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Title	Polar localization and degradation of Arabidopsis boron transporters through distinct trafficking pathways
Author(s)	Takano, Junpei; Tanaka, Mayuki; Toyoda, Atsushi et al.
Citation	Proceedings of the National Academy of Sciences of the United States of America, 107(11), 5220-5225 <a href="https://doi.org/10.1073/pnas.0910744107">https://doi.org/10.1073/pnas.0910744107</a>
Issue Date	2010-03-16
Doc URL	<a href="https://hdl.handle.net/2115/49101">https://hdl.handle.net/2115/49101</a>
Type	journal article
File Information	PNAS107-11_5220-5225.pdf



**Classification:** BIOLOGICAL SCIENCES, PLANT BIOLOGY

**Title:** Polar localization and degradation of Arabidopsis boron transporters through distinct trafficking pathways

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**Manuscript information:**

The number of text pages: 25

The number of figures: 5

The number of tables: 0

## **Abstract**

Boron (B) is essential for plant growth, but is toxic when present in excess. In the roots of *Arabidopsis thaliana* under B limitation, a boric acid channel, NIP5;1, and a boric acid/borate exporter, BOR1, are required for efficient B uptake and subsequent translocation into the xylem, respectively. However, under high B conditions, BOR1 activity is repressed through endocytic degradation, presumably to avoid B toxicity. In this study, we investigated the localization of GFP-tagged NIP5;1 and BOR1 expressed under the control of their native promoters. Under B limitation, GFP-NIP5;1 and BOR1-GFP localized preferentially in outer (distal) and inner (proximal) plasma membrane (PM) domains, respectively, of various root cells. The polar localization of the boric acid channel and boric acid/borate exporter indicates the radial transport route of B toward the stele. Furthermore, mutational analysis revealed a requirement of tyrosine residues, in a probable cytoplasmic loop region of BOR1, for polar localization in various cells of the meristem and elongation zone. The same tyrosine residues were also required for vacuolar targeting upon high B supply. The present study of BOR1 and NIP5;1 demonstrates the importance of selective endocytic trafficking in polar localization and degradation of plant nutrient transporters for radial transport and homeostasis of plant mineral nutrients.

'\body'

## **Introduction**

Plant growth depends on nutrient acquisition from the soil. Roots are multicellular organs and are responsible for nutrient uptake and transport to shoots. For the directional transport of nutrients, localization of transporters to specific membrane domains in specific cell types is required. In animal epithelial cells, polar localization of transporters at the apical or basolateral plasma membrane (PM) allows directional transcellular transport of nutrients. Several recent studies have demonstrated polar localization of mineral nutrient transporters in plant cells. The silicic acid channel Lsi1 (OsNIP2;1) and the silicon exporter Lsi2 localize to the outer PM domain and inner PM domain, respectively, in the exodermis and endodermis, thus driving directional transcellular transport of silicon in rice roots (1, 2).

Boron (B) is an essential nutrient for plants, and B deficiency is a major cause of reduced crop production (3). Boron is essential for the structure and function of the plant cell wall, as it cross-links the pectic polysaccharide rhamnogalacturonan II (4). Boron is also known to be toxic when present in excess, and B toxicity is a worldwide problem in food production, especially in arid areas (5). In solution at physiological pH, and in the absence of interactions with biomolecules, B mainly exists as uncharged boric acid ( $\text{B(OH)}_3$ ). Boric acid is a weak Lewis acid with a  $pK_a$  of 9.24 [ $\text{B(OH)}_3 + \text{H}_2\text{O} = \text{B(OH)}_4^- + \text{H}^+$ ] (6). It has long been believed that passive diffusion of boric acid across the lipid bilayer constitutes the major and possibly the only mechanism for membrane transport of B.

In the last several years, two different types of boric acid/borate transporters were discovered to be operating under B limitation in *Arabidopsis thaliana* (reviewed in 7). One is NIP5;1, a member of the major intrinsic protein (MIP) family; it functions as a major boric acid channel in the PM. NIP5;1 is essential for efficient B import into roots under conditions of B limitation (8). The other transporter is BOR1, a PM boric acid/borate exporter, and is essential for efficient xylem loading of B (9). BOR1 homologs are also involved in B toxicity tolerance in plants (10, 11). BOR4, one of six BOR1 paralogs (BOR2-BOR7) in *A. thaliana*, is also a PM boric acid/borate exporter; its overexpression results in efficient efflux of B from the roots and significant growth improvement at toxic concentrations of B (mM range) (10). Interestingly, GFP-tagged BOR4 localizes in a polar manner to the PM of the outer (distal) side of root epidermal cells.

To avoid problems related to B deficiency or toxicity, the transport of B must be tightly regulated in order to maintain an acceptable level of radial transport of B in roots. Under B limitation, the uptake of B into *A. thaliana* roots was shown to be upregulated mainly through the upregulation of *NIP5;1* mRNA (8). In addition, the rate of root-to-shoot translocation of B was upregulated under B limitation and was rapidly downregulated after exposure to high levels of B (12). This downregulation was attributed to the post-transcriptional regulation of BOR1 (12). The level of *BOR1* mRNA was largely unaffected by the B conditions tested. In root tip cells of transgenic *A. thaliana* plants constitutively expressing BOR1-GFP fusion protein under the control of the cauliflower mosaic virus 35S RNA promoter, BOR1-GFP localized to the PM under B

limitation and was transferred to endosomes and then degraded at high levels of B supply.

Here, we investigated the localization of GFP-tagged NIP5;1 and BOR1 expressed under the control of their native promoters. In the PM of root cells, GFP-NIP5;1 and BOR1-GFP localized preferentially in outer (distal) and inner (proximal) domains, respectively. At high B supply, BOR1-GFP, but not GFP-NIP5;1, was selectively transported to the vacuole for degradation. The differential polar localization of B transporters in *A. thaliana* and their responses to B conditions provide a model for studying mechanisms of lateral polar localization and endocytic degradation of PM proteins in plants.

In animal epithelial cells, the mechanisms for polar localization of nutrient transporters at the apical or basolateral PM have been intensively studied. The apical and basolateral PMs are separated by a tight junction, which provides a diffusion barrier, and polar sorting or retention involves sorting signals embedded in the cytosolic parts of membrane proteins, glycosylation, or binding to scaffold proteins (13). Sorting signals are also involved in selective internalization and lysosomal targeting for degradation of PM proteins (14). Our mutational analysis suggested that tyrosine-based sorting signals embedded in a probable cytoplasmic loop region of BOR1 are required for its polar trafficking and endocytic degradation.

## **Results**

### **Polar localization of NIP5;1 and BOR1**

The subcellular localization of NIP5;1 was examined using transgenic plants expressing

NIP5;1 that was N-terminally tagged with GFP (GFP-NIP5;1), under control of the *NIP5;1* promoter. Confocal laser scanning microscopy revealed that GFP-NIP5;1 was localized preferentially in the outer (distal) PM of lateral root cap cells and epidermal cells of the meristem and elongation zones (Fig. 1A-C). Consistent with data obtained using a *NIP5;1* promoter: $\beta$ -glucuronidase (GUS) construct (8), the GFP signal decreased toward the basal region. To confirm the activity of the GFP-NIP5;1 protein, we introduced the construct into the *nip5;1-1* mutant. The expression of GFP-NIP5;1 under the control of the *NIP5;1* promoter partially complemented the growth defect of the mutant under B limitation in all transgenic lines tested (Fig. S1A) although the accumulation of *GFP-NIP5;1* mRNA were lower than that of *NIP5;1* mRNA in the wild-type plants (Fig. S1B). These results suggest that GFP-NIP5;1 is functional *in planta* and that the pattern of GFP-NIP5;1 expression represents, at least in part, the native pattern of NIP5;1 expression. The polar localization of GFP-NIP5;1 to the outer PM domain in epidermal cells is likely to be important for efficient uptake of B from the soil solution into roots (8).

We then analyzed the subcellular localization of BOR1 using transgenic plants that expressed BOR1-GFP under the control of the *BOR1* promoter. The expression of BOR1-GFP in the *bor1-1* mutant fully complemented the growth defect under limited B supply (Fig. S2). Remarkably, BOR1-GFP was localized to the inner (proximal) PM domain in the columella, lateral root cap, epidermis, and endodermis in the root tip region, and in the epidermis and endodermis in the elongation zone (Fig. 1D, E). This localization pattern suggests that B is directionally transported toward the stele by the

boric acid/borate exporter in the meristem and elongation zone. BOR1-GFP was also observed in the epidermis, cortex, endodermis, and stele cells of the root hair zone (Fig. 1F). Interestingly, polar localization to the inner PM domain was clearly observed in the endodermis, but was less evident in other cell types in the root hair zone (Fig. 1F). At the cell wall of the endodermis, the Casparian strip develops to prevent apoplastic flow of solutes in and out of the stele. The blockage of apoplastic flow was visualized with cell wall staining using a membrane-impermeable dye, propidium iodide (PI), applied in the medium (Fig. 1G). The relatively strong localization of BOR1 at the inner side of mature endodermal cells, which is not reached from the outside by PI (Fig. 1F, G), suggests a role for BOR1 in B loading to the stele and, at the same time, preventing B leakage from the stele. This is consistent with our previous observation that BOR1 is required for efficient loading of B into the xylem (9).

