ATP-Binding Cassette B4, an Auxin-Efflux Transporter, Stably Associates with the Plasma Membrane and Shows Distinctive Intracellular Trafficking from That of PIN-FORMED Proteins

Misuk Cho, Zee-Won Lee, and Hyung-Taeg Cho*
Department of Biological Sciences and Genomics and Breeding Institute, Seoul National University, Seoul 151–742, Korea (M.C., H.-T.C.); and Division of Life Science, Korea Basic Science Institute, Daejeon 305–333, Korea (Z.-W.L.)

Intracellular trafficking of auxin transporters has been implicated in diverse developmental processes in plants. Although the dynamic trafficking pathways of PIN-FORMED auxin efflux proteins have been studied intensively, the trafficking of ATP-binding cassette protein subfamily B proteins (ABCBs; another group of auxin efflux carriers) still remains largely uncharacterized. In this study, we address the intracellular trafficking of ABCB4 in Arabidopsis (Arabidopsis thaliana) root epidermal cells. Pharmacological analysis showed that ABCB4 barely recycled between the plasma membrane and endosomes, although it slowly endocytosed via the lytic vacuolar pathway. Fluorescence recovery after photobleaching analysis revealed that ABCB4 is strongly retained in the plasma membrane, further supporting ABCB4’s nonrecycling property. The endocytosis of ABCB4 was not dependent on the GNOM-LIKE1 function, and the sensitivity of ABCB4 to brefeldin A required guanine nucleotide exchange factors for adenosyl ribosylation factor other than GNOM. These characteristics of intracellular trafficking of ABCB4 are well contrasted with those of PIN-FORMED proteins, suggesting that ABCB4 may be a basic and constitutive auxin efflux transporter for cellular auxin homeostasis.

Cell-to-cell auxin transport produces local auxin gradients for diverse plant developmental processes such as cell and tissue differentiation and organogenensis (Petrásek and Friml, 2009). This auxin transport is mediated by several membrane transport proteins such as PIN-FORMED (PIN), AUXIN RESISTANT1 (AUX1)/LIKE-AUXIN RESISTANT1, and ABCB/PGP/MDR (for ATP-binding cassette protein subfamily B/P-glycoprotein/multidrug resistance; Vanneste and Friml, 2009). Directional auxin transport is achieved by asymmetric localization of PINs in the plasma membrane (PM), and the dynamic subcellular trafficking is responsible for the regulation of PIN protein localization (Petrásek and Friml, 2009).

Previous studies have demonstrated that both common and unique trafficking pathways cooperate for the subcellular localization of PIN1, PIN2, and AUX1. PIN1 and PIN2 proteins undergo recycling, and their basal localization is regulated by brefeldin A (BFA)-sensitive GNOM, a guanine nucleotide exchange factor for adenosyl ribosylation factor (ARF-GEF; Geldner et al., 2001, 2003; Kleine-Vehn et al., 2008a). However, trafficking of AUX1 and apical localization of PIN2 are regulated by ARF-GEFs other than GNOM (Geldner et al., 2003; Kleine-Vehn et al., 2006, 2008a). In addition to ARF-GEFs, retromer complexes such as SORTING NEXIN 1 (SNX1) and vacuolar protein-sorting proteins also affect the recycling of PINs from the lytic vacuolar pathway (Kleine-Vehn et al., 2008b). Whereas GNOM is involved in the recycling of PIN1 (Geldner et al., 2003), GNOM-LIKE1 (GNL1; a BFA-resistant ARF-GEF) is required for endocytosis of PIN2 (Teh and Moore, 2007). In the presence of BFA, BEN1 (for BFA-visualized endocytic trafficking defective 1; an ARF-GEF) affects the early step of endocytic trafficking for both PIN1 and V-type ATPase (Tanaka et al., 2009). These previous studies indicate that different ARF-GEFs are involved in the regulation of endocytosis and recycling processes of different PINs and other auxin transporters.

Of the many ABCB family members, only ABCB1, ABCB4, and ABCB19 have been implicated in auxin transport (Noh et al., 2001; Multani et al., 2003; Geisler et al., 2005; Terasaka et al., 2005; Cho et al., 2007). For
example, the ABCB4 loss-of-function mutants show several root phenotypes, such as agravitropism, abnormal lateral root formation, and enhanced root hair elongation (Santelia et al., 2005; Terasaka et al., 2005; Cho et al., 2007; Lewis et al., 2007). These ABCBs (AT-ABCBs, where AT stands for auxin transporting) localize to the PM in either a polar or nonpolar manner, depending on the protein and the cell type in which they are expressed (Geisler et al., 2005; Terasaka et al., 2005; Blakeslee et al., 2007; Cho et al., 2007; Wu et al., 2007; Mravec et al., 2008). Although the effect of BFA on the trafficking of AT-ABCBs has been studied, only intracellular trafficking of ABCB19 was examined in relatively more detail (Cho et al., 2007; Titapiwatanakun et al., 2009; Wu et al., 2010). Insensitivity to short-term treatment of BFA and the long recovery time after photobleaching of ABCB19 indicated that ABCB19 is stably anchored in the PM and that the recycling from PM to endosomes is not dynamic, which is different from PINs and ABCB1 (Titapiwatanakun et al., 2009). In addition, trafficking of ABCB19 to the PM is dependent on GNL1 (Titapiwatanakun et al., 2009). It was also shown that endoplasmic reticulum-localized TWISTED DWARF1 is required for the proper targeting of the three AT-ABCBs to the PM, whereas PIN2 localization is not affected by TWISTED DWARF1 (Wu et al., 2010).

