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P-Glycoprotein4 Displays Auxin Efflux Transporter–Like Action in *Arabidopsis* Root Hair Cells and Tobacco Cells [W] [OA]

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ATP binding cassette (ABC) transporters transport diverse substrates across membranes in various organisms. However, plant ABC transporters have only been scantily characterized. By taking advantage of the auxin-sensitive *Arabidopsis thaliana* root hair cell and tobacco (*Nicotiana tabacum*) suspension cell systems, we show here that *Arabidopsis* P-glycoprotein4 (PGP4) displays auxin efflux activity in plant cells. Root hair cell–specific overexpression of PGP4 (PGP4ox) and known auxin efflux transporters, such as PGP1, PGP19, and PIN-FORMEDs, decreased root hair elongation, whereas overexpression of the influx transporter AUXIN-RESISTANT1 enhanced root hair length. PGP4ox-mediated root hair shortening was rescued by the application of auxin or an auxin efflux inhibitor. These results indicate that the increased auxin efflux activity conferred by PGP4 reduces auxin levels in the root hair cell and consequently inhibits root hair elongation. PGP4ox in tobacco suspension cells also increased auxin efflux. PGP4 proteins were targeted to the plasma membrane of *Arabidopsis* root hair cells and tobacco cells without any clear subcellular polarity. Brefeldin A partially interfered with the trafficking of PGP4 reversibly, and this was rescued by pretreatment with auxin. These results suggest that PGP4 is an auxin efflux transporter in plants and that its trafficking to the plasma membrane involves both BFA-sensitive and -insensitive pathways.

INTRODUCTION

Plant ABC transporters constitute a large gene family, which in *Arabidopsis thaliana* is composed of 131 members (Theodoulou, 2000; Sánchez-Fernández et al., 2001; Martinoia et al., 2002; Jasinski et al., 2003). These transporters have been implicated in diverse physiological functions, such as xenobiotic detoxification (Thomas et al., 2000; Ito and Gray, 2006), ion regulation (Gaedeke et al., 2001; Suh et al., 2007), heavy metal tolerance (Lee et al., 2005), stomatal regulation (Gaedeke et al., 2001; Klein et al., 2004), disease resistance (Urban et al., 1999; Campbell et al., 2003), lipid catabolism (Pighin et al., 2004), and plant growth (Sidler et al., 1998; Noh et al., 2001; Multani et al., 2003; Geisler et al., 2005). Although we now possess considerable accumulative information about the genome-wide organization and phylogenetic relationship of plant ABC transporters, as well as their physiological roles, little is known about the biochemistry of ABC transporters, such as their substrate specificities.

Certain developmental roles of plant ABC transporters are associated with their auxin-transporting activities. The long-distance transport of auxin occurs either through phloem or in a cell-to-cell manner. The intercellular transport of auxin requires transmembrane transporters (also called carriers or permeases; hereafter, they will be referred to as transporters) whose polarized localization in certain cell types gives rise to the directional flow of auxin and the generation of local auxin gradients. Bacterial amino acid transporter–like AUXIN-RESISTANT1 (AUX1) and its homolog LIKE-AUX1 are known as auxin influx transporters, and PIN-FORMED (PIN) proteins are known as auxin efflux transporters (for recent reviews, see Leyser, 2006; Teale et al., 2006; Cho et al., 2007; Kerr and Bennett, 2007; Vieten et al., 2007). Several plant ABC transporters have been implicated in intercellular auxin transport. *Arabidopsis* multidrug resistant–related protein5 is thought to transport auxin conjugates (Gaedeke et al., 2001), and maize (*Zea mays*) P-glycoprotein1/Brahytic2 (PGP1/BR2) and its sorghum (*Sorghum bicolor*) ortholog, which are probable orthologs of *Arabidopsis* PGP1, are involved in polar auxin transport (Multani et al., 2003). However, only three *Arabidopsis* PGPs, PGP1, PGP19, and PGP4, have been biochemically characterized (Geisler and Murphy, 2006), and all three of them can bind to the auxin efflux inhibitor 1-*N*-naphthylphthalamic acid (NPA) with high affinity (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2003; Terasaka et al., 2005). PGP1 and PGP19 showed auxin efflux activities in plant, yeast, and animal cells (Geisler et al., 2005; Bouchard et al., 2006; Petrášek et al., 2006; Blakeslee et al., 2007), and auxin influx activity for PGP4 was observed in yeast and animal cells (Santelia et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007). A recent study also demonstrated that PGPs interact physically with PINs to modulate polar auxin transport, transport specificity, and directionality (Bandyopadhyay et al., 2007; Blakeslee et al., 2007).

Unlike PGP1 and PGP19, however, the biochemical activity of PGP4 has been demonstrated only in yeast or animal cells.

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Expression of PGP4 in animal HeLa cells enhanced the retention of radiolabeled indole-3-acetic acid (IAA) inside the cells (Terasaka et al., 2005). In yeast cells, the introduction of PGP4 conferred an IAA hypersensitivity to an IAA-sensitive *yap1-1* mutant and increased the sensitivity of the JK93da strain to the auxin analog 5-fluoro-IAA (Santelia et al., 2005). These results indicate that PGP4 has auxin influx activity in yeast and animal cells. In another heterologous expression study using HeLa cells, coexpression of PGP4 with AUX1 additively enhanced auxin influx, whereas the coexpression of PGP4 with the auxin efflux transporter PIN2 synergistically enhanced PGP4-mediated auxin influx (Blakeslee et al., 2007). This latter result implies that heterologous systems may not always furnish the same molecular environments for plant-origin membrane transporters. It has been observed that plant proteins often are nonfunctional or mislocalized in heterologous expression systems, which has resulted in their attribution of molecular or cellular functions that they do not have in plants (Bassham and Raikhel, 2000; Noh et al., 2001; Geisler and Murphy, 2006).

In the *Arabidopsis* root, PGP4 is expressed mainly in the outer tissues, such as the lateral root cap, epidermis, and cortex, and loss-of-function *pgp4* mutants show several root phenotypes, including longer root hairs (Santelia et al., 2005; Terasaka et al., 2005; Lewis et al., 2007). The root hair is an outgrowth of the root epidermal cell. In a recent study, we demonstrated that root hair elongation can be used as a biological marker to study the activity of auxin transporters (hereafter referred to as the root hair model) (Lee and Cho, 2006; Cho et al., 2007). Auxin is a well-defined positive effector for root hair elongation (for review, see Okada and Shimura, 1994; Schiefelbein, 2000). The root hair model for cellular auxin transport relies on the simple fact that auxin transporter-mediated changes in intracellular auxin levels can influence root hair elongation: shorter root hairs by the enhanced auxin efflux (or reduced influx) activity, and longer root hairs by the enhanced auxin influx (or reduced efflux) activity. For example, overexpression of PIN3 (the auxin efflux transporter) or PINOID (the positive regulator of PINs) in the root hair cell greatly reduced root hair length (Lee and Cho, 2006). As far as they are major auxin transporters in the root hair cell, the effects of the loss of those auxin transporters also can be explained by the root hair model. Within the context of the root hair model, the maintenance of strong PGP4 expression in root hair cells and the enhancement of root hair elongation following the loss of PGP4 might imply that PGP4 catalyzes auxin efflux in root hair cells.

In this study, we tested the directionality of PGP4-mediated cellular auxin transport by taking advantage of the root hair cell system. We analyzed the effect of overexpression of PGP4 and other biochemically defined auxin influx or efflux transporters in the context of the root hair model. In order to ensure that the transgene effects were restricted to root hair cells, a root hair cell–specific promoter (Cho and Cosgrove, 2002; Kim et al., 2006) was employed. Transgenic tobacco (*Nicotiana tabacum*) suspension cells were also adopted as an additional plant cell system to directly measure the auxin-transporting activity of PGP4. In addition to determining the directionality of PGP4-mediated auxin transport, we also examined the subcellular dynamics of PGP4 proteins during membrane trafficking.

RESULTS

PGP4 Acts Negatively on Root Hair Growth in Association with Auxin Transport

Several mutants defective in auxin transport show alterations in root hair growth that are most likely due to either a defect in auxin supply to the root hair cell or a loss of auxin-transporting activity in the hair cell (for review, see Cho et al., 2007). To know whether PGP4 also affects root hair growth, we examined the root hair phenotype of an *Arabidopsis* T-DNA insertion mutant line (SALK_063720) (Terasaka et al., 2005). The homozygous *pgp4* mutant root had 25% (at $P < 0.0001$) longer root hairs than the wild-type root (Figures 1A and 1B). A previous study with two other *PGP4* T-DNA insertion lines showed 36 and 42% more root hair elongation in each of these lines, respectively (Santelia et al.,

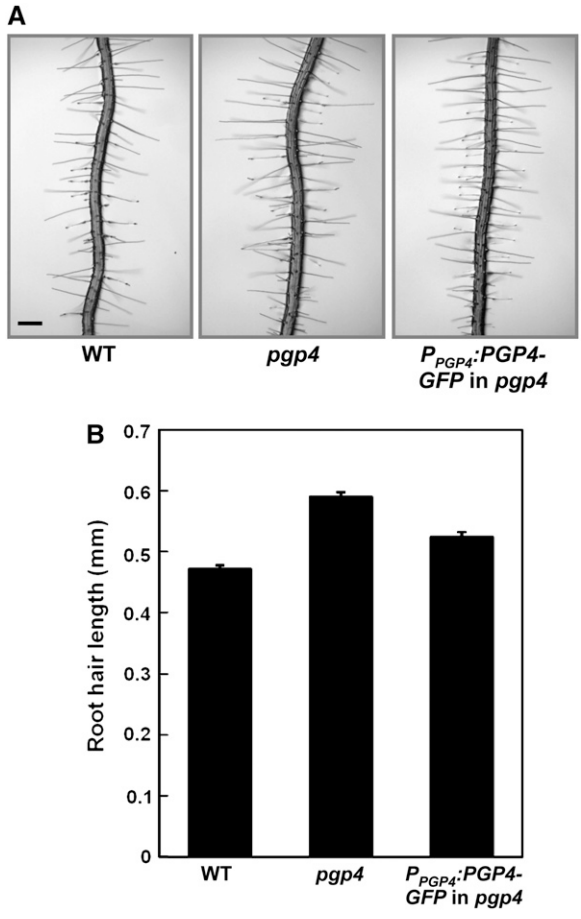

Figure 1. The Loss of PGP4 Enhances Root Hair Elongation.