The distinct inner PM localization of BOR1-GFP in mature endodermal cells implied the existence of a specialized mechanism for polar localization in this cell type. In accordance with this, the fluorescent lipophilic styryl dye FM4-64, which does not permeate the membrane and is routinely used as an endocytic tracer, stained the outer side of the PM, but barely labeled the inner PM in mature endodermal cells, even when the dye was applied for 3 h (Fig. 1H). In the endodermis, the PM tightly adheres to the cell wall where Casparian strips develop (15). It is likely that this region functions similarly to the tight junction in animal epithelial cells to limit lateral diffusion of PM proteins. This suggestion is intensively investigated and discussed in the accompanying paper by Alassimone *et al.*

We previously reported that BOR1-GFP accumulated in brefeldin A (BFA)-induced aggregated endosomes, the so-called BFA compartments, within 30 min under both low and high B conditions (12). BFA inhibits a subclass of ARF-GEFs, inhibits the recycling of PM proteins such as PIN auxin efflux carriers to the PM, and causes endosomal aggregation in root cells of *A. thaliana* (16). Here we confirmed that BOR1-GFP accumulates in BFA compartments under low B condition and showed that the response is reversible (Fig. S3). In contrast to BOR1-GFP, little GFP-NIP5;1 accumulated in BFA compartments in the presence of cycloheximide (CHX), a protein synthesis inhibitor (Fig. S4A-F). It remains to be determined whether the different sensitivities of GFP-NIP5;1 and BOR1-GFP to BFA reflect different rates or routes of recycling between the PM and endosomal compartments.

### **Vacuolar trafficking of BOR1, but not NIP5;1, is dependent on boron nutritional status**

The transport activities of NIP5;1 and BOR1 are required for plant growth under B limitation (8, 9), whereas these activities may be detrimental under high B conditions. As previously demonstrated for BOR1-GFP expressed under the control of the cauliflower mosaic virus 35S RNA promoter (12), BOR1-GFP expressed under the control of the *BOR1* promoter in various root cells disappeared within 2 h of exposure to 100  $\mu$ M B (+B, Fig. 2A). Before being degraded, BOR1-GFP was observed in punctate structures, most of which co-labeled with mCherry-RabF2a/Rha1, a multivesicular body (MVB)/late endosome (LE) marker (Fig. 2B) (17). Based on results using concanamycin A, a specific

V-ATPase inhibitor that inhibits GFP degradation in the vacuole, we suggested that BOR1-GFP is finally degraded in the vacuole (12). Here, we confirmed this using a simple method based on the fact that GFP is relatively stable in the vacuole in the absence of light (18). When plant roots were incubated in low-B medium (−B) in the absence of light for 3 h, BOR1-GFP fluorescence was detected in the PM, but not in the vacuole, of epidermal cells (Fig. 2C). However, in high-B medium (+B) in the absence of light, BOR1-GFP fluorescence was detected in the vacuole (Fig. 2C), which was surrounded by a tonoplast that stained with the endocytic dye FM4-64 (Fig. S5A). These results confirm that BOR1-GFP is internalized from the PM and is transported to the vacuole through the MVB/LE pathway. As was shown for the PIN2 auxin carrier and BRI1 steroid receptor kinase (19, 20), BFA treatment inhibited the appearance of BOR1-GFP fluorescence in the vacuole, resulting in the accumulation of BOR1-GFP in aggregated endosomes, together with FM4-64 (Fig. S5B). These results suggest that vacuolar trafficking of BOR1 involves BFA-sensitive endosomes in a pathway shared with PIN2 and BRI1.

We then examined whether B conditions affect GFP-NIP5;1 localization. In contrast to BOR1-GFP localization, GFP-NIP5;1 localization to the PM did not appear to be affected by exposure to high B for 2 h (Fig. 2D). After 5 h in high B, GFP-NIP5;1 was still observed in the PM, although the GFP fluorescence level had apparently decreased. These results suggest that NIP5;1 is not regulated by a rapid change of trafficking toward the vacuole, as found for BOR1, but is likely controlled by constitutive degradation following B-dependent regulation of *NIP5;1* mRNA levels (8).

## **Tyrosine-based signals contribute to polar localization of BOR1**

The tyrosine-based signal YxxΦ, where Y is tyrosine, x is any amino acid, and Φ is any bulky hydrophobic residue, is well established as a membrane protein signal for endocytosis and sorting to lysosomes in mammals (14). The tyrosine-based signal is also responsible for basolateral protein sorting in polarized epithelial cells (13). Tyrosine-based signals recognized by a medium (μ) subunit of adaptor protein (AP) complexes sort proteins into coated vesicles. In *A. thaliana* cells, a tyrosine-based signal in the cytosolic tail of a vacuolar sorting receptor was shown to interact with a μ subunit of AP complexes (21). Mutational analysis of tyrosine-based signals has revealed their roles in Golgi-to-prevacuolar compartment transport of a vacuolar sorting receptor (22) and in ligand-induced endocytosis of a leucine-rich repeat receptor-like protein, LeEix2 (23). Three tyrosine-based signals (Y373QLL, Y398DNM, and Y405HHM) were deduced in the predicted largest loop of BOR1 (Fig. 3A). BOR1 and its homologs are predicted to have 9–11 transmembrane domains, depending on the prediction programs used (<http://aramemnon.botanik.uni-koeln.de>). For BOR1, the consensus prediction of 16 programs was two transmembrane domains between the largest loop region and the C-terminal region. A large-scale phospho-proteome approach in *A. thaliana* identified phosphorylation in the C-terminal tail of BOR3, a BOR1 homolog, suggesting the cytosolic location of the C-terminal tail of BOR1 homologs (24). Therefore, the largest loop regions of BOR1, containing putative tyrosine-based signals, are likely located in the cytosol. The NIP5;1 sequence contains two potential tyrosine-based signals, but these are located in a predicted transmembrane domain and an extracellular loop region.

To investigate the role of each putative tyrosine-based signal in endocytic degradation and polar localization of BOR1, transgenic plants expressing mutant-type BOR1 proteins fused to GFP were generated. In these mutants, tyrosine residues in the putative tyrosine-based signals were substituted with alanine. Under low B conditions, BOR1(Y373A)-GFP showed polar localization similar to that of WT BOR1-GFP (Fig. 3B; Fig. 1D, E). However, the polar localization of BOR1(Y398A)-GFP and BOR1(Y405A)-GFP was less clear (Fig. 3C, D), indicating the importance of Y398 and Y405 for polar localization of BOR1. Mutant BOR1-GFP in which both Y398 and Y405 were substituted with alanine, BOR1(Y398A/Y405A)-GFP, showed even less polar localization (Fig. 3E). Strikingly, BOR1(Y373A/Y398A/Y405A)-GFP, in which tyrosine was substituted with alanine at all three putative tyrosine-based signals, showed almost apolar localization (Fig. 3F). However, in endodermal cells of the root hair zone, BOR1(Y373A/Y398A/Y405A)-GFP preferentially localized to the inner PM domain (Fig. 3G), with a distribution similar to that of wild-type BOR1-GFP (Fig. 1F). Thus, the tyrosine-based mechanism is important, but may not be the only mechanism of BOR1 polar localization.

To investigate the trafficking step that requires tyrosine residues, we treated plant roots expressing BOR1(Y373A/Y398A/Y405A)-GFP with BFA in the presence of CHX. With 10 or 50  $\mu$ M BFA, BOR1(Y373A/Y398A/Y405A)-GFP accumulated in BFA compartments (Fig. 3I; S4G-I), as did wild-type BOR1-GFP (Fig. 3H; S4D-F), in epidermal cells. Therefore, tyrosine residues may not to be involved in endocytosis, but rather in post-endocytic trafficking of BOR1. However, the existence of multiple

mechanisms of BOR1 endocytosis, dependent and independent of tyrosine-based signals, cannot be excluded.

### **Slow lateral diffusion of BOR1 and NIP5;1 in the PM**

In *Saccharomyces cerevisiae*, slow lateral diffusion and endocytic cycling maintain polar localization of PM proteins in the mating projection without the need for diffusion barriers (25). It is reasonable to hypothesize that the polarity of B transporters in plants is kinetically maintained by similar mechanisms and that the tyrosine-based signals of BOR1 are involved in this process. To measure the lateral diffusion velocity of BOR1-GFP and BOR1(Y373A/Y398A/Y405A)-GFP, we performed fluorescence recovery after photobleaching (FRAP) analyses in elongating epidermal cells. After 2- $\mu$ m regions of the apical and basal PMs were bleached, the recovery of fluorescence into these regions was monitored. BOR1-GFP and BOR1(Y373A/Y398A/Y405A)-GFP had similar recovery rates, each reaching a constant level within ~20 min (Fig. 4A-C). Incubation with CHX did not significantly affect the rate of BOR1-GFP diffusion (Fig. 4C), indicating little contribution of biosynthetic trafficking. Thus, the non-polarized distribution of the mutant protein is not simply due to faster lateral diffusion in the PM, but is likely attributable to defective polar trafficking to the PM. FRAP analysis of GFP-NIP5;1 in the outer PM of elongating epidermal cells gave a recovery rates similar to that of BOR1-GFP (Fig. 4D). These recoveries were comparable to that of PIN2 auxin carrier and much slower than the rapid recovery of a small PM marker protein, GFP-Lti6a, which occurred within ~2 min (26). Slow diffusion may be a basis for maintaining the

polarity of PM proteins by endocytic recycling in plant cells without a diffusion barrier.