To characterize the trafficking of auxin-transporting ABCB proteins in greater detail, we compared the subcellular behavior of ABCB4 with PINs using a variety of cytological, genetic, and pharmacological tools in Arabidopsis (Arabidopsis thaliana). Our study suggests that ABCB4 proteins are strongly retained in the PM and show a distinct regulation mechanism relative to PIN proteins.

RESULTS
ABCB4 Proteins Exhibit Lower Levels of Endosomal Localization Than PINs

To characterize the intracellular trafficking of ABCB4, its subcellular localization in root epidermal cells was observed. Colocalization analysis of ProABCB4:ABCB4-GFP transformants with the endocytic tracer FM4-64 revealed that only a small number (less than five in a cell; n = 20 seedlings) of ABCB4-containing vesicles overlapped with FM4-64-positive endosomes (Fig. 1A). Because the abundance of ABCB4 in endosomes is low, we tested ABCB4 endosomal localization in the ABCB4 overexpression line and compared this with PIN2 localization. Similar low abundance of ABCB4 in endosomal vesicles was also observed in the root tissues of Pro-35S (cauliflower mosaic virus 35S promoter): ABCB4-YFP transformants and with leaf protoplast cells (Fig. 1, B and C; n = 50 seedlings). However, in root epidermal cells, PIN2 exhibited higher levels of endosomal localization (less than 15 in a cell; n = 12) compared with ABCB4 (Fig. 1D).

In order to identify the endosomes in which ABCB4 localizes, we performed colocalization studies with certain known endosomal markers. ABCB4-GFP partially overlapped with the scattered cytoplasmic particles of SNX1-mRFP (approximately 54%; n = 21 seedlings; Fig. 1E). SNX1 is known as a prevacuolar compartment (PVC; a synonym of multivesicular bodies) marker (Jaillais et al., 2008; Kleine-Vehn et al., 2008b) that acts in the degradation pathway of PIN2 and BOR1 (a PM boron transporter) (Jaillais et al., 2008; Kleine-Vehn et al., 2008b) and is also responsible for redirecting the proteins to the trans-Golgi network (TGN) and the PM (Jaillais et al., 2007, 2008). However, SNX1 seems to localize not only to the PVC but also predominantly in the TGN, as the recycling point for vacuolar sorting receptors from the PVC to the TGN (Niemex et al., 2010). Next, we examined whether ABCB4 colocalizes with Rab5 orthologs ARABIDOPSIS RAB GTPASE HOMOLOG F2B (ARA7/RabF2b) and ARABIDOPSIS RAB HOMOLOG F2A (RHA1/RabF2a), other PVC markers (Lee et al., 2004; Haas et al., 2007). The F1 double transgenic seedlings of ProABCB4: ABCB4-GFP and Pro-35SmRFP-ARA7/RabF2b failed to show any distinct ABCB4 localization in the ARA7/RabF2b-positive endosomes (Fig. 1F). Like ARA7/RabF2b, ABCB4 was also not observed in the RHA1/RabF2a-positive endosomes (Fig. 1G). Additionally, internal ABCB4-GFP signals (ProABCB4:ABCBC4-GFP) did not overlap with the Golgi marker sialyl transferase (ST)-DsRed (Saint-Jore et al., 2002; Fig. 1H).

To test whether ABCB4 localized in the endosomes that are involved in PIN2 recycling, the trafficking inhibitor ENDOSIDIN1 (ES1) was employed. ES1 blocks early endocytosis of PIN2 as well as AUX1 and BRI1, but not PIN1 or PIN7, resulting in the internal accumulation of characteristic endosidin bodies defined by two TGN/early endosome markers, syntaxin SYP61 and the V-ATPase subunit VHA-a1 (Robert et al., 2008). Although ES1 caused internal PIN2 aggregations, it failed to form similar endosidin bodies with ABCB4 (Supplemental Fig. S1), which indicates that ABCB4 trafficking is predominantly ES1 independent.

BFA blocks the retargeting of endocytosed vesicles from recycling to the PM as well as the degradation pathway to the vacuole and results in aggregates (BFA compartments) inside the cell (Geldner et al., 2001; Kleine-Vehn et al., 2008b). Because ABCB4 generated BFA compartments like PIN (Cho et al., 2007), BFA treatment analysis together with the use of subcellular compartment markers could provide information about the trafficking pathways of ABCB4. Thus, we tried to identify the subcellular organelles that are present in the ABCB4-containing BFA compartments.

Although ABCB4 did not show strong endosomal localization, BFA induced its aggregation into BFA compartments that merged closely with FM4-64 positive endosomes (Fig. 2, A and B). BFA treatment caused both ABCB4 and SNX1 to form ball-shaped BFA compartments, and these two signals appeared to be merged (Fig. 2C), which is consistent with the results shown in Figure 1E. However, ARA7/RabF2b was partially merged with the BFA compartments of the double transgenic line ProABCB4:ABCBC4-GFP/Pro-35SmRFP-ARA7/RabF2b.
ABC4 in response to BFA, and some of them were located at the edge of the ABC4 aggregates (Fig. 2D). Another PVC marker, RHA1/RabF2a, did not merge with BFA compartments of ABC4 (Fig. 2E). The RHA1 endosomes did not aggregate together upon BFA treatment like ABC4 but surrounded the ABC endo- aggregates.

