incubated at 4°C for various times. Immediately after the cold incubation, the roots were gravistimulated at 23°C by reorienting the plate 90°. To measure the curvature 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 by image analyzing software Image J.

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 a new agar plate and grown vertically at 4 or 23°C under continuous white light. After 12 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 for1 h. To observe the effect of exogenous auxin on GUS expression, the control and cold-treated seedlings were incubated in 1 μM IAA for 30 min and subjected to GUS staining as described above. The cells 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 and 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 in 40, 20, and 10% ethanol for 5 min and vacuum infiltrated for 15 min in 5% ethanol and 25% glycerol. The roots were imaged with a light microscope (Nikon Diaphot) equipped with a digital camera control unit (Digital Sight [DS-L2]; Nikon).

To image actin, we used the protocol described earlier by Rahman et al. (2007). Five-day-old *Arabidopsis* seedlings were transferred to new agar plates and incubated either at 23 or 4°C for 12 h. After the 12-h incubation period, roots were immediately fixed in PIPES buffer (50 mM PIPES, 4% paraformaldehyde, 1.2% glutaraldehyde, 5 mM $CaCl_2$, 0.4 mM maleimidobenzoyl-*N*-hydroxy succinimide), permeabilized in PME buffer (50 mM PIPES, 5 mM EGTA, and 2 mM $MgSO_4$), Triton, and cold methanol, successively, and subjected to brief cell wall digestion with pectinase (0.01% w/v) and pectolyase (0.001% w/v). The primary antibody was a mouse monoclonal anti-(chicken gizzard) actin (C4; Chemicon) diluted 1:200 in PBS, 1% BSA, and 0.01% sodium azide (PBA) and the secondary was Cy-3-goat anti-mouse IgG (1:200; Jackson Immunoresearch). To observe the effect of TIBA on actin, 5-d-old *Arabidopsis* seedlings were incubated in 5 or 25 μM TIBA for 30 min and subjected to immunostaining as described above. The imaging was done on a spinning-disc confocal microscope (Olympus BX-61 equipped with Bx-DSU) equipped with a ×60 oil immersion objective.

For live cell microscopy, 5-d-old GFP transgenic seedlings were used. For BFA treatment, 5-d-old control or cold-stressed seedlings were incubated in 20 or 50 μM BFA for 30 min. The roots were imaged using a confocal laser microscope (Olympus BX-61 equipped with confocal section unit) equipped with a ×60 oil immersion objective. To minimize the effect of room temperature during imaging, the imaging of cold-treated seedlings was completed within 30 min.

Auxin Transport Assay

Five-day-old vertically grown *Arabidopsis* seedlings were transferred to agar plates and incubated at 23°C or at 4°C for 12 h. For measuring root basipetal auxin transport, a 2-mm-wide strip of Parafilm was placed on the surface of the 2% agar plates containing MOPS buffer solution (5 mM MOPS, 5 mM KNO_3, 2 mM $CaNO_3$, 2 mM $MgSO_4$, and 1 mM KH_2PO_4, pH 6.7). The seedlings were transferred to the agar plate with the root tip aligned on the top of the parafilm strip. A donor block was prepared by mixing 5 μM [^{3}H] IAA (3.7 MBq mL^{-1}) in 1.5% agar containing MES buffer solution (5 mM MES, 5 mM KNO_3, 2 mM $CaNO_3$, 2 mM $MgSO_4$, and 1 mM KH_2PO_4, pH 5.7). The donor block was cut into 1 mm^3 and placed on the edge of the parafilm strip so that the agar block just touched the root tip of the seedlings. Plates were then incubated vertically under nearly saturating humidity for 1 h. To measure auxin transport, 5-mm root segments away from the apical 2 mm were carefully cut and soaked overnight in 5 mL of liquid scintillation fluid (ACSII; Amersham Biosciences), and the radioactivity was measured with a scintillation counter (model LS6500; Beckman ACSII USA Instruments). Eight root segments were used per count.

Uptake Assay

Five-day-old vertically grown *Arabidopsis* seedlings were transferred to 23 or 4°C for 12 h. After the incubation, root tips of 3 mm in length were excised and placed on nylon mesh (1 cm^2) with 250-μm openings. The nylon mesh containing 10 root tips was transferred to a 2.6-cm Petri dish on a piece of filter paper of 1.2 cm^2 (Advantec No. 2) wetted with 75 μL of MES buffer solution supplemented with 2 μM [ring-^{14}C^1]BA (4.4 KBq mL^{-1}) and incubated for 1 h under nearly saturating humidity. After incubation, the root tips were carefully transferred to a 2.6-cm Petri dish containing 3 mL of buffer without labeled compound and washed for 2 min with gentle shaking, three times. The root tips were then soaked for overnight in 5-mL liquid scintillation fluid (ACSII; Amersham Biosciences), and the radioactivity was measured with a scintillation counter.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AXR1 (At1g05180), TIR1 (At3g62980), PIN2 (At5g57090), PIN3 (At1g70940), LTI6B (At3g05890), ARA7 (At4g19640), NAG (At4g38240), IAA2 (At3g23030), TUB6 (At5g12250), and FIMBRIN (At4g26700).

Supplemental Data

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

Supplemental Figure 1. Kinetics of Root Tip Orientation and Root Elongation at Early Time Points of Recovery.

Supplemental Figure 2. Comparison of Uptake of Benzoic Acid in Control and Cold-Treated Roots.

Supplemental Figure 3. Quantitative Analysis of Formation of PIN2-BFA Body in the Transition Zone of PIN2-PIN2–GFP Transgenic Plants in Presence or Absence of Cold Stress.

Supplemental Figure 4. Quantitative Analysis of Effect of BFA on Endosome and Golgi Markers.

Supplemental Figure 5. Cold Stress Inhibits the Intracellular Dynamic Cycling of PIN2 in the Transgenic Line Where PIN2 Is Expressed under the 35S Promoter.

Supplemental Figure 6. Effect of Cold Stress on General Endocytosis Traced by Styryl Dye FM 4-64.

Supplemental Figure 7. Effect of Cold Stress and TIBA on Actin: Chemical Fixation.

Supplemental Figure 8. Effect of TIBA on Endosomal Movement.

Supplemental Figure 9. Effect of DMSO Preincubation on Root Elongation and Gravity Responses of Cold-Stressed Seedlings.

Supplemental Movie 1. Dynamics of Endosome in *Arabidopsis* Root Cells.

Supplemental Movie 2. Dynamics of Endosome in *Arabidopsis* Root Cells.

Supplemental Movie 3. Dynamics of Golgi in *Arabidopsis* Root Cells.

Supplemental Movie 4. Dynamics of Golgi in *Arabidopsis* Root Cells.

Supplemental Movie 5. Dynamics of Endosome in *Arabidopsis* Root Cells.

Supplemental Movie 6. Dynamics of Endosome in *Arabidopsis* Root Cells.

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