### **Tyrosine-based signals control B-induced BOR1 turnover by endocytic traffic to vacuoles**

We tested the effect of mutations of the tyrosine residues on vacuolar trafficking of BOR1 by transferring plants from low-B to high-B medium. Upon high B treatment, BOR1(Y373A)-GFP showed degradation similar to that of BOR1-GFP (Fig. 5A; Fig. 2A). However, BOR1(Y398A)-GFP and BOR1(Y405A)-GFP showed slower degradation (Fig. 5B, C). Strikingly, the double mutant BOR1(Y398A/Y405A)-GFP and triple mutant BOR1(Y373A/Y398A/Y405A)-GFP remained at the PM for 5 h after transfer to high-B medium (Fig. 5 D, E). Immunoblot analysis of the microsomal fraction of roots confirmed that BOR1(Y373A/Y398A/Y405A)-GFP did not undergo B-dependent degradation after treatment with high B for 2 h (+B2h, Fig. 5F). These results indicate that multiple tyrosine residues in the large loop region are required for the polar localization and vacuolar targeting of BOR1. When plants were grown with a continuous supply of high B (+B, 100  $\mu$ M), BOR1(Y373A/Y398A/Y405A)-GFP was significantly accumulated, whereas BOR1-GFP was barely detectable (Fig. 5F; Fig. S6), suggesting that vacuolar targeting dependent on tyrosine-based signals is a major mechanism controlling the BOR1 level under sufficient B conditions. We then tested the importance of the tyrosine residues under toxic B conditions. When plants were continuously supplied with 1 mM or 3 mM B, neither BOR1(Y373A/Y398A/Y405A)-GFP nor BOR1-GFP was significantly accumulated in the PM (Fig. S6). However, when plants

grown in low B were supplied with 3 mM B for 5 h, BOR1(Y373A/Y398A/Y405A)-GFP remained at the PM, but BOR1-GFP disappeared (Fig. S6). These results suggest that BOR1 biosynthesis is controlled in response to B conditions and that vacuolar targeting dependent on tyrosine-based signals rapidly and efficiently downregulates BOR1 accumulated in the PM.

To investigate the physiological relevance of polar localization and B-dependent degradation of BOR1, we observed the B-dependent growth characteristics of transgenic *bor1-1* lines expressing BOR1(Y373A/Y398A/Y405A)-GFP. Despite its apolar localization in root tip cells (Fig. 3F), BOR1(Y373A/Y398A/Y405A)-GFP rescued the shoot growth defect of the *bor1-1* mutant under low B conditions (Fig. S2). This may be attributable to the polar localization of BOR1(Y373A/Y398A/Y405A)-GFP in the inner PM domain of endodermal cells of the root hair zone (Fig. 3G) and a possible redundant function of a BOR1 homolog in root tip cells. We failed to find different sensitivities to B toxicity in the transgenic lines with BOR1(Y373A/Y398A/Y405A)-GFP and BOR1-GFP (Fig. S2), possibly owing to an additional mechanism of BOR1 downregulation independent of the tyrosine-based mechanism in toxic B conditions (Fig. S6) and B extrusion from root surfaces by BOR4 (10).

## **Discussion**

In this study, we demonstrated the lateral polar localization of GFP-NIP5;1 and BOR1-GFP to opposite PM domains. GFP-NIP5;1 localized to the outer PM domain in lateral root cap cells and epidermal cells (Fig. 1A-C). In contrast, BOR1-GFP localized to

the inner PM domain of columella and lateral root cap, epidermal, and endodermal cells, when expressed under the control of the *BOR1* promoter (Fig. 1D-H). These patterns of NIP5;1 and BOR1 localization illustrate the radial transport route of B toward the stele. Boric acid, the main form of B in physiological solutions, permeates the lipid bilayer by passive diffusion and by facilitated diffusion via members of the MIP family (27-30). In roots of *A. thaliana* under B limitation, the MIP protein NIP5;1 functions as a major boric acid channel for B uptake, and the boric acid/borate exporter BOR1 mediates efficient translocation of B to the xylem (8, 9). BOR1 and its homologs are boric acid/borate exporters, capable of generating a concentration gradient of B across the PM (9, 31, 32). It is conceivable that boric acid/borate export by BOR1 at the inner PM domain indirectly drives boric acid import through NIP5;1 located at the outer PM domain of the same cell or of cells symplastically connected by plasmodesmata. In this regard, our observation that BOR1 was downregulated faster than NIP5;1 in response to high B (Fig. 2) provides a reasonable mechanism for the immediate shut-down of radial B transport to avoid B toxicity.

In animal epithelial and nerve cells, PM domains are separated by diffusion barriers, i.e., tight junctions in epithelial cells and axonal hillocks in neurons, which maintain the polarized distribution of PM proteins in these cells. In contrast, yeast cells have no diffusion barriers during mating, and the localization of PM proteins to the tips of polarized mating intermediates is maintained kinetically by localized recycling, which occurs faster than protein diffusion in the PM owing to the slow lateral diffusion rates of PM proteins in yeast (25). In plant root cells without a diffusion barrier, the apical-basal

polarity of PIN auxin carriers is maintained by post-endocytic trafficking (33). Specifically, PINs are secreted non-polarly and polarity is subsequently generated by endocytosis and recycling (34). Here, we propose a recycling-based mechanism for the polar localization of BOR1 to the inner PM domain. In mature endodermal cells, the diffusion barrier at the Casparian strip domain appears to help maintain the polar localization of BOR1 (Fig. 1*F-H*; Fig. 3*G*; also see accompanying paper by Alassimone *et al.*). However, polar localization of BOR1 is also maintained in cell types without a diffusion barrier (Fig. 1*D, E*). In epidermal cells, the lateral diffusion rate of BOR1-GFP estimated by FRAP analysis (Fig. 4*A, C*) was comparable to that of PIN2-GFP, but was much slower than that of the small non-polarized PM protein GFP-Lti6a (26). This implies that BOR1 lateral diffusion is sufficiently slow to allow polar localization by a recycling-based mechanism, as shown for PINs (34). The mutational analysis revealed that tyrosine residues in putative tyrosine-based sorting signals of BOR1 are required for polar localization in elongating epidermal cells and root cap cells (Fig. 3*B-F*). Given that BOR1(Y373A/Y398A/Y405A)-GFP accumulated in BFA compartments in a manner similar to WT BOR1-GFP (Fig. 3*H, I*; Fig. S4*E, F, H, I*), it is likely that the tyrosine residues are not involved in endocytosis. Considering these findings, a plausible model is that BOR1 polarity is maintained by post-endocytic recycling to the inner PM domain via a pathway requiring the tyrosine-based signals (Fig. S7). Inhibition of a polar targeting pathway due to tyrosine mutations might have resulted in non-polar recycling and thus non-polar distribution of BOR1-GFP. However, BOR1(Y373A/Y398A/Y405A)-GFP still preferentially localized to the inner PM domain in endodermal cells (Fig. 3*G*),

suggesting that polar recycling dependent on tyrosine residues is not the only mechanism of polar localization of BOR1. An alternative mechanism may be polar biosynthetic trafficking to the inner PM domain from the TGN, or preferential endocytosis from the outer PM domain (Fig. S7). Further studies, including the identification of adaptor proteins recognizing the tyrosine-based signals of BOR1, are needed to define the polar trafficking pathway of BOR1.

The present study also confirmed our previous finding that BOR1 is transported via endosomes to the vacuole for degradation upon high B supply (Fig. 2A-C) (12) and revealed that the tyrosine residues in the large loop region of BOR1 are a requirement common to the vacuolar and polar trafficking pathways (Figs. 3 and 5; Fig. S7). In epithelial cells, a tyrosine-based signal located in aquaporin 4 (AQP4) determines its clathrin-dependent endocytosis through an interaction with the  $\mu$  subunit of AP2 adaptor complex and its lysosomal targeting through interactions with the  $\mu$  subunit of AP3 in epithelial cells (35); the stress-induced kinase CKII phosphorylates the serine immediately preceding the tyrosine motif of AQP4, increasing its interaction with  $\mu$ 3 and enhancing its lysosomal targeting and degradation. We propose a similar switch for the B-dependent regulation of BOR1 trafficking.

In contrast to BOR1, NIP5;1 does not contain a potential tyrosine-based signal in an intracellular loop region. In animal epithelial cells, tyrosine-based signals are involved in basolateral localization, but apparently not in apical localization. Apical localization signals include extracellular *N*- and *O*-linked glycosylation, a glycosyl phosphoinositol (GPI)-lipid anchor, and cytoplasmic domains that interact with scaffolding proteins (13).