(A) Root hair phenotypes of the wild type, the *pgp4* mutant, and the complemented transformant (*PPGP4:PGP4-GFP* in the *pgp4* mutant background). Bar = 100 μm for all.

(B) Root hair length of the wild type, *pgp4*, and *PPGP4:PGP4-GFP* roots. Error bars indicate SE ($n = 380$). The averages are significantly different between the wild type and *pgp4* and between *pgp4* and *PPGP4:PGP4-GFP* at $P < 0.0001$ in Student's *t* test.

2005). These results suggested that PGP4 would act as an auxin efflux transporter, resulting in increased auxin retention inside the hair cell and the stimulation of root hair elongation. Here, this hypothesis was tested intensively using transgenic, gene expression, and pharmacological approaches as well as a direct auxin transport assay.

To infer the role of PGP4 in the root hair cell, first we examined the expression of *PGP4* by the PGP4-GFP (for green fluorescent protein) reporter system and by RT-PCR analysis using a root hair cell–specific RNA sample (Lee and Cho, 2006). The *PGP4-GFP* fusion gene construct, driven by the *PGP4* promoter (*PPGP4*), was introduced into the *pgp4* mutant plant, and the phenotypic complementation and the expression pattern of the fusion gene were observed. Introduction of *PPGP4:PGP4-GFP* into the loss-of-function mutant partially restored the wild-type level of root hair length (Figures 1A and 1B). This partial penetrance (45% restoration) of *PPGP4:PGP4-GFP* could be due to some additional *cis*-regulatory elements that are missing in our transgene construct but are remotely operative in the endogenous gene or to the partial functionality of the fusion protein. Expression of *PPGP4:PGP4-GFP* occurred from the root cap cells to the hair differentiation zone and was confined to the epidermis and outer cell layers of the columella and lateral root cap tissues (Figures 2A to 2C). The apical/basal subcellular polarity of the PGP4-GFP fusion protein was not obvious in these tissues, while a previous immunohistochemical study reported a polar localization of PGP4 in certain epidermal cells (Terasaka et al., 2005). The expression of *PGP4* in the root hair cell was also confirmed by RT-PCR analysis of a root hair cell–specific RNA sample. *PGP4*, together with other auxin-transporting *PGP*s and *PIN2*, was expressed in root hair cells (Figure 2D).

If the loss of PGP4 caused longer root hairs due to a defect in auxin efflux activity, overexpression of the gene should shorten root hair length. To verify this hypothesis, we overexpressed PGP4 (*PE7:PGP4* or PGP4ox) in the root hair cell using a root hair cell–specific promoter of the *Arabidopsis EXPANSIN A7* gene (*PE7*) (Cho and Cosgrove, 2002; Kim et al., 2006). In order to obtain visual confirmation of protein expression, the *PGP4* coding region was fused to a fluorescent protein gene (either *PGP4-YFP* [yellow] or *PGP4-RFP* [red]). The PGPox transformants showed significantly reduced root hair elongation compared with control plants (*PE7:YFP*) (Figure 3; see Supplemental Figure 1 online). On average, a 25% reduction in root hair elongation was observed in a sample of 10 randomly chosen independent PGP4ox lines, and no single PGP4ox line showed more root hair elongation than the control plants (see Supplemental Figure 1 online). On the other hand, a strong transgenic line (PGP4ox line 36; Figures 3A and 3B) showed a reduction of as much as 62% in root hair length. A similar result was obtained with cauliflower mosaic virus 35S promoter–driven *PGP4* overexpression lines (*P35S:PGP4*) (see Supplemental Figure 2 online). PGP4 expression was confined only to the root hair cell file in the *PE7:PGP4-YFP* transformant (Figure 3F). To further support the notion that the inhibition of root hair elongation depends on PGP4, we checked for a correlation between phenotypic strength and PGP4 protein levels in three homozygous PGP4ox lines that had distinctive differences in root hair elongation (Figures 3A to 3C). The degree of inhibition of root hair elongation

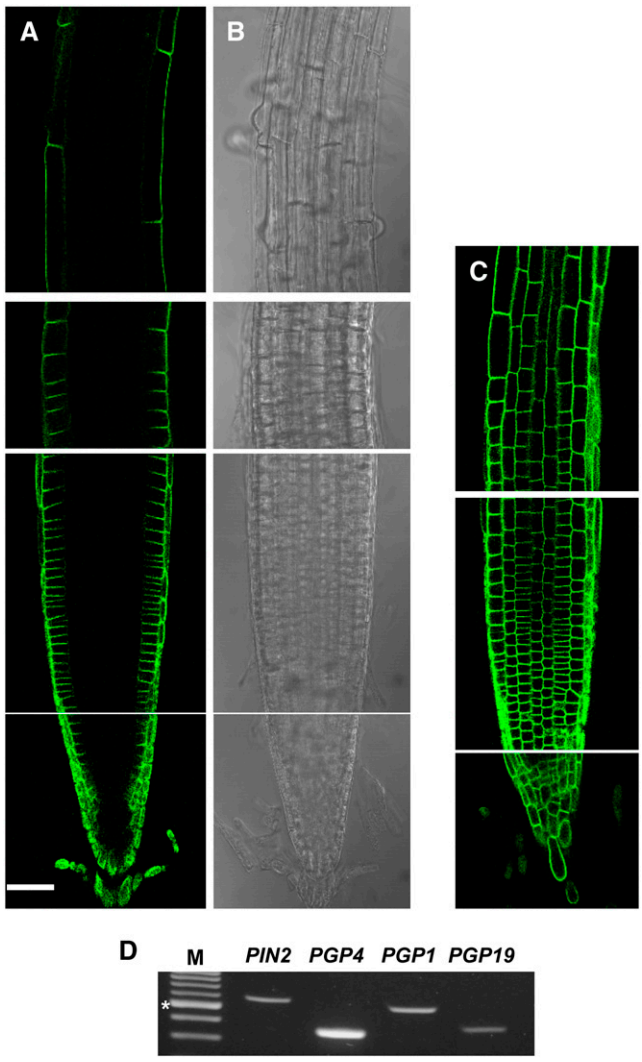

Figure 2. Expression Pattern of *PGP4* in the *Arabidopsis* Root.

(A) to **(C)** Confocal microscopy images of the *pgp4* mutant root harboring *PPGP4:PGP4-GFP*. Bar = 50 μm for all.

(A) and **(B)** Median sectional views of the root: **(A)** fluorescence; **(B)** bright field.

(C) Optical sections of the root surface tissues (root cap and epidermis).

(D) RT-PCR analysis of RNA from *Arabidopsis* root hair cells. M, 100-bp size maker (* 500 bp).

showed a clear positive correlation with the expression level of the PGP4-YFP fusion protein (Figures 3B and 3C).