Also, ABC4-containing BFA compartments did not colocalize with that of ST-DsRed (Fig. 2F). Upon BFA treatment of this line, ABC4-GFP and ST-DsRed signals formed large BFA compartments in a similar way: a round ball-like shape for ABC4-GFP and a ring-like organization for ST-DsRed. However, the ABC4-GFP signals resided within the circular Golgi structure, and the two signals barely overlapped (Fig. 2F).

In summary, part of ABC4 endocytoses through SNX1-positive endosomes, and the number of ABC4-containing endosomes is less abundant compared with that of PIN2.

ABC4 Is Unlikely to Recycle between the PM and Endosomes

Whereas PIN2 recycling between the PM and endosomes was demonstrated using a green-to-red photoconvertible version of PIN2 (PIN2-EosFP; Dhonukshe et al., 2007), the recycling status of ABC4 has not been demonstrated. From the results in Figure 1, we considered that the recycling pathways of ABC4 from the PM and endosomes are not active; instead, an endocytic pathway to the vacuole could be the relevant trafficking pathway for ABC4, which is also responsive to BFA (Kleine-Vehn et al., 2008b). In order to address this hypothesis, we examined from which pathway ABC4-positive BFA compartments are produced. For this purpose, we also used a UV-induced green-to-red photoconvertible protein (Kaede) for tracing the trafficking of PM proteins (Ando et al., 2002). The abcb4 mutant root grows slightly longer hairs, most likely due to the partial lack of ABCB4-mediated auxin efflux in the root hair cell (Cho et al., 2007). The N-terminal Kaede fusion of ABC4 (ProABCB4:Kaede-ABCB4) partially rescued the abcb4 mutant phenotype as the C-terminal GFP version did (Cho et al., 2007; Supplemental Fig. S2), indicating that the fusion construct is functional.

UV (364 nm) radiation under a 40× object lens effectively converted the fluorescence of Kaede-ABCB4 fusion proteins from green to red in root epidermal cells (Fig. 3, A and B). Only a small part of the green-Kaede-ABC4-containing PM was activated, and then the formation of ABC4-containing BFA compartments was observed (Fig. 3, A–E). Although derived from limited endocytic resources (i.e. a small photoactivated portion of the PM), BFA compartments containing the weak red-Kaede signals were formed near the activated (red signal) PM regions (Fig. 3, C and D). BFA compartments that were formed distal to the photoactivated PM section (Fig. 3, D, right panel, nos. 4–6, and E) exhibited weaker red-Kaede signals
than proximal compartments (Fig. 3, D, right panel, nos. 1–3, and E), whereas the opposite pattern was observed for green-Kaede-ABCB4 (Fig. 3D, left panel). The same tendency was observed in nine out of 12 independent seedlings, but the other three contained only one BFA compartment in the cells adjacent to photoactivated PM; thus, we excluded them from the comparison. These findings suggest that endocytosis is responsible for the PM-derived vesicles that reside in the ABCB4-containing BFA compartments.

To address whether ABCB4-containing BFA compartments are derived by blocking ABCB4 recycling to the PM after endocytosis and/or trafficking to the vacuole, we tracked the ABCB4 signal from BFA compartments after washing out the BFA. Kaede-ABCB4-containing cells were treated with BFA for 2 h before photoconversion (Fig. 3F). One large BFA compartment of green-Kaede-ABCB4 was marked as a region of interest (ROI) and converted to red-Kaede-ABCB4 upon 364-nm radiation (Fig. 3G). After a 2-h washout, the BFA compartment disappeared (Fig. 3H) and the red-Kaede-ABCB4 membrane signals (gray arrowheads) including neighbor membranes (yellow arrowheads, background) were compared before and after washing out the BFA. The sum of red-Kaede signal intensity for the gray and yellow arrowheads marking the PM are 36.3 and 20.1 before the washout (Fig. 3G) and 34.0 and 17.7 after washout of BFA (Fig. 3H), respectively. The red-Kaede signal level was not significantly changed after the washout of BFA and was seen repeatedly in nine independent cells from nine trials. Our data suggest that ABCB4 recycling between the PM and endosomes is unlikely, and the BFA compartments of ABCB4 seem to be generated mostly by blocking endocytosis to the vacuole. However, the signal of green-Kaede-ABCB4 in the BFA compartment was much weaker than the ABCB4 signal at the PM (Figs. 2B and 3, C and D), because only approximately 21.5% of the ABCB signal at the PM is BFA sensitive (see Fig. 6A below). This affected the intensity of the photoconverted red-Kaede-ABCB4 BFA compartment and sequentially the red-Kaede-ABCB4 signal at the PM after washout. Thus, the possibility that some undetectable amounts of red-Kaede-ABCB4 recycles to the PM cannot be completely ruled out.