NIP5;1 contains no apparent extracellular *N*- or *O*-linked glycosylation sites and no GPI-lipid anchor. The slow lateral diffusion rate of GFP-NIP5;1 in the PM, comparable to the rates of BOR1-GFP and PIN2-GFP (Fig. 4) (26), may be responsible for maintaining its polar localization, although this must be verified by further studies. In the apical membrane of renal epithelial OK cells, FRAP analysis showed that the lateral mobility of a Na<sup>+</sup>/H<sup>+</sup> exchanger, NHE3, was limited by interactions with NHERF1/2 (36), which are PDZ domain-containing scaffold proteins that interact with a cytoplasmic C-terminal domain of NHE3. Future analysis of protein complexes that include NIP5;1 and BOR1 may reveal a mechanism for limited lateral diffusion and polar localization in the PM of plant cells.

In conclusion, our study demonstrated distinct polar localization of NIP5;1 and BOR1 to outer and inner PM domains, respectively, and the importance of selective endocytic trafficking for the polar localization and vacuolar targeting of BOR1. NIP5;1 and BOR1 provide valuable models for understanding mechanisms of lateral polar localization and endocytic degradation, which are presumably common to a number of plant nutrient transporters, in controlling radial transport and homeostasis of nutrients.

## **Materials and Methods**

### **Plant material and growth conditions**

Col-0 wild-type, the *bor1-1* mutant (37), and the *nip5;1-1* mutant (8) of *A. thaliana* (L.) Heynh. were from our laboratory stocks. The *NIP5;1 promoter::GFP-NIP5;1* construct (described in *Supplemental Methods*) was used to transform Col-0 for GFP imaging and

to transform the *nip5;1-1* mutant (8) for plant growth assays. The *BORI promoter:BORI-GFP* construct and its derivatives (described in *Supplemental Methods*) were used to transform the *bor1-1* mutant. The mCherry-RabF2a/Rha1 line (17) was provided by Niko Geldner and was crossed with the transgenic *BORI promoter:BORI-GFP* line. Plants were grown in growth chambers at 22°C under fluorescent lamps with a 16-h/8-h light/dark cycle. MGRL medium (38) containing 50 µM Fe-EDTA and 0.3 µM boric acid (–B) or 100 µM boric acid (+B) was used. For further details, see *Supplemental Methods*.

### **Acknowledgment**

We are grateful to Y. Kawara and K. Konishi for excellent technical assistance; D. Eide for critical reading of the manuscript; T. Ueda, S. Naramoto, K. Sahara, and M. Watahiki for discussions and advice on imaging; and N. Geldner and J. Alassimone for discussions and sharing of unpublished data. This work was supported in part by a Grant-in Aid for JSPS Fellows from the Japanese Society for the Promotion of Science (to J.T.), a Grant-in Aid for Young Scientists (A, to J.T.), a Grant-in-Aid for Scientific Research (S, to T.F.), and a Grant-in-Aid for Scientific Research Priority Areas (to T.F.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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### Figure legends

**Fig. 1.** Polar localization of GFP-NIP5;1 and BOR1-GFP in root cells.

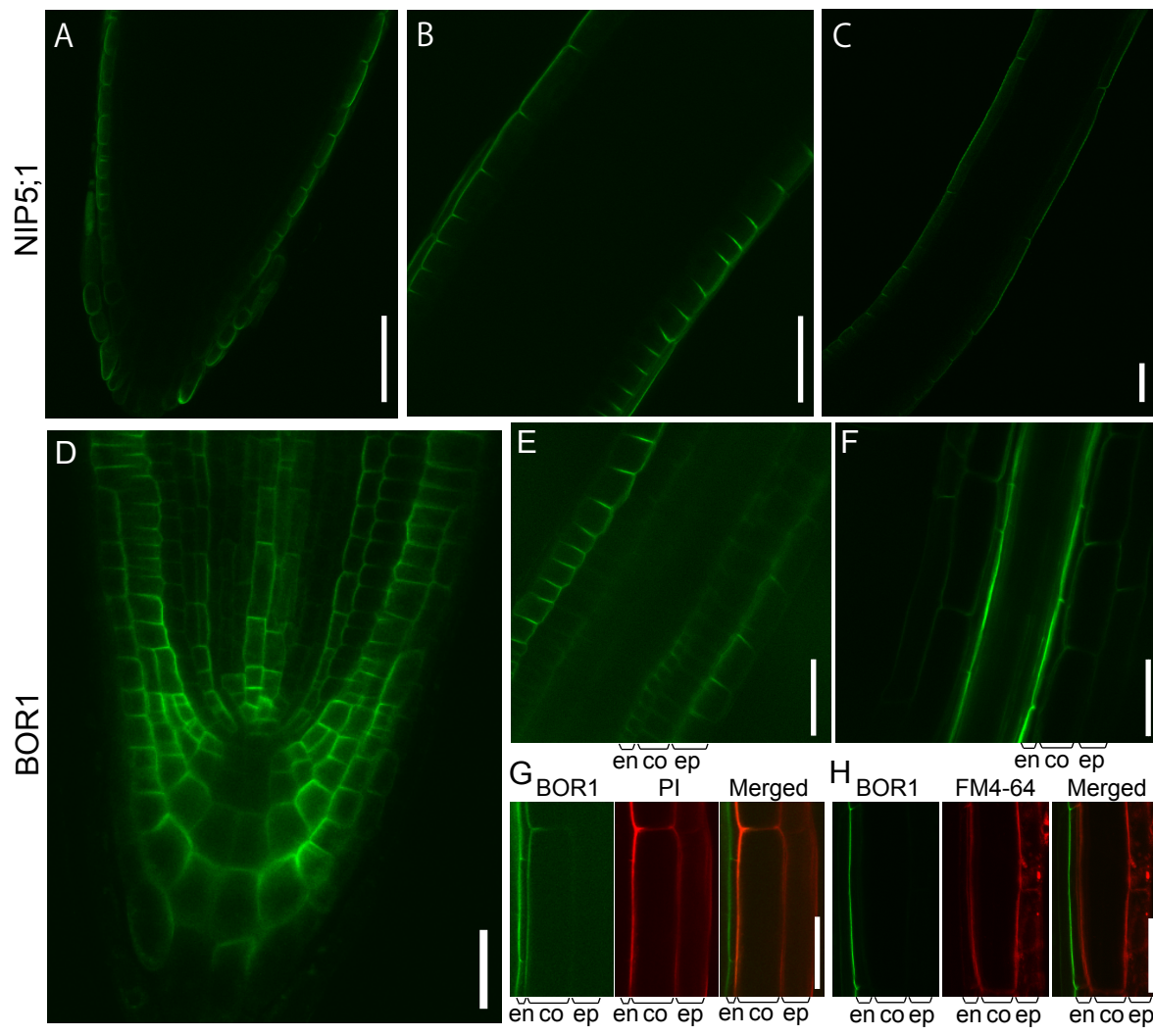
(A-C) GFP-NIP5;1 in the root tip (A) and elongation zone (B, C) of roots under low B conditions. (D-F) BOR1-GFP in the root tip (D), elongation zone (E), and root hair zone (F) of roots under low B conditions. (G) BOR1-GFP and propidium iodide (PI)-stained cell wall in the root hair zone. (H) BOR1-GFP and FM4-64-stained membranes in the root hair zone. FM4-64 was applied for 3 h. en, endodermis; co, cortex; ep, epidermis. Bars = 50  $\mu$ m.

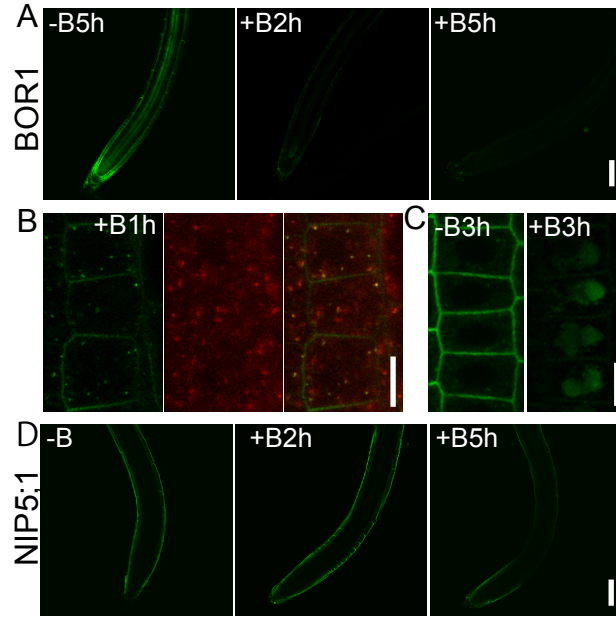
**Fig. 2.** Boron-dependent degradation of BOR1-GFP, but not GFP-NIP5;1, in roots. BOR1-GFP (A-C) or GFP-NIP5;1 (D) in plants grown on low-B medium and then transferred to low-B medium (-B) or high-B medium (+B) for the indicated time periods. (B) Colocalization of BOR1-GFP (green) and mCherry-RabF2a/Rha1 (red) in epidermal cells. (C) GFP fluorescence derived from BOR1-GFP in the vacuole of epidermal cells in the absence of light under high B conditions, but not under low B conditions. Bars = 100  $\mu$ m (A and D) or 10  $\mu$ m (B and C).