Root hair–specific overexpression of PGP1 and PGP19 (Figure 3F), which have been demonstrated to be auxin efflux transporters in plant cells (Geisler et al., 2005; Petrášek et al., 2006), also decreased root hair elongation (Figures 3A and 3D). For seven to nine randomly selected independent transgenic lines, the average reduction of root hair elongation by PGP1ox or PGP19ox was in both cases ~25% compared with the control level (see Supplemental Figure 3 online). In strong phenotypic lines, the reductions were as great as 54 and 43%, respectively,

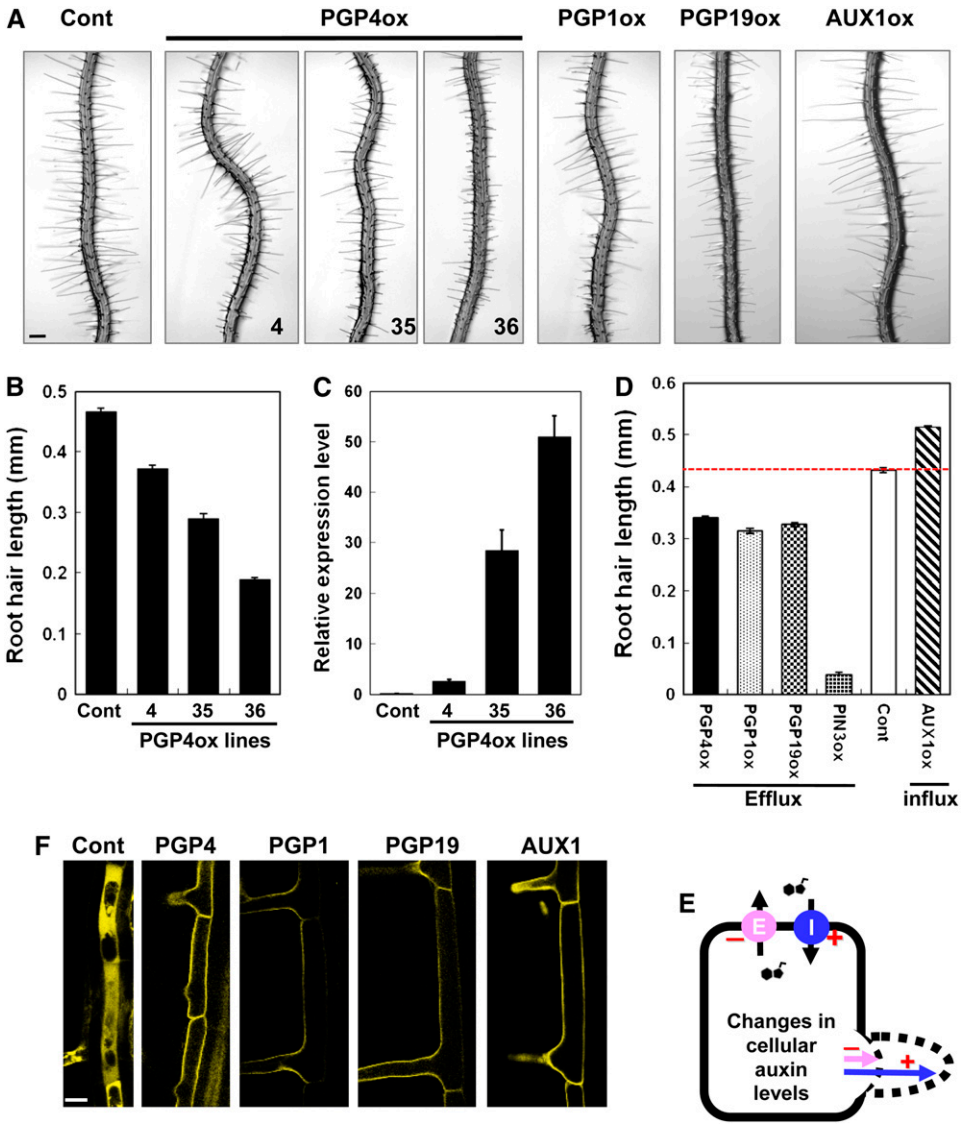

Figure 3. Overexpression of *PGP4* in the Root Hair Cell Decreases Root Hair Elongation.

(A) Roots of control (Cont; *PE7:YFP*), PGP4ox (*PE7:PGP4-YFP*), PGP1ox (*PE7:PGP1-YFP*), PGP19ox (*PE7:PGP19-YFP*), and AUX1ox (*PE7:AUX1-YFP*) transgenic *Arabidopsis* seedlings. Different numbers for PGP4ox indicate independent transgenic lines. Bar = 100 μm for all.

(B) Root hair length of control (Cont) and PGP4ox lines. Error bars indicate SE (n = 369 to 414; average = 396).

(C) Relative expression levels of PGP4-YFP in terms of yellow fluorescence intensity from PGP4-YFP fusion proteins in the roots of independent transgenic lines. Error bars indicate SE (n = 27).

(D) Root hair length of control (Cont), PGPox, PIN3ox (*PE7:PIN3-YFP*), and AUX1ox lines. Error bars indicate SE (n = 963 for Cont, 1782 for PGP4ox [from 10 independent lines], 1557 for PGP1ox [from 9 independent lines], 1152 for PGP19ox [from 7 independent lines], 430 for PIN3ox [from 2 independent lines], and 2187 for AUX1ox [from 11 independent lines]). The dashed line indicates the control level of root hair length. The averages for PGP4ox, PGP1ox, PGP19ox, PIN3ox, and AUX1ox are significantly different from that for Cont (at $P < 0.0001$ in Student's t test).

(E) The root hair model illustrating the effects of efflux (E) and influx (I) activities of auxin transporters on root hair elongation. While efflux activity lowers the cellular auxin level and shortens (−) the root hair, influx activity elevates the auxin level inside the cell and stimulates (+) root hair elongation.

(F) Confocal microscopy images of root hair cells of control (Cont), PGPox, and AUX1ox transformants. Bar = 10 μm for all.

for PGP1ox and PGP19ox (see Supplemental Figure 3 online). PIN3 has been shown to catalyze auxin efflux in plant cells (Lee and Cho, 2006). Overexpression of PIN3 in the root hair cell (PIN3ox) revealed an even greater inhibitory effect on root hair elongation than did PGPox lines, probably indicating the stronger auxin efflux activity of PIN3 in the root hair cell. These results showing that overexpression of auxin efflux transporters, whose activities have already been verified in plant cells, consistently inhibited root hair elongation suggest that PGP4 also acts as an auxin efflux transporter.

In order to further confirm the relationship between the directionality of cellular auxin transport (influx/efflux) and root hair elongation (longer/shorter), we also overexpressed the auxin influx transporter AUX1 (AUX1ox) in the root hair cell using the *PE7* promoter (Figure 3F). As AUX1 has auxin influx activity (Yang et al., 2006), it was expected that AUX1ox would lead to increased uptake of auxin into the root hair cell and enhanced elongation of the root hair. AUX1ox indeed significantly increased root hair elongation (Figures 3A and 3D). Compared with control plants, an average increase of 26% in root hair elongation was observed in a random selection of 11 independent AUX1ox lines (see Supplemental Figure 4 online). One of the strong lines showed a 46% increase of root hair length. The average values for root hair length of these 11 AUX1ox lines were all higher than that of the control line. If PGP4 possessed auxin influx activity, PGP4ox should have enhanced root hair elongation like AUX1ox. The root hair analysis with AUX1ox indicates that PGP4 does not function as an auxin influx transporter in the root hair cell.

IAA and NPA Rescue PGP4ox- or PGP1ox-Mediated Inhibition of Root Hair Growth

If the inhibition of root hair elongation by PGP4ox was caused by PGP4-mediated increases in auxin efflux activity, auxin in the medium should be able to restore root hair elongation in the PGP4ox transformants. We treated control (*PE7:YFP*), PGP4ox, and PGP1ox seedlings with different auxin (IAA) concentrations of between 0 and 3 nM. IAA increased hair elongation of all three transformants, but the rate was higher in the PGPox lines, where it restored root hair length to almost the control level when used at a concentration of 1 to 3 nM (Figure 4A).

The auxin efflux inhibitor NPA significantly enhanced the root hair elongation of the PGP4ox transformant. In the strongest PGP4ox line (line 36), 1 μM NPA enhanced root hair elongation by threefold more than in untreated plants (Figure 4B). This value is in contrast with the only 50% increase seen in control plants treated with the same concentration of NPA. PGP1ox lines also showed greater restoration of root hair length following NPA treatment (1 μM) than did the control plants. Root hair length was restored by approximately twofold in lines 12 and 24 by NPA. This result further suggests that PGP4 functions as an auxin efflux transporter in the root hair cell.

PGP4ox Is Unable to Rescue PIN2ox- or PIN4ox-Mediated Inhibition of Root Hair Growth

We further tested PGP4's directionality in cellular auxin transport by crossing a PGP4ox line with PINox lines. If PGP4 functions as an auxin influx carrier, it should be able to rescue the shortened root hairs induced by PINox. In particular, if PIN2 synergistically facilitates PGP4-mediated auxin influx, as shown in animal HeLa cells (Blakeslee et al., 2007), the coexpression of PGP4 and PIN2 should greatly enhance root hair elongation compared with the expression of PGP4 or PIN2 alone. To test this, we made PIN2ox (*PE7:PIN2-GFP*) transformants and crossed the PGP4ox (*PE7:PGP4-RFP*) homozygote with a PIN2ox homozygote. Root hair phenotype and protein localization were examined in the double

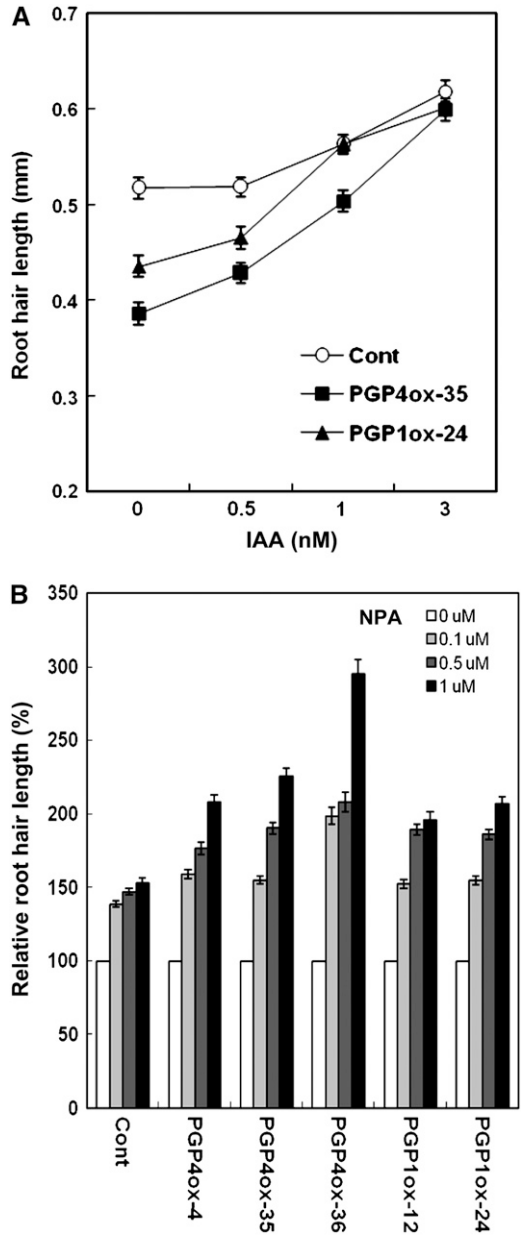

Figure 4. Exogenous IAA or NPA Restores Root Hair Elongation of PGP4ox and PGP1ox Lines.