Fluorescence Recovery after Photobleaching Analysis Reveals a Stable Association of ABCB4 in the PM

BFA resistance and the low endocytosis rate of ABCB4, as indicated by its stable residence in the PM, raise the question of whether ABCB4 associates strongly with the PM. Fluorescence recovery after photobleaching (FRAP) analysis was performed to assess the strength of association between ABCB4 and the PM. This technique determines protein mobility in living cells by measuring the rate of fluorescence recovery at a bleached site (Sprague and McNally, 2005). The mobility of ABCB4-GFP and PIN2-GFP was compared using FRAP analysis. The GFP signals

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**Figure 2.** BFA compartments of ABCB4 colocalized with SNX1. A and B, Colocalization of ABCB4-GFP and FM4-64 in the BFA compartment. Transgenic seedlings were treated with 50 μM BFA for 2 h and then cotreated with 2 μM FM4-64 for 15 min (B). The same volume of DMSO was administered as a control (A). C, Colocalization analysis of ABCB4-GFP and SNX1-mRFP with BFA (50 μM, 2 h). D, Colocalization analysis of ABCB4-GFP and mRFP-ARA7 with BFA (50 μM, 2 h). E, Colocalization analysis of ABCB4-GFP and YFP-RHA1 with BFA (50 μM, 2 h). F, Colocalization analysis of ABCB4-GFP and ST-DsRed with BFA (50 μM, 2 h). Bars = 10 μm.
of ABCB4-GFP and PIN2-GFP were bleached from 2- or 4-μm-wide PM regions of the root epidermal cells, and fluorescence recovery was recorded over time (Fig. 4D). This experiment was designed to test the following three possibilities. First, if the PM fluorescence recovers only by lateral diffusion of unbleached proteins, then the recovery time will be proportional to the width of the bleached area. This parameter can indicate whether the protein has strong residence in the PM or low recycling between the PM and endosomes. Second, if recycling processes are the only contributors to recovery, then the recovery rate will be similar regardless of the size of the bleached area. Third, if both lateral diffusion and recycling contribute to recovery, then the recovery rate will vary according to their relative contribution levels (Rotblat et al., 2004; Goodwin and Kenworthy, 2005).

Fluorescence was observed for approximately 60 min after bleaching. ABCB4-GFP fluorescence was observed to recover faster with a 2-μm bleach compared with a 4-μm bleach (Fig. 4A). To estimate the process of recovery, the maximum recovery value (%) from the recovery curves of ABCB4 and PIN2 after photobleaching was extrapolated using SPSS software, and then the ratio of the time required to attain 50% recovery (t_{1/2}, 4 μm versus 2 μm) of the extrapolated maximum recovery was calculated (Supplemental Table S1). Theoretically, a t_{1/2} ratio of 2 should indicate...
that fluorescence has recovered by lateral diffusion within the PM, whereas a ratio of 1 would indicate recovery by the recycling process (Niv et al., 2002; Goodwin and Kenworthy, 2005). Values between 1 and 2 can be considered to arise from a mix of the two processes, where both diffusion and recycling contribute to recovery. However, because cycloheximide (CHX) was not employed for these experiments, the contribution of recycling and de novo secretion of the ABCB4-GFP and PIN2-GFP recovery could not be distinguished. Both ABCB4 and PIN2 showed faster recovery with the 2-μm bleach than with the 4-μm bleach. However, the difference between the two recovery curves of ABCB4 was distinctively larger than that of PIN2 within 20 min (Fig. 4, A and B), and they exhibited different 4 μm:2 μm ratios of t½, namely 1.81 for ABCB4 and 1.48 for PIN2 (Fig. 4C; Supplemental Table S1). ABCB4 recovery by lateral diffusion is more predominant than recovery by recycling or de novo ABCB4 secretion, suggesting that the ABCB4 protein is associated with the PM and that its intracellular trafficking is less compared with PIN2.

**ABCB4 Is Slowly Trafficked to the Vacuole**

In general, stable PM-resident proteins degrade more slowly than actively recycling PM proteins, because recycling PM proteins create more opportunities to contact other compartments and proteolytic enzymes located in the cytoplasm (Hare and Taylor, 1991). The rate of ABCB4 degradation was examined to confirm that most ABCB4 proteins reside in the PM. Because light causes the degradation of GFP due to conformational changes and also affects GFP signal by inducing a pH change in the vacuole (Tamura et al., 2003), we transferred ABCB4-GFP and PIN2-GFP transgenic lines to the dark, the respective transgenic lines were observed for ABCB4-GFP and PIN2-GFP signals, and the time required for these proteins to be detected in the vacuole was measured. This time could reflect the degradation rate of ABCB4 and PIN2.

Sizable ABCB4-GFP-containing vesicles were detected in the intracellular space of root epidermal cells 5 h after transfer to the dark, and the earliest vacuolar accumulation was observed after 7 h of darkness (Fig. 5, A–C). In contrast, PIN2-GFP appeared in the vacuole within 3 h of darkness (Fig. 5, D–F). The PM and endocytic tracer FM4-64 have been shown to reach the vacuolar tonoplast within 2 h of dark treatment (Dettmer et al., 2006). When the roots of transgenic seedlings were treated with FM4-64 for the final 3 h in the dark, ABCB4- and PIN2-GFP showed colocalization with FM4-64 in the epidermal cell vacuoles (Fig. 5, G and H).