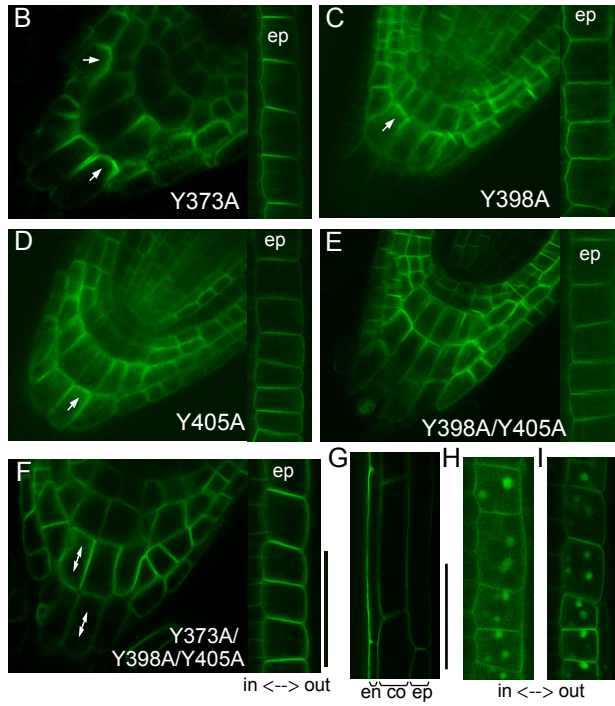
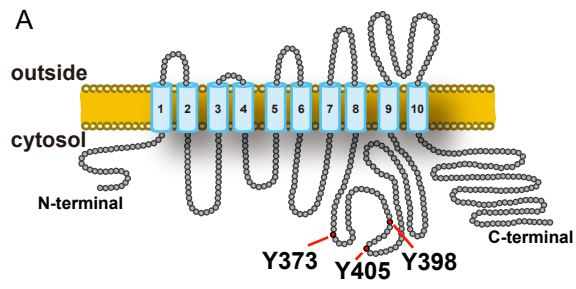
**Fig. 3.** Involvement of Y373, Y398, and Y405 in polar localization of BOR1. (A) A topology model of BOR1. (B-F) BOR1-GFP with Y-to-A mutations in root tips (left) and elongating epidermal cells (right) under low B conditions. (G) BOR1(Y373A/Y398A/Y405A)-GFP in the root hair zone. en, endodermis; co, cortex; ep, epidermis. BOR1-GFP (H) and BOR1(Y373A/Y398A/Y405A)-GFP (I) in elongating epidermal cells of roots incubated with 50  $\mu$ M cycloheximide (CHX) for 30 min and then with 50  $\mu$ M CHX and 50  $\mu$ M brefeldin A (BFA) for 2 h. Bars = 50  $\mu$ m.

**Fig. 4.** Lateral diffusion of BOR1-GFP, BOR1(Y373A/Y398A/Y405A)-GFP, and GFP-NIP5;1. (*A, B, D*) FRAP analysis after bleaching of 2- $\mu$ m regions of interest (ROIs). Recovery of BOR1-GFP (*A*) and BOR1(Y373A/Y398A/Y405A)-GFP (*B*) fluorescence at the apical and basal PMs and recovery of GFP-NIP5;1 fluorescence (*D*) at the outer PM in elongating epidermal cells. (*C*) Quantitative FRAP analysis. Data points and error bars represent averages and SD from 8-12 ROIs in 2-3 roots. Cycloheximide (CHX; 50  $\mu$ M) was applied for 60 min before FRAP analysis (*C* and *D*).

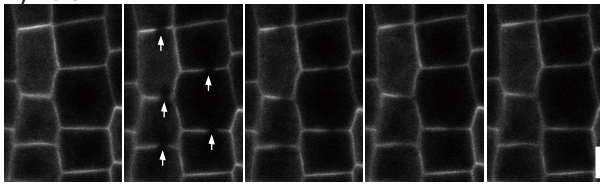
**Fig. 5.** Involvement of Y373, Y398, and Y405 in B-dependent degradation of BOR1. (*A-E*) BOR1-GFP with Y-to-A mutations in plants grown on low-B medium and then transferred to low-B medium for 5 h (-B) or high-B medium for 2 h (+B2h) or 5 h (+B5h). Bar = 100  $\mu$ m. (*F*) Immunoblot analysis of BOR1-GFP and BOR1(Y373A/Y398A/Y405A)-GFP. The plants were grown on low-B medium (-B) and transferred to high-B medium for 2 h (+B2h) or grown on high-B medium (+B).