(A) Restoration of root hair elongation by IAA. Error bars indicate SE (n = 81 to 126; average = 113).

(B) Restoration of root hair elongation by NPA. The root hair length of untreated (0 μM NPA) seedlings was taken to be 100% for each transgenic line. Error bars indicate SE (n = 126 to 252; average = 206).

transgenic hemizygous F1 plants. PIN2ox completely blocked root hair elongation, and the transformant roots developed only a few hair bulges (Figure 5A), similar to PIN3ox (Lee and Cho, 2006) (Figure 3D). When the homozygous PIN2ox line was crossed with a homozygous PGP4ox line, the F1 hybrid transgenic plant showed the same root hair phenotype as that of the PIN2ox line (Figure 5A). In the double transformant, the localizations of both

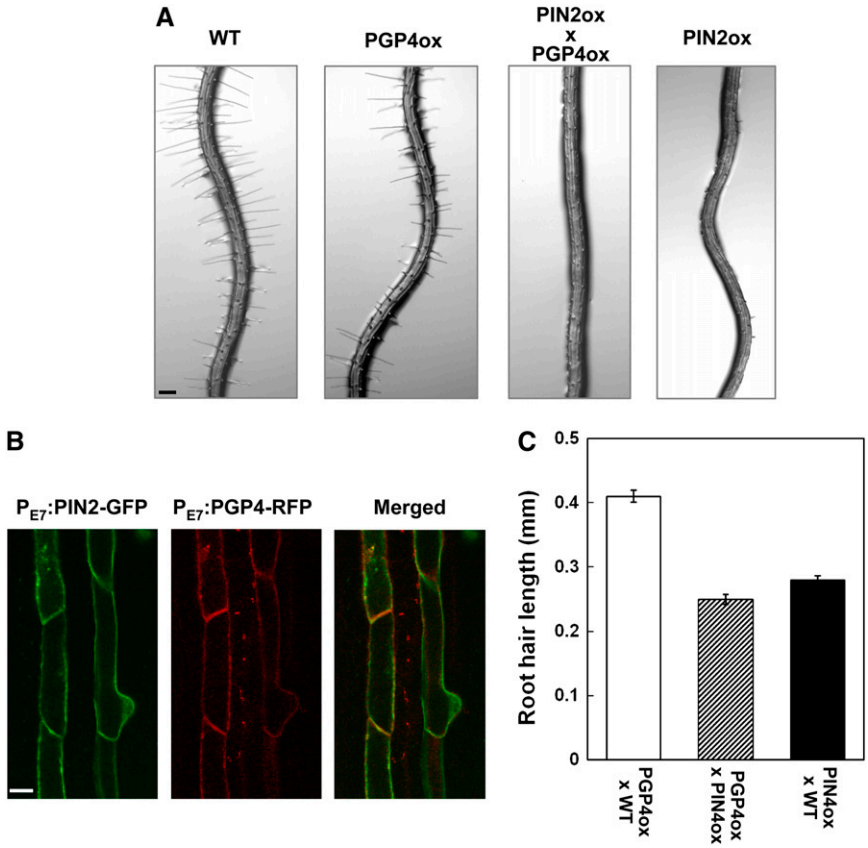

Figure 5. PGP4ox Does Not Rescue the PINox-Mediated Inhibition of Root Hair Elongation.

(A) Root hair phenotypes of the wild type, *PE7:PIN2-GFP* (PIN2ox), *PE7:PGP4-RFP* (PGP4ox), and the cross line of both transformants (*PE7:PIN2-GFP* × *PE7:PGP4-RFP*). Bar = 100 μm for all.

(B) Confocal microscopy images of the same root region of the double transgenic line (*PE7:PIN2-GFP* × *PE7:PGP4-RFP*). Bar = 10 μm for all.

(C) Root hair growth of hemizygous transgenic lines of *PE7:PGP4-RFP* (PGP4ox), *PE7:PIN4-GFP* (PIN4ox), and a cross line (PGP4ox × PIN4ox) for both transformants. Error bars indicate SE (n = 162 to 207; average = 196). The average for [PGP4ox × PIN4ox] is significantly different from that for [PIN4ox × the wild type] at $P < 0.005$ in Student's t test.

PIN2-GFP and PGP4-RFP proteins overlapped with each other in the plasma membrane (PM) of the root hair cell (Figure 5B). As the overwhelming auxin efflux activity of PIN2ox could have prevented the rescue of hair elongation by PGP4ox, we made a second hybrid with the homozygous point-mutated PIN4ox (*PE7:PIN4-GFP*) line, which showed only a mild reduction in root hair elongation. However, the inhibition of root hair elongation was slightly (9% to the PIN4ox hemizygote) enhanced in the F1 hybrid of PGP4ox and PIN4ox compared with that of each single transgenic line (Figure 5C). These data again collectively suggest that PGP4 acts as an auxin efflux transporter rather than an influx transporter in root hair cells.

Overexpression of PGP4 Decreases the Accumulation of Auxin in Tobacco BY-2 Cells

In addition to the root hair system, we adopted the tobacco suspension cell culture system to further confirm the directionality of cellular auxin transport by PGP4. A *P35S:PGP4-YFP* construct was introduced into tobacco Bright Yellow-2 (BY-2) cells via *Agrobacterium tumefaciens*–mediated transformation. The PGP4-YFP fusion proteins were localized to the PM, and no particular alterations of cell morphology or growth were observed in the transgenic cells (Figure 6A).

In order to assay the cellular transport of auxin, the retention of [^{3}H]1-naphthalene acetic acid (NAA) in the cell was measured. PGP4ox cells retained less [^{3}H]NAA than did control cells over the whole incubation time period (Figure 6B). The PGP4ox tobacco cells accumulated only 67% [^{3}H]NAA compared with the control cells after a 25-min incubation (Figure 6B). In contrast with the free diffusion of NAA into cells, NAA export is mostly an active process mediated by efflux transporters (Delbarre et al., 1996). Thus, the decreased retention of NAA in PGP4ox cells can be attributed to the increased auxin efflux activity afforded by PGP4ox. We also examined the sensitivity of PGP4-mediated auxin efflux to the auxin efflux inhibitor NPA. After a 20-min NPA treatment, the NAA retention of the control cells increased by 3.3-fold, whereas that of the PGP4ox cells showed a 2.5-fold

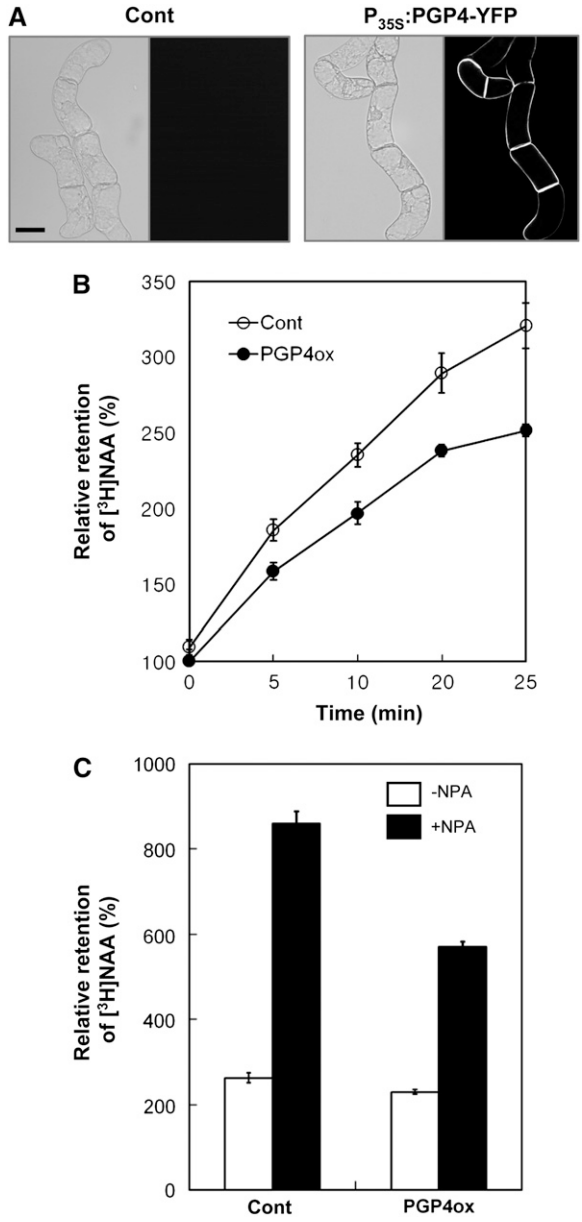

Figure 6. PGP4 Shows Auxin Efflux Activity in Tobacco Cells.