Wortmannin is an inhibitor of phosphatidylinositol-3-kinase and interferes with the endocytic process from the PM to the vacuole (Matsuoka et al., 1995; Kleine-Vehn et al., 2008b). Wortmannin was used to acquire additional evidence for the vacuolar targeting of ABCB4 and also to compare the vacuolar targeting of ABCB4 and PIN2. In all of the seedlings observed, treatment with 33 μM wortmannin for 2 h caused a relocalization of PIN2-GFP to wortmannin-induced compartments (previously described as PVC/multivesicular bodies by Kleine-Vehn et al., 2008b; Fig. 5I). However, the wortmannin sensitivity of ABCB4 was different. Only four out of 21 seedlings responded to wortmannin within 2 h (Fig. 5, J and K), whereas 10 out of 21 seedlings responded within 5 h (Fig. 5L). In addition, fewer wortmannin compartments were observed for ABCB4 than for PIN2. These results indicate that, as with PIN2, the lytic vacuolar pathway of ABCB4 is dependent upon the phosphatidylinositol-
dependent pathway and the different sensitivities to wortmannin are likely due to differences in rates (or amounts) of endocytosis to the vacuole. Kaede-ABCB4 was used to quantify ABCB4 signals in the PM of root epidermal cells over time (Fig. 5, M and N). Green-Kaede-ABCB4 in the PM signals remained at 89.5% of their initial value after 9 h. However, red-Kaede-ABCB4 signals in the PM were reduced to 45.1% at 5 h after UV photoactivation, and this value continued until 9 h with slight reduction (Fig. 5O). Because we measured ABCB4 signals in the PM without considering the ABCB4 signal in the vacuole, these data could restrictively provide the $t_{1/2}$ of ABCB4 in the PM, which is approximately 2.5 h.

In summary, the ABCB4 endocytic event to the vacuoles is a slow process that is related to the stable anchoring of ABCB4 in the PM.

BFA Sensitivity of ABCB4 Trafficking Requires ARF-GEFs Other Than GNOM, and Its Endocytosis Is Independent of GNL1

Although ABCB4 trafficking is responsive to BFA, which induces the formation of internal ABCB4-containing compartments, a substantial proportion of the ABCB4 signal remains in the PM (Figs. 1 and 3; Cho et al., 2007). To determine how much of the ABCB4 residing in the PM is resistant to BFA, a quantitative confocal microscopy program was used to compare the intensities of ABCB4 (ProABCB4:ABCB4-GFP), PIN1 (ProPIN2:PIN1-GFP), and PIN2 (ProPIN2:PIN2-GFP) signals in the respective areas of localization in the PM of BFA-treated root epidermal cells. Compared with the control (i.e. only dimethyl sulfoxide [DMSO] treatment), 94.6% of the ABCB4-GFP signal was retained in the PM after treatment with CHX only and
78.0% with cotreatments of CHX and BFA (Fig. 6, A, D, E, and F). The PM PIN2-GFP signal was decreased to 86.7% of the control value after CHX treatment (Fig. 6, B, G, and H), and cotreatment of CHX and BFA reduced the PIN2-GFP signal in the PM to 66.9% (Fig. 6, B, G, and I). In contrast, cotreatment of CHX and BFA reduced the PIN1-GFP signal in the PM to 42.8% of the control level (Fig. 6, C, J, and L), whereas CHX alone slightly decreased the PIN1-GFP signal to 92.9% (Fig. 6, C, J, and K). The differential sensitivities to CHX and/or BFA of ABCB4, PIN2, and PIN1 indicate that their traffickings are differently regulated.

Because ARF-GEFs such as GNOM and GNL1 have been implicated in PIN trafficking, their potential roles in ABCB4 trafficking were investigated using the same BFA concentration as was used for individual experiments. ProABCB4:ABCB4-GFP was introduced into the GNOMM696L-myc line, which is resistant to BFA (Geldner et al., 2003). Similar to observations in the wild-type background, application of 50 μM BFA for 1 h caused the formation of ABCB4-containing BFA compartments in the BFA-resistant GNOMM696L-myc line (Fig. 7, A and B). In the GNOMM696L-myc line, PIN1 did not aggregate in response to 50 μM BFA because BFA sensitivity of PIN1 trafficking is regulated only by GNOM (Geldner et al., 2003). Thus, these data suggest that other BFA-sensitive ARF-GEFs are required for the BFA sensitivity of ABCB4 trafficking, although the involvement of GNOM could not be completely ruled out. Endocytosis of PIN2 is more affected by GNL1, a BFA-insensitive ARF-GEF, than by PIN1, and PIN2 is not properly internalized to the BFA compartments in the gnl1 mutant (Teh and Moore, 2007). If GNL1 mediates the endocytosis of ABCB4, BFA would not be expected to cause ABCB4 internalization. The response of ABCB4 to 25 μM BFA for 1 h did not differ significantly in the GNL1 and gnl1-2 backgrounds (Fig. 7, C and D). These data indicate that, unlike the PIN proteins, ABCB4 trafficking operates independently of GNL1. It will be the next challenge to identify and characterize the responsible ARF-GEFs for ABCB4 trafficking.

Cytochalasin D Treatment Shows That ABCB4 Trafficking Is Actin Dependent But Slower and Less Active Than PIN

To determine whether the cytoskeleton plays an important role in ABCB4 trafficking, we examined the effects of actin and microtubule inhibitors on the localization and the intracellular trafficking of ABCB4. Cytochalasin D (CytD) is an actin-depolymerizing drug that affects the polar localization and trafficking of PIN1 (Geldner et al., 2001). ABCB4 signal barely responded to 30 μM CytD for 2 h (Fig. 8, A and B), whereas PIN1 and PIN2 signals showed intracellular aggregation under the same conditions (Fig. 8, E–H). However, an extended treatment of up to 3 h induced the intracellular aggregation of ABCB4 (Supplemental Fig. S3). In addition, the effect of CytD on ABCB4 trafficking was observed when CytD was cotreated with BFA; the formation of ABCB4-containing BFA compartments was considerably reduced in the presence of CytD (Fig. 8, C and D).
2,3,5-Triiodobenzoic acid (TIBA), a weak auxin as well as an auxin transport inhibitor, also inhibits endocytosis and vesicle motility by interfering with actin stabilization (Dhonukshe et al., 2008a), although ABCC4 localization seemed to be unaffected by prolonged treatment (2 h) with TIBA alone (Fig. 8, I and J). However, pretreatment with TIBA (50 μM for 30 min) followed by cotreatment with BFA inhibited the formation of ABCC4-containing BFA compartments in the root epidermis (Fig. 8K). Similarly, the BFA compartments of ABCC4 were unable to disappear after washing out with TIBA (Fig. 8L).