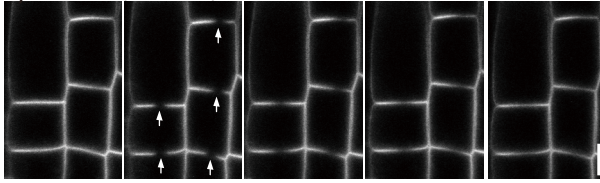




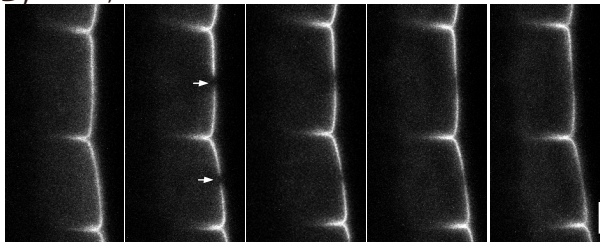
A, BOR1



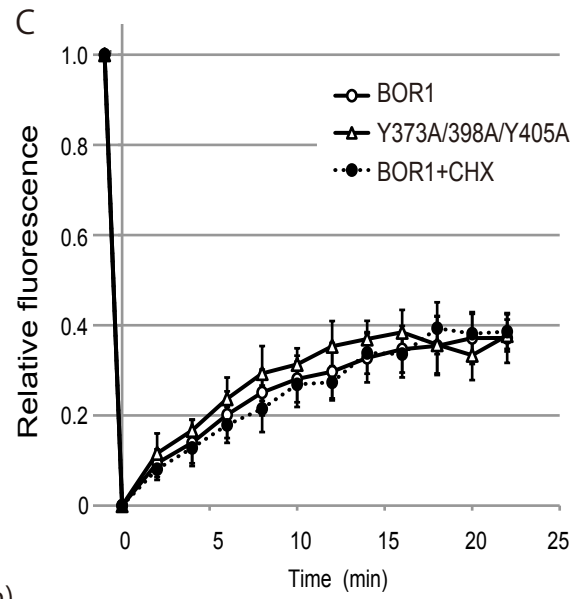
B, Y373A/Y398A/Y405A

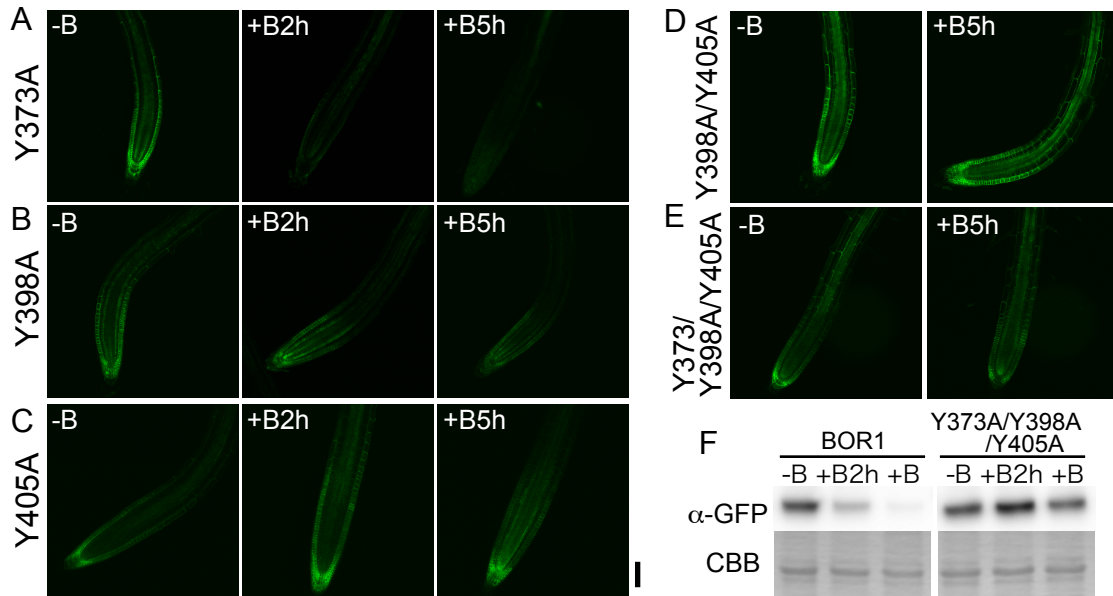


D, NIP5;1

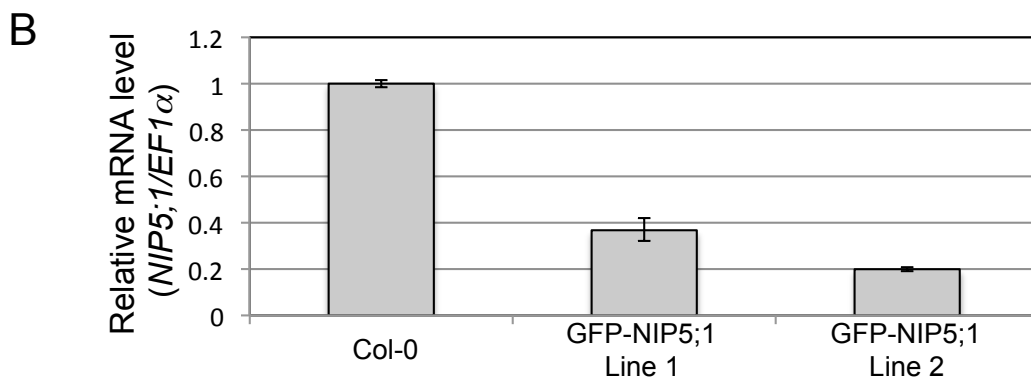
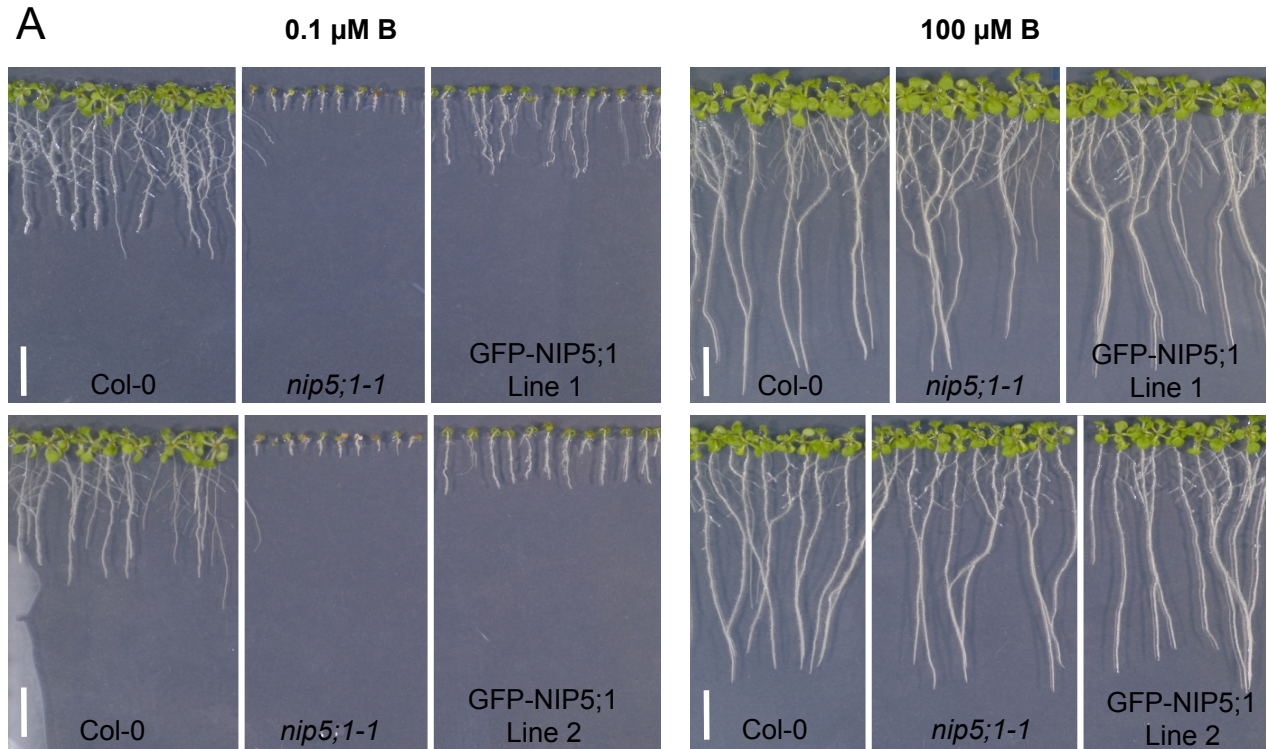


pre 0 5 10 20 (min)



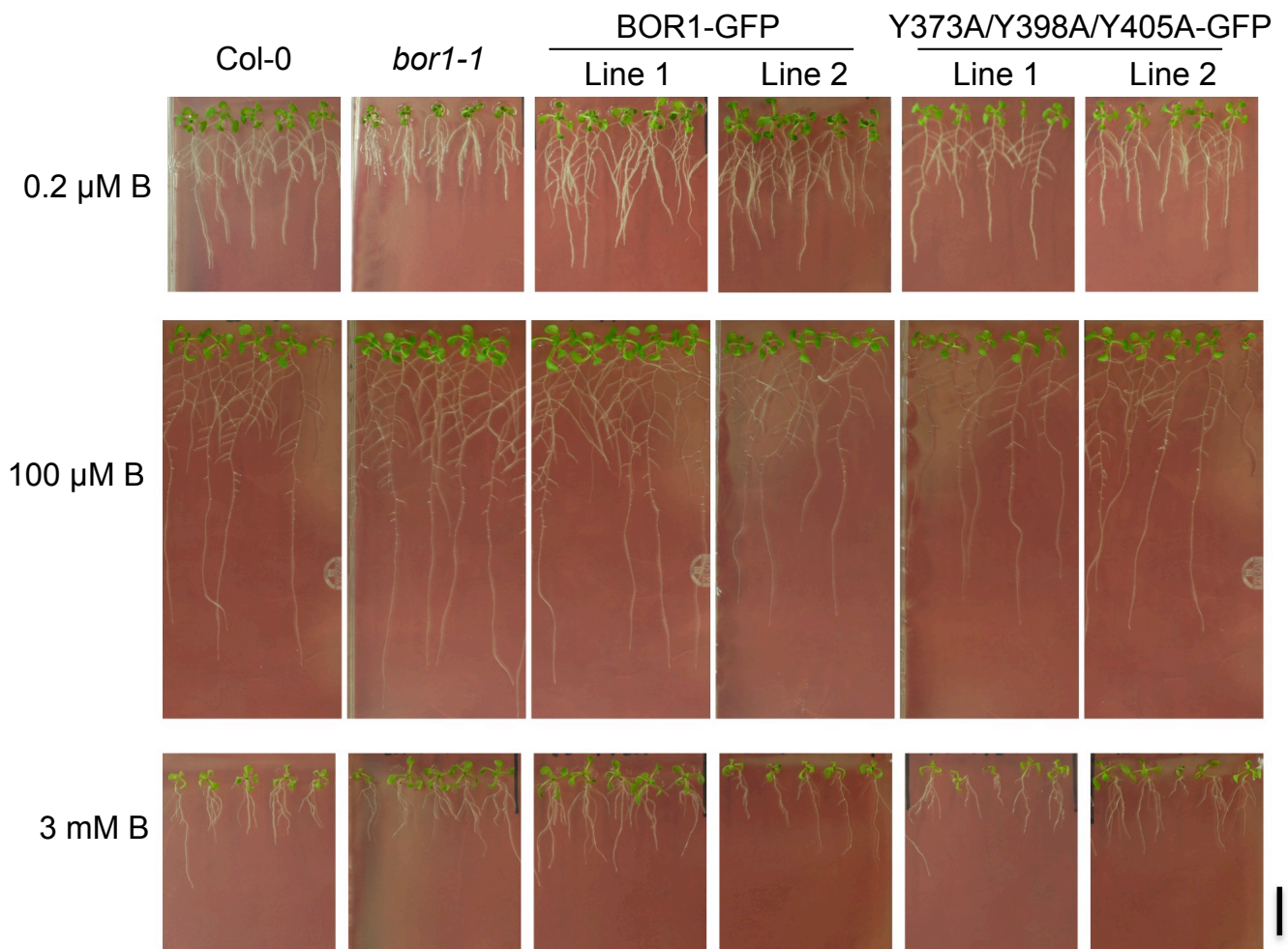


Supplemental figure 1



**Fig. S1.** The low-B sensitive growth of the *nip5;1* mutant was partially rescued by GFP-NIP5;1, expressed under the control of the *NIP5;1* promoter. (A) Col-0 wild type, *NIP5;1* T-DNA insertion lines (*nip5;1-1*), and two T3 homozygous *NIP5;1* promoter:GFP-NIP5;1/*nip5;1-1* lines were grown for 12 days on solid medium containing 0.1  $\mu\text{M}$  or 100  $\mu\text{M}$  boric acid. Bars = 10 mm. (B) RT-mediated real-time PCR analysis of *NIP5;1* or *GFP-NIP5;1* mRNA accumulation in roots of Col-0 and two T3 homozygous *NIP5;1* promoter:GFP-NIP5;1/*nip5;1-1* lines. Plants were grown for 12 days on solid medium containing 0.3  $\mu\text{M}$  boric acid. Mean values and standard deviations are presented for independent reverse transcription reactions followed by real-time PCR (n=3). The mRNA level of *NIP5;1* in the *nip5;1-1* mutant was negligible (8).