(A) Confocal microscopy images of control (Cont) or *P35S:PGP4-YFP* (PGP4ox) transgenic tobacco BY-2 cells. PGP4-YFP is localized to the cell membrane (right panels). Bar = 30 μm for all.

(B) The cellular accumulation of [^{3}H]NAA is reduced by PGP4ox. Data points are percentages of PGP4ox at time 0 after the application of [^{3}H]NAA.

(C) Inhibition of cellular auxin efflux by 10 μM NPA. NAA retention was measured at 20 min after incubation.

Data represent means ± SE from five independent experiments (two replicates for each) for **(B)** and three independent experiments (two replicates for each) for **(C)**.

increase (Figure 6C). In NPA-treated cells, the NAA retention level of PGP4ox was ~66% of that of the control. This indicates that a portion (34%) of PGP4-mediated auxin efflux is NPA-insensitive. This is reminiscent of PGP19, in which 20% of the auxin efflux mediated by this transporter was NPA-insensitive (Petrášek et al., 2006).

PGP4-YFP Accumulates in Internal Compartments by BFA but Not by Staurosporine

Brefeldin A (BFA) induces the formation of internal compartments that include recycling membrane transporter proteins such as PINs and AUX1 (Steinmann et al., 1999; Grebe et al., 2002). However, it is not known whether the membrane trafficking of plant PGPs can be altered by BFA treatment. Here, we show that BFA induces the accumulation of PGP4 proteins in the characteristic BFA compartments. When *PE7:PGP4-YFP* transformant seedlings were treated with 10 μM BFA for 2 h, typical BFA compartments were formed (Figure 7B; see Supplemental Figure 5A online). A similar result was also obtained with the *PPGP4:PGP4-YFP* transformant (see Supplemental Figure 5B online). The internal compartments disappeared following a 2-h washout (Figure 7C), indicating that the BFA-mediated internal accumulation of PGP4 is reversible. This result suggests that the trafficking pathway of PGP4 at least partially overlaps with those of PINs.

The polar localization and activity of PINs are regulated by a Ser/Thr protein kinase, PINOID, which most likely occurs through the modulation of PIN trafficking (Friml et al., 2004; Lee and Cho, 2006). A protein kinase inhibitor, staurosporine (ST), causes PIN3 proteins to accumulate inside the cell, which was previously shown to rescue the PIN3ox-mediated inhibition of root hair elongation (Lee and Cho, 2006). We tested whether ST also affects the trafficking of PGP4 proteins. However, while 2 μM ST was enough to lead to an accumulation of PIN3 (Lee and Cho, 2006), PGP4 did not accumulate at 10 μM (Figure 7E) or even 50 μM (our unpublished data) ST. This indicates that, unlike PIN3, the trafficking of PGP4 is not affected by protein kinases.

We next examined whether PIN and PGP4 colocalize to the same BFA compartments. For this purpose, we crossed a *PPIN2:PIN2-GFP* transformant with a *PE7:PGP4-RFP* transformant. Because the expression of the *PE7:PGP4-RFP* transgene was extended down to the PIN2 expression domain but with the trichoblast cell specificity, the overlapping expression of PIN2-GFP and PGP4-RFP could be visualized in the same trichoblast cells (Figure 8). While PIN2 showed a clear polar localization at the upper side of the cell, the subcellular polarity of PGP4 in the same region was not obvious. Treatment with 50 μM BFA resulted in the formation of typical BFA compartments in the root hair cells of the transformants (Figure 8B). These internal compartments were observed by means of both PIN2-GFP and PGP4-RFP signals, and the signals overlapped with each other. This result suggests that BFA-sensitive components are involved in the trafficking pathway of both PIN2 and PGP4. However, because the PM localization of PGP4 was less affected than the PM localization of PIN2 by BFA treatment (Figures 7 and 8; see Supplemental Figure 5 online), PGP4 would also seem to take a BFA-resistant trafficking pathway to the PM.

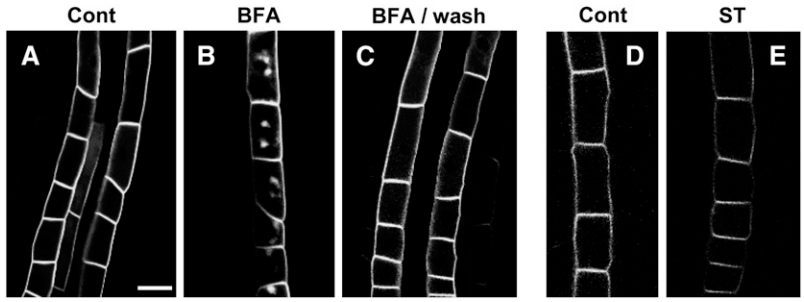

Figure 7. Effects of BFA and ST on the Subcellular Localization of PGP4-YFP.

Confocal microscopy images of *PE7:PGP4-YFP* transformant roots. The transformant seedlings were treated with 0.5× Murashige and Skoog (MS) medium for 2 h **([A]** and **[D])**, 10 μM BFA for 2 h **(B)**, 10 μM BFA for 2 h followed by a washout with 0.5× MS medium for 2 h **(C)**, and 10 μM ST for 2 h **(E)** before observation. Bar = 10 μm for all.

NAA Inhibits the BFA-Induced Internalization of PGP4-YFP

Auxin inhibits the BFA-induced internal accumulation of PINs, implying a positive feedback mechanism for auxin efflux by the hormone itself (Paciorek et al., 2005). All of the major auxins, such as IAA, NAA, and 2,4-D, showed such an inhibitory effect. We examined the auxin effect on the trafficking of PGP4 proteins in response to BFA. A 30-min pretreatment and coincubation with 5 μM NAA was enough to prevent BFA (10 μM)-induced internal accumulation of the PGP4-YFP protein in the trichoblast cells (Figure 9B). The inhibitory effect of NAA was still apparent after a 1-h incubation with BFA (Figure 9D), but after 1.5 h the BFA compartments started to reappear (data not shown). This result demonstrates that the residence of PGP4 in the PM is enhanced by auxin, as observed previously for PIN proteins.

DISCUSSION

PGP4 Facilitates Auxin Efflux in Plant Cells

In this study, we have shown that *Arabidopsis* PGP4 displays auxin efflux transporter properties in two independent plant cell

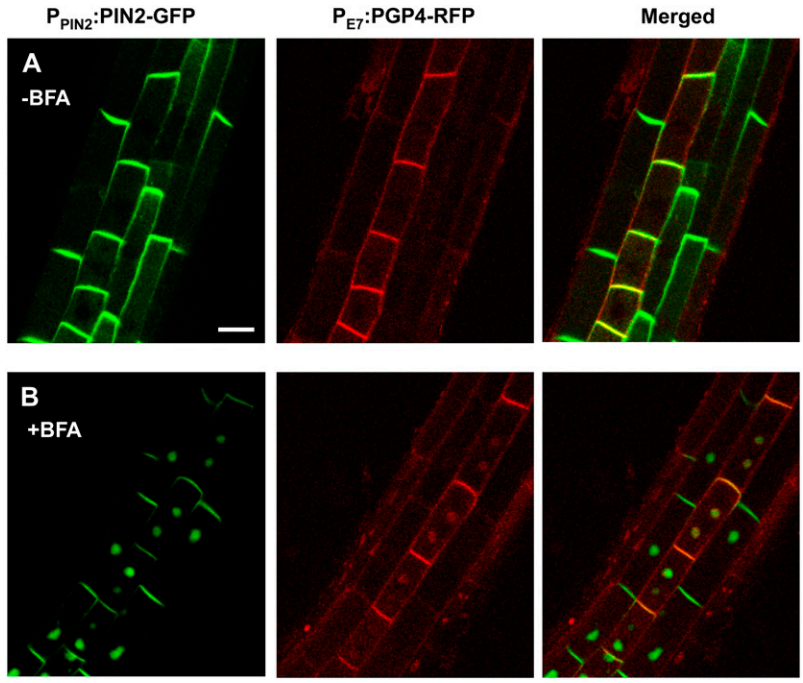

Figure 8. PGP4 and PIN2 Are Colabeled in the Same BFA-Induced Intracellular Compartments.

Confocal microscopy images of the same root region of a double transgenic plant (*PPIN2:PIN2-GFP* × *PE7:PGP4-RFP*) treated with 0 μM **(A)** or 50 μM **(B)** BFA for 2 h. The green fluorescence was from whole epidermal cells expressing PIN2-GFP from the PIN2 promoter, and the red fluorescence was from the trichoblast cell files expressing PGP4-RFP from the *EXPA7* promoter. The merged images with a yellowish tone are shown only in the trichoblast cell files. Bar = 10 μm for all.