In contrast to actin, microtubules do not play a key role in ABCC4 trafficking. The microtubule-disrupting agent oryzalin (20 μM for 3 h) did not significantly affect ABCC4 trafficking. Only higher concentrations of oryzalin (40 μM for 3 h) could interfere with the ABCC4 signal in the PM, presumably by causing morphological changes to the cell (Supplemental Fig. S4).

These data demonstrate that actin is involved in the endocytosis of ABCC4 as well as in ABCC4’s targeting to the PM, but the reaction time and degree of intracellular aggregation of ABCC4 after treatment with CytD are different from those of PIN1 and PIN2.

DISCUSSION

Although the intracellular trafficking of PIN proteins has been actively studied, the trafficking of ABCC-type auxin efflux proteins remains poorly understood. This study revealed both similarities and differences in the trafficking of ABCC4 and PINs. PIN proteins show dynamic intracellular trafficking and polar localization in the PM. By contrast, ABCC4 localizes in a nonpolar manner and is stably retained in the PM. The primary intracellular trafficking of ABCC4, which is the degradation pathway to the vacuole, is slow. This endocytic pathway of ABCC4 is routed through SNX1-positive endosomes like PIN2 (Kleine-Vehn et al., 2008b). However, the subcellular localization of SNX1 still remains controversial. Two groups reported that SNX1 localized to the PVC (Jailais et al., 2008; Kleine-Vehn et al., 2008b), whereas another group showed partial colocalization of SNX1 with both PVC and TGN (Phan et al., 2008). Moreover, the specific localization of SNX1 to TGN has been reported recently (Niemes et al., 2010). Our result showed that SNX1 likely localizes to TGN, because the core BFA compartment of ABCC4, which is derived by TGN, is SNX1 positive (Robinson et al., 2008). However, FRAP analysis and the results using Kaede-ABCC4 suggest that ABCC4 is stably localized in the PM and that only undetectable levels of ABCC4 might recycle between the PM and endosomes. So, trafficking of ABCC4 to the vacuole is via SNX1-positive endosomes, and ABCC4-containing BFA compartments...
might be generated by the BFA blocking the degradation pathway. Like PIN2, the BFA sensitivity of ABCB4 is dependent on GNOM and also other ARF-GEFs, whereas the BFA sensitivity of PIN1 trafficking predominantly depends on GNOM (Geldner et al., 2003; Kleine-Vehn et al., 2008b). On the other hand, the endocytosis of ABCB4 was not affected by GNL1, a protein important for the endocytosis of PIN2 (Teh and Moore, 2007). In addition, the endocytic process of ABCB4 is a slow process compared with PIN2. This likely resulted in the slow response of ABCB4 trafficking to CytD and wortmannin.

Although some features of ABCB4 trafficking are characterized in this study, two new questions are raised. First, if ABCB4 trafficking is very slow, how does ABCB4 quickly respond to BFA to make aggregates? We traced the ABCB4 signals right after BFA treatment. Interestingly, BFA initially increased the number of intracellular vesicles from the PM (Supplemental Fig. S5, A and B; Supplemental Movie S1). The BFA action was very fast. The internalizing effect of BFA on ABCB4 vesicles was already observed within 2 min after BFA treatment and lasted up to 20 min, after which they aggregated with each other to form BFA compartments (Supplemental Fig. S5, A and B; Supplemental Movie S1). The BFA-mediated blocking of ABCB4 trafficking to the degradation pathway alone hardly can explain this prompt ABCB4 response to BFA. We speculate that BFA initially promotes the endocytosis of ABCB4 and then blocks the endocytosis of ABCB4 to the vacuole, resulting in endosomal aggregates. The increased endocytosis of PM proteins or tracers into endosomes by BFA treatment has been demonstrated with BR11 (the brassinosteroid receptor) in the Arabidopsis root, FM1-43 in tobacco (Nicotiana tabacum) BY-2 cells, and FM4-64 in the pollen tube of Picea meyeri (Emans et al., 2002; Wang et al., 2005; Geldner et al., 2007).

