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Polar localization and endocytic degradation of a boron transporter, BOR1, is dependent on specific tyrosine residues

Author's name(first, MI, last)

Akira Yoshinari[1], Koji Kasai[2], Toru Fujiwara[2], Satoshi Naito[1, 3], Junpei Takano[3]*

Affiliations

[1] Division of Life Science, Graduate School of Life Science, Hokkaido University, Sapporo 060-8589, Japan

[2] Division of Applied Biological Chemistry, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo 113-8657, Japan

[3] Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

*** Coresponding; jtakano@abs.agr.hokudai.ac.jp**

Key words (5-10)

mineral transporter, polar localization, tyrosine-based signal, ubiquitination, membrane trafficking, endocytosis

Abstract

Boron (B) is essential for plants, but is toxic in excess. Plants have to strictly regulate the uptake and translocation of B. In *Arabidopsis thaliana* root cells, a boric acid channel, NIP5;1, and a boric acid/borate exporter, BOR1, localize to the outer (facing soil) and inner plasma membrane domains, respectively, under B limitation. The opposite polar localizations of the importer and exporter would enable plant roots to transport B efficiently towards the xylem. In addition, accumulation of the B transporters is controlled by B conditions. When plants are shifted from low to high B conditions, NIP5;1 transcript accumulation is down-regulated through mRNA degradation. The BOR1 protein is transported to the *trans*-Golgi network/early endosome and multivesicular body and finally degraded in the vacuole. We have recently shown that both the polar localization and the endocytic degradation of BOR1 are controlled by at least two tyrosine residues in a large loop located in the cytosol. We also showed that ubiquitination is required for the endocytic degradation of BOR1. Here, we analyzed possible involvement of an additional tyrosine residue (Y414) in the loop region and discuss the pathway of the BOR1 trafficking for polar localization and

endocytic degradation of BOR1.

Polar Localization of Boron Transporters

Transporters are required for uptake and translocation of essential minerals in plants. For the directional transport of minerals across root cells, polar localization of transporters in the plasma membrane (PM) appears to be important. For example, a potato phosphate transporter, PT2, and a maize ferric-phytosiderophore transporter, YS1, localize in the outer PM domain (facing soil) of root epidermal cells[1, 2]. This outer polar localization would be beneficial for uptake of minerals from the soil solution. Furthermore, there are examples of opposite polar localization of an importer and an exporter in root cells. A silicic acid channel, Lsi1 (OsNIP2;1), and a silicon exporter, Lsi2, localize to the outer and inner PM domains, respectively, in both the exodermis and endodermis of rice roots[3]. A boric acid channel NIP5;1 and a borate exporter BOR1 also localize to outer and inner PM domains, respectively, in various types of root cell in *Arabidopsis thaliana*[4]. The polar localization of importers and exporters indicates the radial transport route of nutrients from soil to xylem, and possibly represents a common system for other mineral nutrients.

How is outer/inner polarity maintained in the PM of plant cells? One possibility is a separation of outer and inner PM domains accompanying the polar trafficking of the transporters. In differentiated endodermal cells in roots, the Casparian strip functions as an apoplastic diffusion barrier and also as a diffusion barrier for the PM[5, 6]. The Casparian strip clearly separates outer and inner PM domains when visualized with fluorescent-protein tagged NIP5;1 and BOR1, respectively. Importantly, transporters including NIP5;1 and BOR1 show outer/inner polarity also in cells without an apparent diffusion barrier[4, 7, 8], suggesting that additional mechanisms are present for retention or recycling. These mechanisms are largely unknown but may be similar to those for apical/basal polarity of auxin transporters[9].

Boron-dependent Degradation of Boron Transporters

Another interesting feature for localization of boron (B) transporters is B-dependent degradation of BOR1. When plants were transferred from low B conditions to high B conditions, BOR1-GFP was transported from the PM to the multivesicular body (MVB)/late endosome (LE), and then to the vacuole for degradation[4, 10]. Immunogold

electron microscopy detected BOR1-GFP in the *trans*-Golgi network (TGN)/early endosome (EE) and intralumenal vesicles of the MVB/LE after high B supply[11]. These data suggest high B-induced trafficking of BOR1 from the PM to TGN/EE, then to intralumenal vesicles of the MVB/LE, and finally to the vacuolar lumen for degradation. In contrast, accumulation of NIP5;1 is regulated mainly through B-dependent degradation of *NIP5;1* mRNA[12]. Consistently, disappearance of GFP-NIP5;1 after high B supply was slower than that of BOR1-GFP[4]. The down-regulation of B transporters is apparently important for preventing accumulation of B to toxic levels.