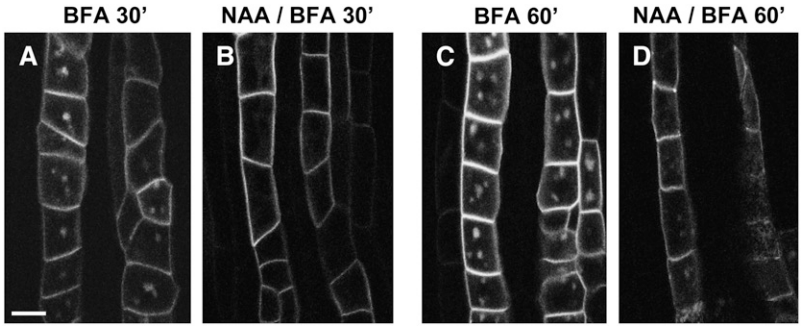

Figure 9. Auxin Inhibits the BFA-Induced Internal Accumulation of PGP4.

Confocal microscopy images of *PE7:PGP4-YFP* transformant roots. Transformant seedlings were incubated with 10 μM BFA for 30 **(A)** and 60 **(C)** min or preincubated with 5 μM NAA for 30 min and then cotreated with 5 μM NAA and 10 μM BFA for 30 **(B)** and 60 **(D)** min. Bar = 10 μm for all.

systems: the root hair cell and tobacco cell suspensions. The root hair cell system for measuring auxin transport activity is based on the fact that root hair length is proportional to the cellular auxin level, which can vary according to auxin-transporting activities (Lee and Cho, 2006). The tobacco suspension cell system provides a direct way to monitor auxin movement across the cell membrane (Delbarre et al., 1996). This study presents seven lines of experimental evidence in support of the view that PGP4 is an auxin efflux transporter in plant cells.

First, the loss of PGP4 enhanced root hair elongation. PGP4 is the only known auxin transporter that maintains a high level of expression in the epidermal cells of the root hair region (Terasaka et al., 2005) (Figure 2A). Our RT-PCR analysis with the root hair cell–specific RNA sample also reveals that the expression level of PGP4 is maintained at a relatively high level in the root hair cell (Figure 2D). Therefore, the loss of PGP4 could result in a considerable reduction in auxin efflux from the hair cell, which in turn would raise the cellular auxin level and enhance root hair elongation.

Second, root hair cell–specific overexpression of PGP4 (PGP4ox) significantly decreased root hair elongation. This can be explained by the increased export of auxin in the presence of high levels of PGP4, which would have lowered the intracellular auxin concentration and inhibited root hair elongation (Figure 3). This hypothesis is strongly supported by the observation that the reduction of root hair elongation in independent PGP4ox lines was proportional to the expression level of PGP4 protein (Figures 3B and 3C). Furthermore, the PGP4ox-mediated reduction of root hair elongation was rescued by the application of exogenous auxin (IAA) or the auxin efflux inhibitor NPA (Figure 4), as observed previously in the case of PIN3, a defined auxin efflux transporter (Lee and Cho, 2006). A mass supply of auxin from the medium would have overcome the increased efflux of auxin from the hair cell by PGP4ox, thus restoring the root hair elongation of the PGP4ox root. NPA is a well-defined inhibitor of cellular auxin efflux (Lomax et al., 1995) and can bind to PGP proteins (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2003; Terasaka et al., 2005). Furthermore, it has been shown that NPA inhibits PGP1-mediated auxin efflux in *Arabidopsis* protoplasts and HeLa cells (Geisler et al., 2005), PGP4-mediated auxin influx in HeLa cells (Terasaka et al., 2005), and PGP19-mediated auxin efflux in tobacco cells (Petrášek et al., 2006). If NPA inhibits PGP4ox-mediated auxin efflux activity, auxin levels in the hair cell will increase and consequently root hair elongation will be restored in the PGP4ox transformant. NPA, at a concentration as low as 1 μM, greatly restored root hair elongation of the PGP4ox transformant root (Figure 4B), indicating that NPA blocked PGP4ox-mediated auxin efflux in the root hair cell.

Third, root hair cell–specific overexpression of AUX1, an auxin influx transporter (Marchant et al., 1999; Yang et al., 2006), significantly enhanced root hair elongation (Figure 3D). The overexpression of an auxin influx transporter is anticipated to accumulate more auxin inside the cell and thus stimulate root hair elongation. While AUX1ox did enhance root hair elongation, PGP4ox inhibited it. This strongly suggests that PGP4 does not function as an auxin influx transporter but rather as an auxin efflux transporter.

Fourth, root hair cell–specific overexpression of PGP1, PGP19, PIN2, PIN3, and PIN4, which already have been biochemically defined as auxin efflux transporters (Geisler et al., 2005; Lee and Cho, 2006; Petrášek et al., 2006; Blakeslee et al., 2007), all decreased root hair elongation. Figure 3D clearly illustrates that auxin influx transporter and efflux transporters affect root hair elongation in a reverse manner, resulting in shorter hairs under conditions of efflux activity and longer hairs under conditions of influx activity (Figure 3E), and that PGP4 belongs to the group of transporters with auxin efflux activity.

Fifth, PGP4ox could not rescue PIN2ox- or PIN4ox-induced inhibition of root hair elongation. If PGP4 acted as an auxin influx transporter, PGP4ox should have rescued the short-hair phenotypes caused by the overexpression of auxin efflux transporters. However, this did not happen. Rather, PGP4ox slightly enhanced the PIN4ox-mediated inhibition of root hair elongation (Figure 5C). A previous study reported that the coexpression of PIN2 with PGP4 synergistically increased auxin influx activity in HeLa cells (Blakeslee et al., 2007). Again, if PIN2 and PGP4 acted synergistically in plants, double transgenic plants for PGP4ox and PIN2ox should have grown much longer root hairs than either of the single transgenic plants. Our result indicates that plant transporter proteins can behave differently in animal cells.

Sixth, PGP4ox facilitated auxin efflux in tobacco suspension cells. In the suspension cell culture system, the auxin transport

assay clearly demonstrated that PGP4 catalyzes auxin efflux in plant cells (Figure 6).

Seventh, the auxin-mediated inhibition of PGP4 accumulation in endomembrane compartments implicates an auxin efflux transporter rather than an influx transporter. Auxin transporters such as PINs and AUX1 constitutively recycle between endosomes and the PM, so that BFA, an inhibitor of endosome-to-PM trafficking, causes the formation of internal BFA compartments (Grebe et al., 2002; Geldner et al., 2003; Kleine-Vehn et al., 2006). While endocytosis of auxin efflux transporters such as PINs is inhibited by their substrate auxin (Paciorek et al., 2005), endocytosis of AUX1 is not affected by auxin (Kleine-Vehn et al., 2006). This is reasonable, as an increased intracellular auxin concentration will induce a positive feedback mechanism that facilitates auxin efflux. Our result demonstrated that auxin (NAA) efficiently blocked the BFA-mediated internal accumulation of PGP4 (Figure 9), as observed previously for other auxin efflux transporters. This suggests that the auxin-mediated inhibition of endocytosis might be a general feature of auxin efflux transporters. Such a mechanism would permit the positive feedback of cellular auxin efflux for the homeostasis of intracellular auxin levels and/or for the maintenance of a constant auxin flow through the cell.

The Directionality of PGP-Mediated Transport Is Flexible Depending on the Expression System

At present, we do not know why PGP4 reveals auxin efflux activity in plant cells but auxin influx activity in yeast and HeLa cells. However, we might be able to infer the mechanism from several other similar cases. One possibility for the different directionality of PGP4-mediated auxin transport in different systems could be the existence of probable modulators for PGPs. The immunophilin-like protein TWISTED DWARF1 has been implicated in modulating the function of PGPs (Geisler et al., 2003; Bouchard et al., 2006). When coexpressed with PGP1, TWISTED DWARF1 showed opposite effects on PGP1-mediated auxin efflux activity in yeast and HeLa cells: inhibition was observed in yeast, whereas activation was seen in HeLa cells (Bouchard et al., 2006). This suggests that PGPs can be modulated in a reverse manner by the same molecule depending on the expression system, which most likely reflects the presence of organism-specific additional modulators. Actually, yeast does not have PGPs; thus, it is possible that the lack of proper modulators in yeast cells made PGP4 molecules behave differently. It is also arguable whether plant proteins are properly expressed and correctly localized to the right subcellular compartments in yeast cells. Some plant and mammal PGPs are not functionally expressed in yeast (Noh et al., 2001; Geisler and Murphy, 2006), and plant proteins frequently mislocalize in yeast due to the presence of different trafficking components and routes in yeast cells (Bassham and Raikhel, 2000).