Supplemental figure 2



**Fig. S2** Boron-dependent growth of *bor1-1* mutant lines expressing BOR1-GFP or BOR1(Y373A/Y398A/Y405A)-GFP under the control of the *BOR1* promoter. Col-0 wild type, *bor1-1* mutant, and T3 homozygous transgenic *bor1-1* lines were grown on solid medium with 0.2  $\mu$ M, 100  $\mu$ M, or 3 mM boric acid. Bar = 10 mm.

Supplemental Figure 3

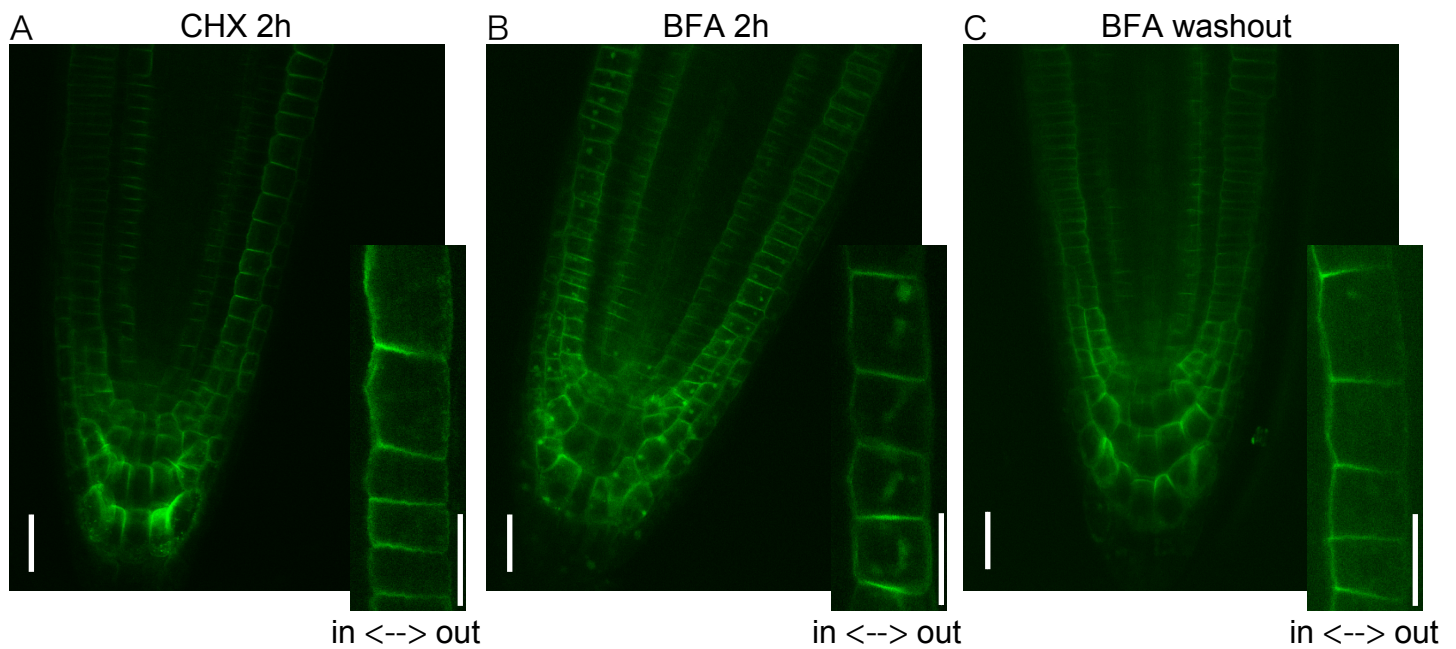


Fig. S3. Reversible effects of brefeldin A (BFA) on localization of BOR1-GFP in low B conditions. (A) Treatment with 50  $\mu$ M cycloheximide (CHX) for 2 h. (B) Treatment with 50  $\mu$ M BFA for 2 h. (C) Treatment with 50  $\mu$ M BFA for 90 min and then 50  $\mu$ M BFA and 50  $\mu$ M CHX for 30 min, followed by washing with 50  $\mu$ M CHX for 2 h. Root tips and elongating epidermal cells are shown. Bars = 25  $\mu$ m.

Supplemental Figure 4

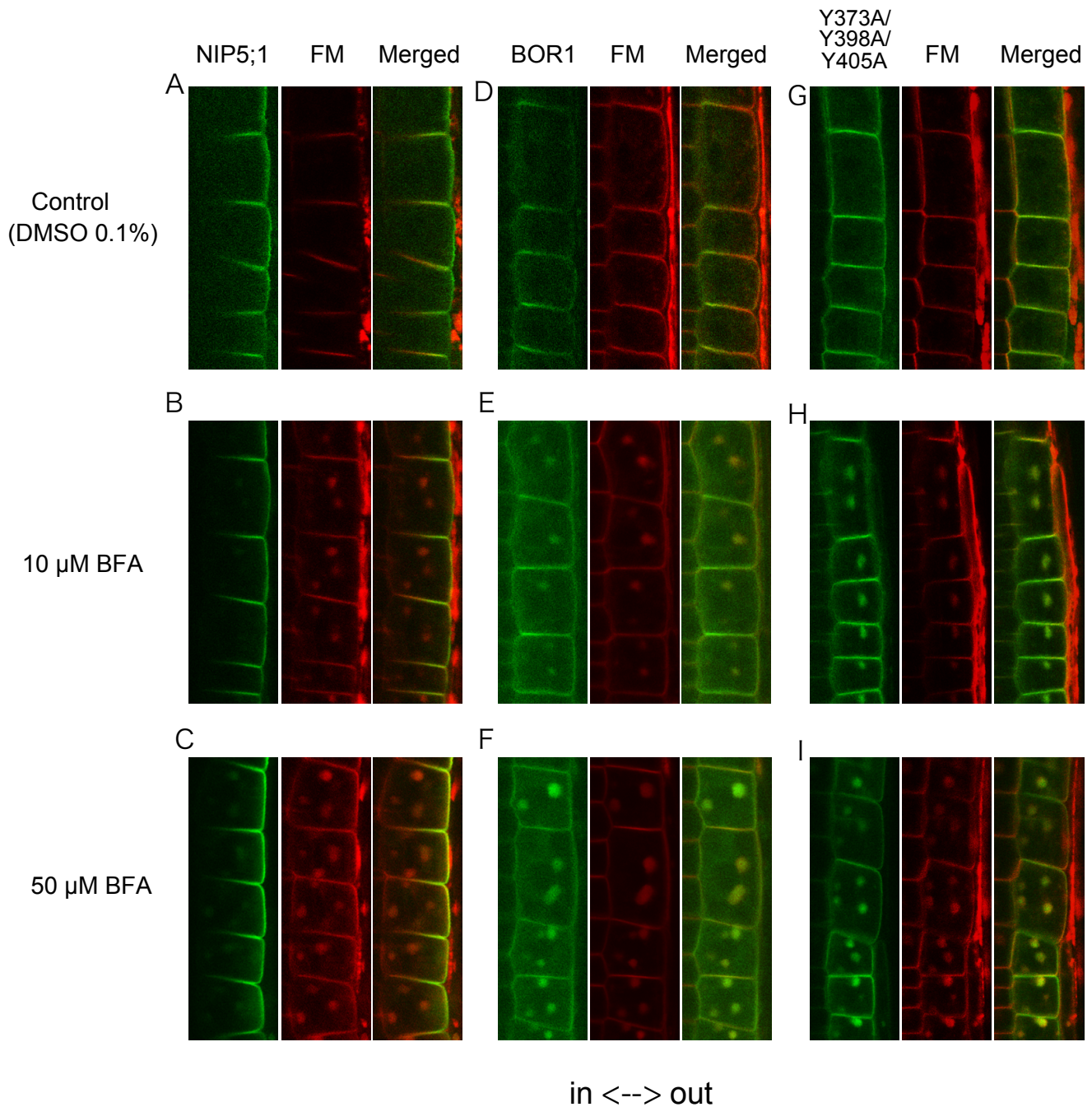


Fig. S4. Effects of brefeldin A (BFA) on localization of GFP-NIP5;1, BOR1-GFP, and BOR1 (Y373A/Y398A/Y405A)-GFP. GFP-NIP5;1 (A-C), BOR1-GFP (D-F), and BOR1 (Y373A/Y398A/Y405A)-GFP (G-I) in elongating epidermal cells of roots incubated with 50  $\mu$ M cycloheximide (CHX) for 30 min and then with 50  $\mu$ M CHX and 0.1% DMSO (control, A, D, G), 10  $\mu$ M BFA (B, E, H), or 50  $\mu$ M BFA (C, F, I) for 2 h. GFP (green), FM4-64 (red), and merged images are shown. Note the polarized localization of BOR1-GFP and GFP-NIP5;1 in the PM and their accumulation in the BFA compartments in comparison with the FM4-64 staining. Bar = 50  $\mu$ m.