Specific Tyrosine Residues in BOR1 Play Important Roles in BOR1 Trafficking

In our previous report, we demonstrated that the polar localization of BOR1 requires tyrosine residues in a large loop region that is most probably located in the cytosol[4]. Interestingly, the same residues are also required for the B-dependent degradation of BOR1. These tyrosine residues (Y373, Y398, and Y414) of BOR1 probably function as tyrosine-based signals. 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, sorting to lysosomes, and also polar PM sorting in mammals[13, 14]. The tyrosine-based signals are recognized by a medium (μ) subunit of adaptor protein (AP) complexes for sorting into coated vesicles and different post-Golgi transport pathways.

To further investigate the regulatory mechanisms of BOR1 trafficking, we compared amino acid sequences of the cytosolic large loop region of BOR1 to those of BOR1 homologs in various plant species (**Fig. 1A**). Consistent with the results that Y398 and Y405 are especially important for BOR1 trafficking[4], Y398 and Y405, but not Y373, were highly conserved among the BOR1 homologs. We also noticed that a fourth tyrosine residue, Y414, in the large loop was highly conserved. Y414QQP may function as a tyrosine-based signal or a tyrosine phosphorylation site. In mammal and yeast systems, Src kinase recognizes YxxP motifs and tyrosine phosphorylation is involved in endocytosis of membrane proteins[15, 16]. Although there is no full-length homolog of Src kinase in the *Arabidopsis* genome, there are genes that encode src homology3 (SH3) -containing proteins [17].

To investigate the possible importance of the Y414 residue, we generated

transgenic plants expressing BOR1(Y414A)-GFP under control of the *BOR1* promoter. Under low B conditions, the BOR1(Y414A)-GFP showed polar localization indistinguishable from wild-type BOR1-GFP in root cap cells and elongating endodermal cells, while the BOR1(Y373A/Y398A/Y405A)-GFP showed apolar localization, as previously reported (**Fig. 1B**). Then we developed a method for time-course analysis of degradation after shifting to high B medium in epidermal cells. Plants grown on solid media containing 0.3 μM B were transferred to glass-bottom dishes, and then the roots were covered with media solidified with 0.5% Gellan gum containing 100 μM B and observed with an inverted microscope. Under this condition, the speckled endosomes stained with wild-type BOR1-GFP apparently increased 15 min after the high B supply, illustrating the rapid response to B (**Fig. 1C**). BOR1(Y414A)-GFP also showed rapid internalization, while BOR1(Y373A/Y398A/Y405A)-GFP was not internalized. Therefore, Y414 does not seem to be involved in polar localization or vacuolar trafficking.

BOR1 Trafficking Pathway

We have shown that at least two well-conserved tyrosine residues of BOR1 are required for both polar localization and B-dependent degradation of BOR1[4]. Furthermore, we recently demonstrated the requirement for ubiquitination at K590 in a C-terminal region for B-dependent degradation but not for polar localization[18]. Ubiquitination of membrane proteins functions as a signal for endocytosis (internalization from PM) and MVB sorting (entry into the intraluminal vesicles of an MVB) in animal and yeast cells[19, 20]. We reported that inhibition of the recycling pathway by brefeldin A visualized accumulation of both BOR1(Y373A/Y398A/Y405A)-GFP and BOR1(K590A)-GFP in endosomal aggregation similar to the case for wild-type BOR1-GFP[4, 18], suggesting that the tyrosine-based signals and ubiquitination are not required for endocytosis from the PM. Although there are various possibilities, we propose a model of BOR1 trafficking considering a recent important finding that the MVB/LE matures from TGN/EE[21] (**Fig. 2**). Under low B conditions, the tyrosine-based signals bind with an AP in the TGN/EE, and BOR1 is directed to the polar recycling pathway. Under high B conditions, the same tyrosine-based signals bind with another AP, and BOR1 is recruited toward a

TGN/EE sub-compartment, which becomes the MVB/LE. Moreover, the ubiquitination of BOR1 at K590 accelerates MVB sorting. The MVB fuses with the vacuole[21] and the intraluminal vesicles containing BOR1 are released into the vacuolar lumen.