In particular, the different subcellular localization of a membrane transporter can give rise to seemingly opposite directionalities of cellular transport. A human PGP is progressively transported from the endoplasmic reticulum to the Golgi and the PM in HeLa cells (Fu et al., 2004). While the PGP stays in the endoplasmic reticulum and Golgi, the PGP accumulates its substrate daunorubicin inside the cell. However, when the PGP localizes to the PM, it lowers the intracellular accumulation of daunorubicin by exporting it out of the cell. In the case of PIN, a point mutation at Ser-97 in PIN2 caused the mislocalization of PIN2 to intracellular compartments in yeast, leading to an increased accumulation of auxin inside the cell (Petrášek et al., 2006). These examples show that the same protein can be shown either as an efflux or an influx transporter at the cellular level, depending on its subcellular localization. We observed that PGP4-YFP fusion proteins predominantly localize to the internal compartments of *Δyap1-1* mutant yeast cells (see Supplemental Figure 6 online). We infer from these studies that the internal mislocalization of PGP4 in yeast cells could have led to the interpretation that it functions as an auxin influx transporter.

Another possibility for the dual directionality of PGP4-mediated auxin transport could be PGP4's functional flexibility in heterologous systems. Coordinated auxin transport by PGP–PIN interactions has been reported (Noh et al., 2003; Blakeslee et al., 2007). Intriguingly, PGP4 showed different auxin transport directionalities depending on its PIN partner. In HeLa cells, coexpression of PIN2 enhanced PGP4-mediated auxin influx activity, whereas coexpression of PIN1 and PGP4 showed auxin efflux activity (Blakeslee et al., 2007). This functional plasticity of PGP4 might reflect the lack of certain plant-specific modulators for PGP4 in animal cells.

Subcellular Dynamics of PGP4 Trafficking

BFA blocks vesicle trafficking from endosomes to the PM by inhibiting the ADP-ribosylation factor (ARF)-guanine-nucleotide exchange factor (Peyroche et al., 1996), resulting in the formation of internal BFA compartments (Steinmann et al., 1999). The trafficking of PM proteins, such as PINs and AUX1, is affected by BFA; thus, they accumulate in BFA compartments (Steinmann et al., 1999; Geldner et al., 2001, 2003; Friml et al., 2002; Grebe et al., 2002; Paciorek et al., 2005; Kleine-Vehn et al., 2006). Because the BFA-mediated internal accumulation of those PM proteins is reversible, washout of BFA restores the trafficking of these proteins. Although the BFA effect on trafficking of PINs and AUX1 has been shown, its effect on the trafficking of plant PGPs has not been studied yet.

This study demonstrates that trafficking of PGP4 to the PM is partially sensitive to BFA, as the PGP4-YFP protein accumulated in typical BFA compartments in a reversible manner (Figure 7). However, because substantial amounts of PGP4-YFP still remained in the PM even in the presence of BFA (see Supplemental Figure 5 online), PGP4 seems to also take a BFA-insensitive route to the PM. The BFA effect on PGP4 contrasts with its effect on PIN2, as shown in Figure 8, in which the expression domains of both PIN2-GFP and PGP4-RFP overlap with each other. While BFA significantly reduced the PIN2-GFP signal in the PM, the signal intensity of PGP4-RFP was little affected by BFA. This double transgenic transformant also demonstrates that two auxin-exporting proteins are colabeled in the same internal compartments in response to BFA. This suggests that PIN2 and PGP4 at least partially share a common membrane trafficking pathway.

On the other hand, the effect of ST, a Ser/Thr protein kinase inhibitor, showed that there was a difference between the

trafficking of PGP4 and that of PINs. ST interferes with the trafficking of PIN3 (Lee and Cho, 2006) and PIN2 (our unpublished observation) to the PM, resulting in their accumulation in cytoplasmic compartments. Therefore, ST treatment results in the restoration of root hair elongation in PINox transformants. However, ST had no effect on PGP4 trafficking (Figure 7E). Although the protein kinase PINOID modulates the subcellular polar localization and activity of PINs (Friml et al., 2004; Lee and Cho, 2006), the mechanism that permits protein kinase–mediated membrane trafficking has not yet been characterized. The different responses of PINs and PGP4 to the protein kinase inhibitor may provide a means to characterize protein kinase–mediated membrane trafficking in plants.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana, Columbia ecotype, was used as the wild type. *pgp4* (SALK_063720) mutant seeds were obtained from the ABRC. *PPIN2:PIN2-GFP* seeds were provided by Jiří Friml. Seedlings were grown on agarose plates containing 2.15 g/L MS nutrient mix (Sigma-Aldrich), 1% sucrose, 0.5 g/L MES, pH 5.7, with KOH, and 0.8% agarose. Seeds were cold-treated for 3 d at 4°C and germinated at 23°C under a 16-h-light/8-h-dark photoperiod. Transformed plants were selected on hygromycin-containing plates (10 μg/mL). For pharmacological experiments, 3-d-old seedlings were transferred to new plates containing the indicated chemicals and incubated for an additional 1 d before observation of root hairs.

Transgene Constructs

The *EXPA7* promoter (*PE7*; −448 deletion) (Cho and Cosgrove, 2002) was used to direct the root hair cell–specific expression of *PGP*, *AUX1*, and *PIN* genes. To construct the YFP fusion, the YFP coding region was amplified by PCR from the *pEYFP-Actin* vector (Clontech) using primers 5′-GCTACCCCGGGCCACCATGGTGAGCAA-3′ and 5′-GATCTGAGCTCTTACTTGTACAGCTCGTG-3′ and replaced *GFP* in the *PE7:GFP* construct (Lee and Cho, 2006), which resulted in *PE7:YFP*. For the *PE7:PGP4-YFP* construct, *PGP4* was obtained from *Arabidopsis* genomic DNA by PCR using primers 5′-ATCTGTCGACCTCTAATGGCTTCAGAGAG-3′ and 5′-AATTCCCGGGGAGAAGCCGCGGTTAGATG-3′. For the *PE7:PGP1-YFP* and *PE7:PGP19-YFP* constructs, cDNAs of *PGP1* and *PGP19* were obtained from the Institute of Physical and Chemical Research. Primers 5′-AGAAGTCGACCGGAAATGGATAATGACG-3′ and 5′-AGTACCCGGGGAGCATCATCTTCCTTAAC-3′ were used for the PCR of *PGP1*, and 5′-TCGGGTCGACAAAAAACCATGTCGG-3′ and 5′-ATCACCCGGGGAATCCTATGTGTTTGAAG-3′ were used for the PCR of *PGP19*. The PCR products of *PGP4*, *PGP1*, and *PGP19* were inserted into the *Sal*I and *Xma*I sites of the *PE7:YFP* vector. For *PE7:PGP4-RFP*, the RFP coding region amplified from the *pDsRed2-N1* vector (Clontech) using primers 5′-CCCGGGATCCACCGGTCGCCA-3′ and 5′-TCTAGAGTCGCGGCCGCTACAGG-3′ was replaced with YFP in the *Xma*I and *Xba*I sites of *PE7:PGP4-YFP*.

PE7:PIN2-GFP and *PE7:PIN4-GFP* constructs were generated by fusing the *PE7* promoter and the genomic *PIN-GFP* coding regions from *PPIN2:PIN2-GFP* (Xu and Scheres, 2005) and *PPIN4:PIN4-GFP* (Vieten et al., 2005) in the binary vector *pCAMBIA1300-NOS*. The *PIN-GFP* coding fragments were obtained by PCR using the following primers: 5′-AAAAGGTCGACAAAATGATCACCGGCAAAG-3′ and 5′-ATACGTCTAGATAATAACTTAAAGCCCCA-3′ for the *PIN2-GFP* fragment; and 5′-AACTCGTCGACCCACCGGAAAAAATG-3′ and 5′-TCTCTCTCTAGAGCCAGTTGATACTG-3′ for the *PIN4-GFP* fragment. These PCR products and the *PE7* fragment that was amplified by PCR using primers 5′-TGAATAAGCTTTTGGTTCTAATG-3′ and 5′-ACCCAGTCGACCCTCTTTTTCTTTAT-3′ were cloned into the corresponding enzyme sites in the binary vector. The *PIN4* gene used in this experiment contained a missense mutation, Ile(102)Asn (ATC to AAC), whose root hair–specific overexpression showed a mild reduction in root hair length.

The *PE7:AUX1-YFP* construct was created as described previously (Swarup et al., 2004) by introducing AUX1-YFP into the *Xho*I and *Xba*I sites right after *PE7* in *pCAMBIA1300-NOS*. The AUX1-YFP fragment was cloned using primer sets 5′-AGCTCTCGAGATGTCGGAAGGAGTAGAAG-3′ and 5′-CCTGTCTAGATCTCTCAAAGACGGTGGTGT-3′ for the N-terminal region of AUX1, 5′-AAGCGGTACCAACCACGTCATTCAGTGG-3′ and 5′-GGTACCTTTGAAGCTTTTGCCTTCTTTTT-3′ for YFP, and 5′-GGTACCGTGAGCAAGGGCGAGGAG-3′ and 5′-GGTACCCTTGTACAGCTCGTCCAT-3′ for the C-terminal region of AUX1.

For the *P35S:PGP4-YFP* construct, the *YFP-NOS* terminator region was obtained by enzyme digestion with *Xma*I and *Eco*RI from *PE7:PGP4-YFP* and inserted into the same enzyme sites of the *pBI121* vector (Clontech) to obtain *P35S:PGP4-YFP*. Next, the genomic *PGP4* coding region was amplified by PCR using primers 5′-ATCTGTCTAGATCTAATGGCTTCAGAGAG-3′ and 5′-AATTCCCGGGGAGAAGCCGCGGTTAGATG-3′ and introduced into the *Xba*I and *Xma*I sites of the vector.