## Supplemental Figure 5

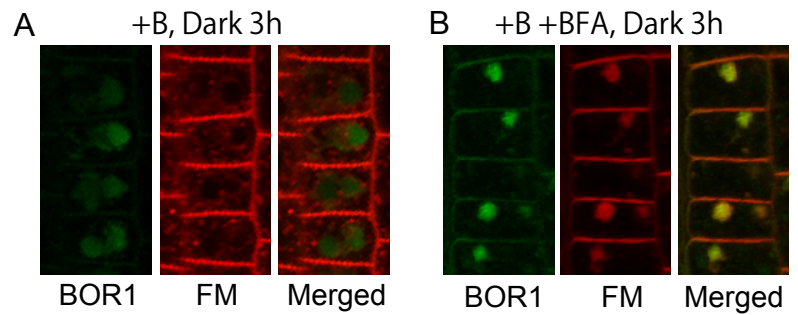


Fig. S5. Effects of brefeldin A (BFA) on vacuolar trafficking of BOR1-GFP under high B conditions. (A) Accumulation of BOR1-GFP in the vacuole of epidermal cells in the absence of light. The diffuse GFP signal was surrounded by tonoplast stained with FM4-64 for 3 h. (B) Incubation with 50  $\mu$ M BFA for 3 h in the absence of light led to the accumulation of BOR1-GFP and FM4-64 in BFA-induced aggregated endosomes and prevented the appearance of BOR1-GFP and FM4-64 in the vacuole. Bar = 10  $\mu$ m.

Supplemental figure 6

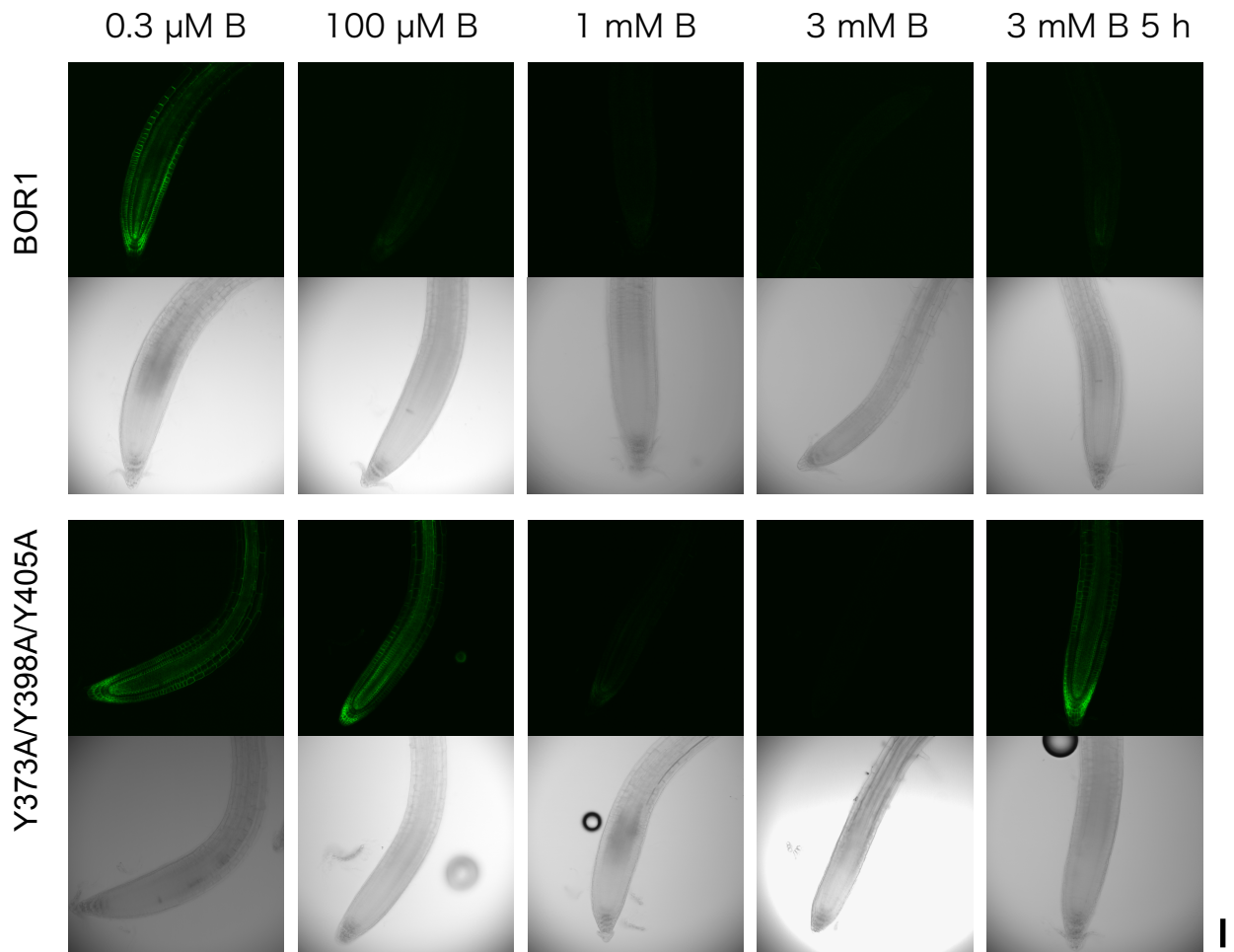
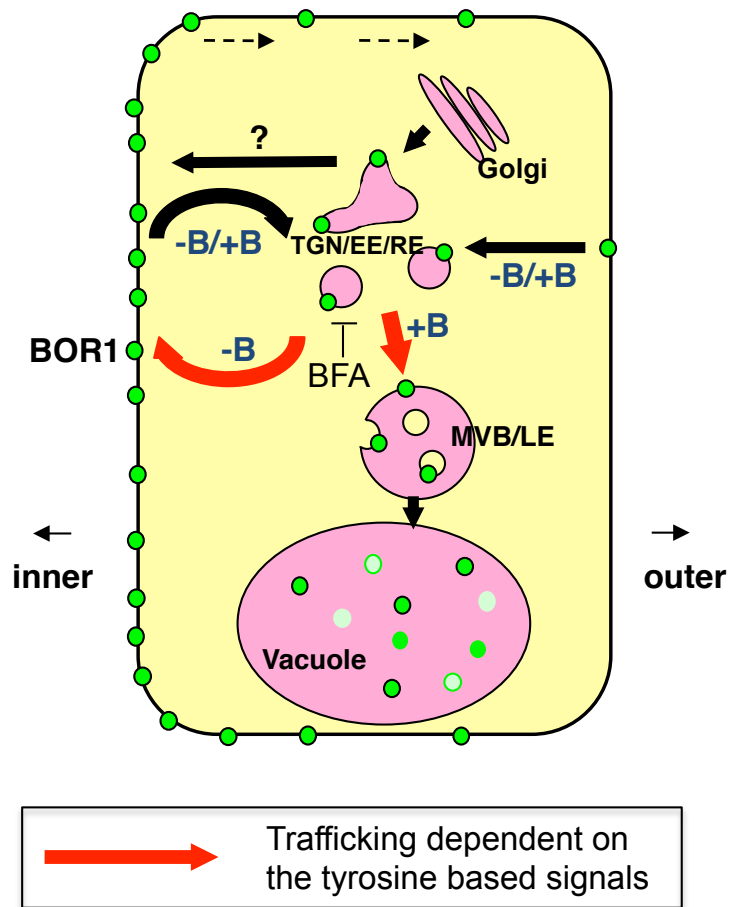


Fig. S6. Accumulation of BOR1-GFP and BOR1(Y373A/Y398A/Y405A)-GFP expressed under the control of the BOR1 promoter. Transgenic plants were continuously supplied with B at concentrations ranging from low to toxic (0.3  $\mu$ M, 100  $\mu$ M, 1 mM, and 3 mM), or were shifted from low B (0.3  $\mu$ M) to a toxic concentration of B (3 mM) for 5 h. GFP-derived fluorescence and transmission image are shown. Bar = 100  $\mu$ m.

Supplemental Figure 7



**Fig. S7.** A hypothetical model of the BOR1 trafficking pathway. Under B limitation (–B), BOR1 polarity at the inner PM domain is maintained by endocytic cycling that involves constitutive endocytosis and polar recycling to the inner PM domain. The polar recycling occurs from BFA-sensitive TGN, early endosomes (EE), or recycling endosomes (RE); this pathway depends on tyrosine-based signals. In addition to polar recycling, polar biosynthetic transport to the inner PM domain and/or preferential endocytosis from the outer PM domain may also occur. At high B, BOR1 is transported to multivesicular bodies (MVB)/late endosomes (LE) and then to the vacuole for degradation; this pathway also requires tyrosine-based signals.