Clearly, identification of regulators for the polar trafficking of BOR1 and the components of B-sensing machinery is required to further elucidate BOR1 trafficking mechanisms. The study of B transporters will help to reveal common mechanisms of transporter trafficking and endocytic pathways in plant cells.

Figure Legends

Figure 1

Effect of Y414A substitution on the polar localization and endocytic degradation of BOR1. (A) Amino acid sequences of the cytosolic large loop region of BOR1 and its homologs. The sequences were aligned using the Clustal W program (http://clustalw.ddbj.nig.ac.jp/). Putative tyrosine-based signals are highlighted. (B) Involvement of tyrosine-based signals in polar localization of BOR1. BOR1-GFP with tyrosine–to–alanine substitution in elongating epidermal cells (ep; left end of each

panel) and root tips under low B conditions. Transgenic lines expressing BOR1-GFP(WT), BOR1(Y414A)-GFP, and BOR1(Y373A/Y398A/Y405A)-GFP under control of the *BOR1* promoter are shown. Plants were grown on vertically placed solid medium containing 0.3 μM boric acid, 1% sucrose, and 1.5% Gellan gum (Wako Pure Chemicals) for 5 days. Other growth conditions were the same as in the previous work[4]. (C) Time-course analysis of the subcellular localization of BOR1-GFP(WT), BOR1(Y414A)-GFP, and BOR1(Y373A/Y398A/Y405A)-GFP after high B supply. Plants were grown on solid medium containing 0.3 μM boric acid for 5 days. The plants were then transferred to solid medium containing 100 μM boric acid and 0.5% Gellan gum. Time after shifting to high B medium is indicated. Laser scanning confocal microscopy was performed using a Zeiss LSM510 META. Excitation and detection wavelengths for GFP were 488 nm and 505–530 nm (band path), respectively. Scale bars indicate 50 μm (B) and 20 μm (C), respectively.

Figure 2

A hypothetical model of the BOR1 trafficking pathway. Under low B conditions, the

polar localization of BOR1 to the inner PM domain is maintained by polar recycling from the *trans*-Golgi network/early endosome (TGN/EE) and the pathway is dependent on the tyrosine-based signals. Relatively slow diffusion in the PM[4] also seems to be important for the polar localization. Under high B conditions, BOR1 is transported to a sub-compartment of the TGN/EE, which becomes a multivesicular body/late endosome (MVB/LE). This pathway also requires the tyrosine-based signals. BOR1 is ubiquitinated under high B conditions and the ubiquitination accelerates BOR1 sorting into the intraluminal vesicles of an MVB. The MVB fuses with vacuoles and the intraluminal vesicles containing BOR1 are released into the vacuolar lumen.