Second, what is the physiological relevance of auxin transporters such as ABCB4s that are stably associated with the PM? The polarity of PINs is generated and maintained by constant polar recycling of nonpolar PM-localized PINs and can be altered in response to internal and external stimuli (Dhonukshe et al., 2008b; Kleine-Vehn and Friml, 2008; Petrásek and Friml, 2009). These dynamic changes in subcellular PIN localization linked with the dynamic intracellular trafficking of PIN proteins contribute to the modulation of local auxin gradients and thus to the alteration of developmental programs in response to internal and environmental cues (Petrásek and Friml, 2009; Zazimalová et al., 2010). On the contrary, AT-ABCBs are retained in the PM in a nonpolar and persistent manner (Blakeslee et al., 2007; Titapiwatanakun et al., 2009; this study). ABCB4, being nonpolar, may not need similar mechanisms for polarization and hence may not need rapid endocytosis and recycling, which may be suitable for functioning as basal auxin efflux transporters to maintain homeostatic cellular auxin levels. For instance, whereas PIN2 is expressed from the root tip to the elongation zone of root epidermal cells, where it is involved in the basipetal auxin stream and root gravitropism, ABCB4 is expressed in the entire root epidermis (Biliou et al., 2005; Cho et al., 2007). ABCB4 might be implicated in supplying auxin throughout the epidermal cells so that the cells maintain a certain auxin concentration necessary for their growth and other auxin-mediated fundamental events. Stable association of ABCB proteins with the PM might also provide a platform for PIN proteins to reside in the PM. Regardless of relocation, the proximity between PINs and AT-ABCBs may enable effective physical interactions between them. The contribution by ABCB19 to PIN1 function is associated with their colocalization in the membrane microdomains (Titapiwatanakun et al., 2009). ABCB4 was also detectable in the detergent-resistant membrane (Borner et al., 2005). However, whether ABCB4 actually stabilizes PIN2 in the detergent-resistant membrane fraction has yet to be demonstrated.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Columbia ecotype was used as the wild type. The following Arabidopsis mutants and transgenic lines were also employed: abcb4/pgp4 (Cho et al., 2007), gnl1 (Teh and Moore, 2007), ProABCB4:ABC2-GFP (Cho et al., 2007), Pro-35S:ABC2-YFP (Cho et al., 2007), ProPIN2:PIN1-GFP (Lee et al., 2010), ProPIN2:PIN2-GFP (Xu and Scheres, 2005), Pro-35S:mRFP-AF(A7)-RabF2b (Haas et al., 2007), Pro-35S:YFP-RHA1/RabF2a (Haas et al., 2007), ST-DsRed (Saint-Jore et al., 2002), SNX-mRFP (Jailia et al., 2006), and GNOM bleached (Geldner et al., 2003). Seedlings were grown on plates containing one-half-strength (1/2) Murashige and Skoog (MS) nutrient mix (Duchefa Biochemie), 1% Suc, 0.5 g l−1 MES (pH 5.7) with KOH, and 0.8% agarose (SeaKem; Cambrex BioScience Rockland). Seeds were cold treated for 3 d at 4°C and germinated at 23°C under a 16-h-light/8-h-dark photoperiod.

Transgene Constructs

The ProABCB4:Kaede-ABC2 construct was generated by sequential cloning. The first ABC2 genomic fragment, containing the region from +1 to +1,583 (relative to the transcription initiation site), was obtained by PCR using the primers 5′-TGAAACCCCGTGTCGAAGAGCGGC-3′ and 5′-TCT-TTGAGGTCCTAATCTGCATTCAATTC-3′. This fragment was used to replace GFP in the pGPTV-GFP vector (Cho and Cosgrove, 2002). The second ABC2 fragment, containing the region from +1,584 to +4,743, was generated by PCR using the primers 5′-AGGGAGATATGAGGCAAGAAGATCTTTAC-3′ and 5′-ATAAGAGCCTCCTAAGAGCCGGTATAC-3′. This fragment was inserted into the SacI site of the vector carrying the first ABC2 fragment. The Kaede-coding region was obtained by PCR amplification of the pkKaede-N1 vector using the primers 5′-ACCCGCGCCGCTGGTGAATTTG-3′ and 5′-CCCGGGATGGCTTCAGAGAGCGGC-3′. This region was inserted upstream of the first ABC2 fragment. The ABC2 promoter region (ProABCB4) from −12 to −2,174, was obtained by PCR using the primers 5′-ACATGTCGACAAAGAAGATTGCTTCA-3′ and 5′-AGGAGTCGACAGAATTTCCGTTATCC-3′. The promoter region was inserted upstream of the Kaede region in the ProABCB4:Kaede-ABC2 construct.

The constructs confirmed by DNA sequencing were transformed into Arabidopsis (ecotype Columbia) via Agrobacterium tumefaciens (strain C58C1)-mediated infiltration (Bechtold and Pelletier, 1998). The insertion of transgenes was confirmed by PCR analysis of genomic DNA from transformants. Homozygous plants exhibiting a 3:1 segregation ratio on antibiotic plates were selected for further analyses.
Observation of Reporter Markers and Chemical Treatments

Subcellular localization and expression of transporter-reporter fusion proteins was observed in 3- to 4-d-old seedlings. For chemical treatments, 4-d-old seedlings were transferred into 1/2 MS liquid medium containing different chemicals and incubated for the time periods indicated. The control liquid medium and treatments included the same amounts of solvent. PM4-64, BFA, TIBA, Cy3D, naphthylacetic acid, wortmannin, and ESI were dissolved in 100% DMSO. Oryzalin and cytokinines were dissolved with 100% ethanol and water, respectively. Fluorescence from reporter proteins (GFP, yellow fluorescent protein [YFP], and red fluorescent protein [RFP]) was observed using a confocal laser scanning microscope (LSM 510; Carl Zeiss). GFP, YFP, and RFP signals were detected using 488/505- to 530-nm, 514/>53-nm, and 543/45-nm excitation/emission filter sets, respectively. To isolate GFP and YFP for double transgenic lines of ProABCB4-ABC4-GFP and Pro-35S:YFP-RHA1/RabF2a, a 488-nm laser line was employed, images were taken in 11-nm bandwidth from 511 to 618 nm, and then GFP and YFP images were separated through linear unmixing. Fluorescence images were digitalized with LSM Image Browser. Overlapping between ABC4-GFP (or PIN-FP) and the fluorescent molecular markers was qualitatively analyzed where overlapping of the intracellular particles was mostly partial but obvious. The relationships of the various trafficking components and chemicals and the molecular markers are illustrated in Supplemental Figure S6.