For the *PPGP4:PGP4-GFP* construct, PGP4 was first obtained by *Sal*I/*Xma*I enzyme digestion of *PE7:PGP4-YFP* and inserted into the *pGPTV-GFP* vector (Cho and Cosgrove, 2002). The *PGP4* promoter region (*PPGP4*) between −12 and −2174 (relative to the transcription initiation site) was obtained by PCR using primers 5′-ACATGTCGACTAAAGGATTTGGGTCTA-3′ and 5′-AGAGGTCGACAGATACCTCACGATCC-3′ and inserted before the *PGP4* region of the vector.

All of the transgene constructs were confirmed by DNA sequencing before plant transformation. The constructs were transformed into *Arabidopsis* ecotype Columbia or tobacco (*Nicotiana tabacum*) BY-2 cells by *Agrobacterium tumefaciens* (strain C58C1)–mediated infiltration (Bechtold and Pelletier, 1998). The insertion of transgenes in the transformants was confirmed by transgene-specific PCR analyses of the genomic DNA from the transformants. Homozygous plants with a 3:1 segregation ratio on the antibiotic plates were selected for further analyses. For each construct, we obtained at least 50 T1 independent lines, and randomly chosen lines were analyzed for the phenotypic significance of transgenes.

Measurement of Root Hair Length

The measurement of root hair length was performed as described previously (Lee and Cho, 2006). For the estimation of root hair length, digital photographs of roots were taken with a stereomicroscope (Leica MZ FLIII) at ×40 to ×50 magnification. Four or five consecutive fully grown hairs protruding perpendicularly from each side of the root, representing a total of nine hairs from both sides of the root, were measured from 9 to 243 (average, 65) roots. Hair bulges of <14 μm were considered arrested at the early bulge stage and were not counted. To know the effect of both PIN2ox and PGP4ox on root hair elongation, homozygous lines of *PE7:PIN2-GFP* and *PE7:PGP4-RFP* were crossed. The F1 double transgenic plants were observed for the measurement of root hair length. The F1 cross of homozygous *PE7:PGP4-RFP* and the wild type was taken as a control.

Culture and Transformation of Tobacco BY-2 Cells

Tobacco BY-2 cells (Nagata et al., 1992) were cultured in darkness at 26°C with shaking (150 rpm). The liquid culture medium included 3% (w/v) sucrose, 4.3 g/L MS salts, 100 mg/L inositol, 1 mg/L thiamin, 0.2 mg/L 2,4-D, and 200 mg/L KH_2PO_4, pH 5.8. The suspension cells were subcultured weekly. Stock BY-2 calli were maintained on solid medium

with 0.8% (w/v) agar and subcultured monthly. Transgenic cells and calli were maintained on the same medium supplemented with 150 μg/mL hygromycin.

For transformation of tobacco BY-2 cells, 50 mL of a 3-d-old culture was cocultivated with 10 mL of *Agrobacterium* harboring *P35S:PGP4-YFP* in a Petri dish in the dark for 3 d at 26°C. Inoculated cells were washed three times and transferred onto solid medium supplemented with 150 μg/mL hygromycin and 200 μg/mL cefotaxime. After 3 weeks, positive calli were transferred onto new medium. Individual calli were then maintained on solid medium or resuspended in the liquid medium to obtain cell suspensions.

Auxin Retention Assay in Tobacco BY-2 Cells

Measurement of auxin retention was performed according to the methods of Delbarre et al. (1996) and Petrášek et al. (2003) with modifications. Transgenic and wild-type BY-2 cells in exponential growth phase were filtrated and equilibrated in uptake buffer (20 mM MES, 40 mM sucrose, and 0.5 mM $CaSO_4$, pH adjusted to 5.7 by KOH) for 45 min with orbital shaking. Equilibrated cells were collected by filtration. One-half gram of cell paste was resuspended in 10 mL of uptake buffer and incubated further for 1.5 h with continuous orbital shaking. [^{3}H]NAA (25 Ci/mmol; American Radiolabled Chemicals) was mixed with 10 mL of the cell suspension to give a final concentration of 2 nM [^{3}H]NAA. At the indicated time point, 0.5-mL aliquots were withdrawn, filtrated rapidly by vacuum pressure on GF/C glass fiber filters (Whatman), and washed twice with 3 mL of ice-cold water. Cell cakes and filters were transferred to a scintillation vial and extracted with 2 mL of ethanol for 30 min, and radioactivity was determined by liquid scintillation counting. Counts were corrected for surface radioactivity by subtracting the initial counts measured immediately after the addition of [^{3}H]NAA. NPA was added to a final concentration 10 μM, and NPA-dependent [^{3}H]NAA accumulation was determined after 20 min. Data are presented as relative values to the [^{3}H]NAA retention of the initial count (100%) for PGP4ox. Three to five independent experiments with two replicates for each were performed.

RT-PCR Analysis of the Root Hair Cell–Specific RNA Sample

The isolation of root hair cell–specific RNA from *Arabidopsis* roots and cDNA synthesis were performed as described previously by Lee and Cho (2006) with modifications. A 25-cycle PCR amplification was performed. Gene-specific primers were used for PCR: 5′-TCATATCCGCGCTACCAGAAGG-3′ and 5′-CCCCAAAATCAAAACAGAAAGAAA-3′ for *PGP1*, 5′-GGGCGCGAGTGGGTCAG-3′ and 5′-AGTGCGCTAGTTGCTTCGTC-3′ for *PGP19*, 5′-AGGCGGACAAAAGCAACGAGTAG-3′ and 5′-TAACGGGGCATAATTGACATAAAA-3′ for *PGP4*, and 5′-TGGGCAAAAAAGGTAGCGACGT-3′ and 5′-CACCTTTGGGTCGTATCGCCTT-3′ for *PIN2*.

Observation of the Reporter Gene Expression

Fluorescence from the reporter proteins (GFP, YFP, and RFP) was observed using a confocal laser scanning microscope (LSM 510; Carl Zeiss). GFP, YFP, and RFP were detected using 488/505 to 530, 514/>530, and 543/>560 nm excitation/emission filter sets, respectively. Fluorescence images were digitized with the Zeiss LSM image browser version 2.80.1123.

Subcellular localization of transporter-reporter fusion proteins was observed from 3- or 4-d-old seedlings. For chemical treatments, 4-d-old seedlings were transferred into half-strength MS liquid medium containing BFA or ST and incubated for the indicated time periods. The control liquid medium included the same amount of the solvent DMSO.

For quantitative analysis of the PGP4-YFP fusion protein in the *PE7:PGP4-YFP* transgenic lines, an epifluorescence stereomicroscope (MZ FLIII; Leica) was used. The fluorescence images from 4-d-old *PE7:PGP4-YFP* transformant roots were taken digitally, and the fluorescence intensity (i.e., the PGP4-YFP expression level) was quantified using the histogram function of Adobe Photoshop (Adobe Systems) as described by Kim et al. (2006). The PGP4-YFP localization in transgenic tobacco BY-2 cells was also observed with a MZ FLIII microscope.

Expression of PGP4-YFP in Yeast

The *PGP4* cDNA insert was amplified using the primers 5′-ATCTGTCGACCTCTAATGGCTTCAGAGAG-3′ and 5′-AATTCCCGGGGAGAAGCCGCGGTTAGATG-3 and cloned into *Sal*I/*Xma*I sites of *pCDL2*, the modified vector of *pYES2* (Invitrogen). The YFP fragment obtained from *PE7:PGP4-YFP* by enzyme digestion with *Xma*I and *Xba*I was inserted into the same enzyme sites of *pCDL2*. This *PGAL1:PGP4-YFP* construct was transformed into the auxin-sensitive *Saccharomyces cerevisiae* strain *Δyap1-1* (Prusty et al., 2004). For the selection of transformants, synthetic minimal medium without uracil, but including 2% glucose (SD-Ura), was used. To observe the subcellular localization of PGP4-YFP, independent transformants were grown in the synthetic minimal medium (SD-Ura) supplemented with 1% galactose. The yellow fluorescence was observed after 3 to 4 d of incubation using a confocal laser scanning microscope (LSM 510).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are At1g12560 (*EXPA7*), At5g57090 (*PIN2*), At1g70940 (*PIN3*), At2g01420 (*PIN4*), At2g36910 (*PGP1*), At2g47000 (*PGP4*), At3g28860 (*PGP19*), and At2g38120 (*AUX1*).

Supplemental Data

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

Supplemental Figure 1. Root Hair Length of *PE7:PGP4-YFP* Lines.

Supplemental Figure 2. Root Hair Length of *P35S:PGP4-YFP* Lines.

Supplemental Figure 3. Root Hair Length of *PE7:PGP1-YFP* (PGP1ox) and *PE7:PGP19-YFP* (PGP19ox) Lines.

Supplemental Figure 4. Root Hair Length of *PE7:AUX1-YFP* Lines.

Supplemental Figure 5. The BFA Effect on Trafficking of PGP4.

Supplemental Figure 6. PGP4-YFP Localizes in the Internal Compartments in Yeast Cells.

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