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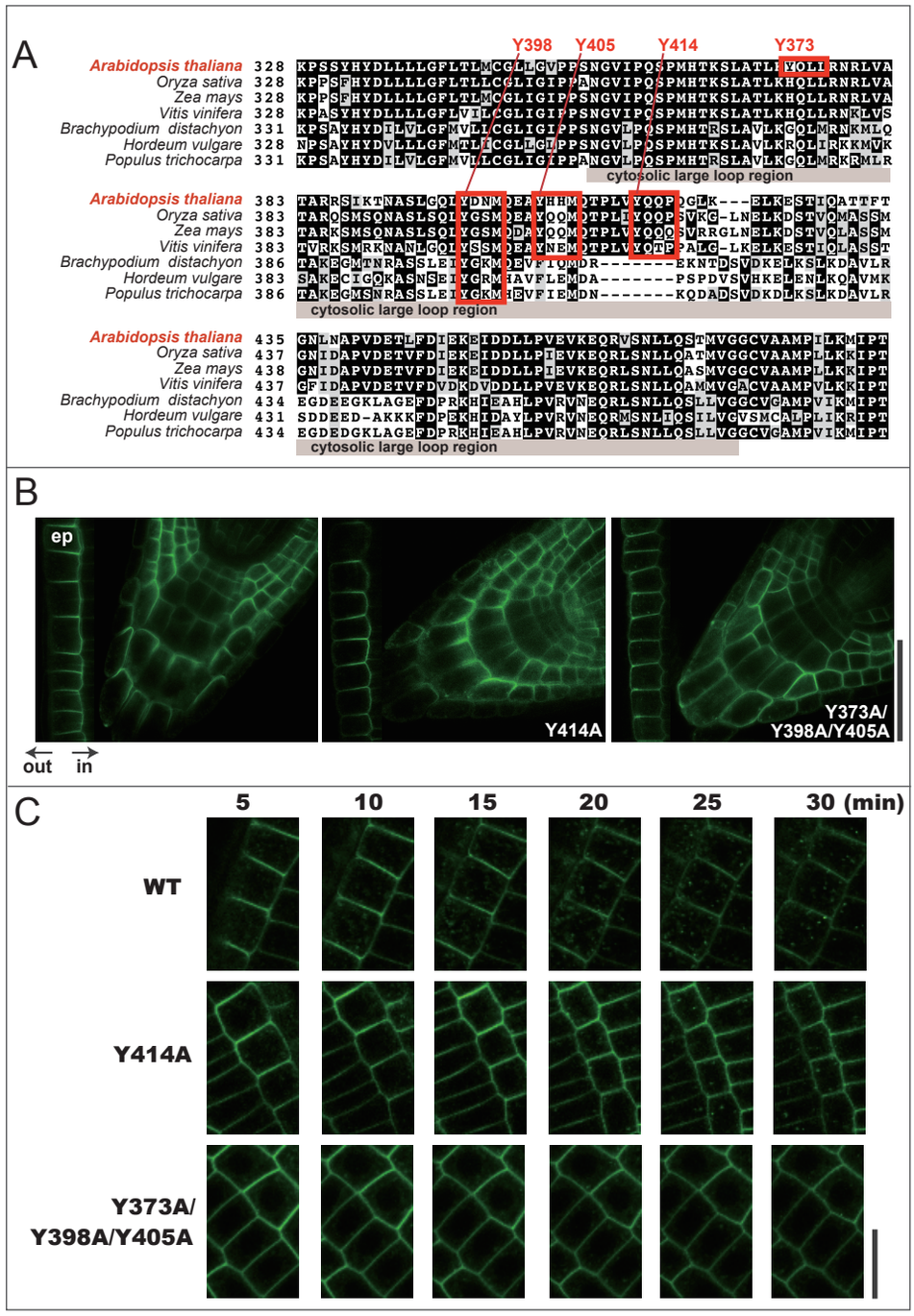
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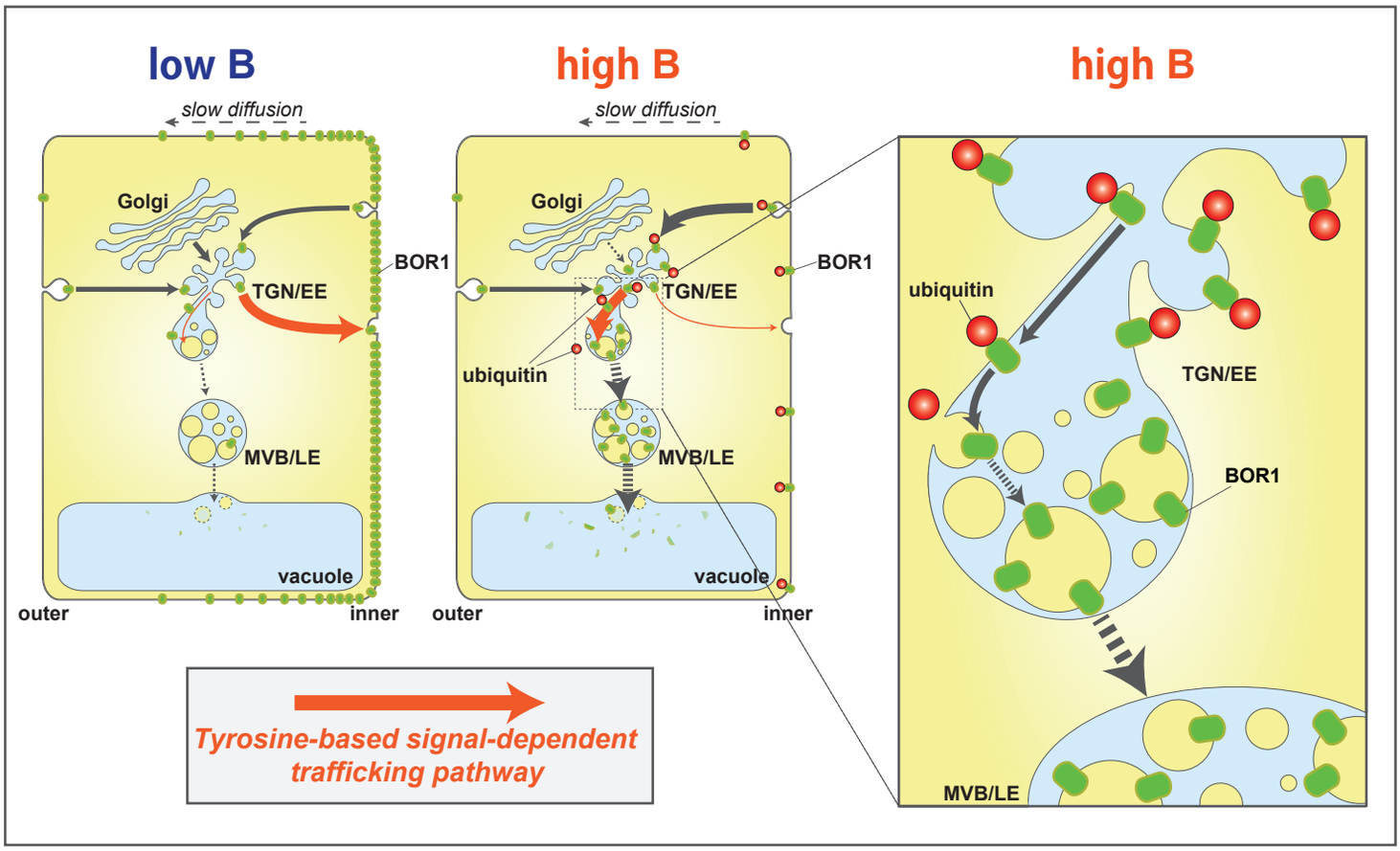
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A
Y398
Y405
Y414
Y373
Arabidopsis thaliana 328 KPSSYHYDLLLLGFLTLMCGLLGVPPSNGVIPQSPMHTKSLATLIYQLLRNRLVA
Oryza sativa 328 KPPSFHYDLLLLGFLTLLCGLIGIPPANGVIPQSPMHTKSLATLKHQLLRNRLVA
Zea mays 328 KPPSFHYDLLLLGFLTLMCGLIGIPPSNGVIPQSPMHTKSLATLKHQLLRNRLVA
Vitis vinifera 328 KPASYHYDLLLLGFLVILCGLIGIPPSNGVIPQSPMHTKSLATLKHQLLRNKLVS
Brachypodium distachyon 331 KPSAYHYDILVLGFMVLICGLIGIPPSNGVLPQSPMHTRSLAVLKGQLMRNKMLQ
Hordeum vulgare 328 NPSAYHYDVLLLGFMTLICGLLGLPPSNGVLPQSPMHTKSLAVLKRQLIRKKMVK