LSM 510 quantification software was used to analyze green fluorescence intensity at the apical and/or basal sides at the PM in the epidermis cells of ProABCB4-ABC4-GFP, ProPIN2:PIN2-GFP, and ProPIN2:PIN1-GFP transgenic lines (Fig. 6). Background signals were subtracted.

Photoconversion of Kaede Protein

For green-to-red photoconversion of ROI as in Figure 3, one side of the PM was marked using a rectangle and illuminated with the 364-nm laser at 12.3% excitation power for 1 min. After the photoconversion, green and red signals were detected using the 488/505- to 530-nm and 543/>53-nm excitation/emission filter sets (LSM 510; Carl Zeiss). Images of green-kaede ABC4 and red-kaede ABC4 were taken using fluorescence microscopy (Olympus BX60) for the degradation rate analysis in the PM (Fig. 5, M-O). For the green-to-red photoconversion, the root tip region of ProABCB4:green-kaede ABC4 transformants was subjected to 1 min of UV illumination under a 20× objective lens. Images of green- and red-kaede ABC4 were taken after 0, 3, 5, 7, and 9 h of incubation in 1/2 MS liquid medium from a different set of seedlings to rule out photobleaching damage. The green and red signals were detected by 488-nm excitation and greater than 505-nm emission filters and measured every 20 s for 20 min after photobleaching and then every 1 min for the next 40 min. After photobleaching, the intensity of green fluorescence was determined using the LSM 510 quantification software. To correct for the variation in expression levels of ABCB4-GFP and PIN2-GFP, fluorescence intensity in the PM were measured using ImageJ.

FRAP Analysis

The transgenic lines ProABCB4-ABC4-GFP and ProPIN2:PIN2-GFP were used for analyses. Seedlings were mounted on a glass slide containing 1.5% agarose and 1/2 MS nutrient mix. For fixing, roots were covered with a coverslip and the edges of the coverslip were attached to the slide with tape. FRAP analysis was performed with an LSM 510 confocal laser scanning microscope equipped with a water-corrected 40× objective, numerical aperture = 1.2 (Carl Zeiss). For bleaching, 2- or 4-μm-wide ROIs were selected and then bleached with 100 iterations of a 488-nm argon laser at full power. GFP recoveries were detected by 488-nm excitation and greater than 530-nm emission filters and measured every 20 s for 20 min after photobleaching and then every 1 min for the next 40 min. After photobleaching, the intensity of green fluorescence was determined using the LSM 510 quantification software. To correct for the variation in expression levels of ABCB4-GFP and PIN2-GFP, values were normalized with the following equation (Goodwin and Kenworthy, 2005):

\[
F(t)_{\text{norm}} = 100 \times \frac{F(t)_{\text{ROI}} - F_{\text{bkgd}}}{F_{\text{ROI}} - F_{\text{bkgd}}}
\]

where \(F(t)_{\text{ROI}}\) = fluorescence of ROI for each time point (t), \(F_{\text{bkgd}}\) = background fluorescence, \(F(t)_{\text{bkgd}}\) = fluorescence of a comparable neighbor PM for each time point, \(F_{\text{ROI}}\) = initial fluorescence of ROI, and \(F_{\text{bkgd}}\) = initial fluorescence of a comparable neighbor PM.

The maximum recoverable fluorescence intensity (\(F_{\text{m}}\) and \(t_{1/2}\)) required to reach the recoverable fluorescence for a Gaussian spot were calculated using nonlinear regression analysis and fitted to the following equation with SPSS (Statistical Package for the Social Sciences; IBM) software (\(r^2 > 95\%\)):

\[
F(t) = F_0 + F_a(t/t_{1/2}) / (1 + (t/t_{1/2}))
\]

where \(F_0\) = initial fluorescence and \(t\) = time. The value derived from 2 μm was used for the \(F_a\) value of the 4-μm width. Statistical analyses were performed using Student’s t test.

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: GN1 (At1g53950), GNOM (At1g3980), ABCB4/PG4 (At2g47000), PIN1 (At1g73590), PIN2 (At5g57090), SMT1 (At5g13710), SNXI (At5g61640), RHA1/RabF2a (At5g45130), ARF7/RabF2b (At4g19640), and ST (At2g03760).

Supplemental Data

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

Supplemental Figure S1. ESI does not alter ABCB4 trafficking.

Supplemental Figure S2. ProABCB4:Kaede-ABCB4 transgenic plants partially complements the abcb4 root-hair phenotype.

Supplemental Figure S3. ABCB4 trafficking is less affected by CytD.

Supplemental Figure S4. ABCB4 trafficking does not depend on microtubules.

Supplemental Figure S5. BFA increases the endosomal abundance of ABCB4.

Supplemental Figure S6. Endomembrane trafficking pathways with the trafficking components and their inhibitors.

Supplemental Table S1. Summary of FRAP analysis.

Supplemental Movie S1. Endocytic trafficking of ABCB4 after BFA treatment.

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