Populus trichocarpa 331 KPSAYHYDILVLGFMVLLCGLIGIPPANGVLPQSPMHTRSLAVLKGQLMRKRMLR
cytosolic large loop region
Arabidopsis thaliana 383 TARRSIKTNASLGQIYDNMQEAYHHMQTPLVYQQPQGLK---ELKESTIQATTFT
Oryza sativa 383 TARQSMSQNASLSQLYGSMQEAYQQMQTPLIYQQPSVKG-LNELKDSTVQMASSM
Zea mays 383 TARKSMSQNASLSQLYGSMQDAYQQMQTPLVYQQQSVRRGLNELKDSTVQLASSM
Vitis vinifera 383 TVRKSMRKNANLGQLYSSMQEAYNEMQTPLVYQTPPALG-LKELKESTIQLASST
Brachypodium distachyon 386 TAKEGMTNRASSLEIYGKMQEVFIQMDR-------EKNTDSVDKELKSLKDAVLR
Hordeum vulgare 383 SAKECIGQKASNSEIYGRMHAVFLEMDA-------PSPDVSVHKELENLKQAVMK
Populus trichocarpa 386 TAKEGMSNRASSLEIYGKMHEVFIEMDN-------KQDADSVDKDLKSLKDAVLR
cytosolic large loop region
Arabidopsis thaliana 435 GNLNAPVDETLFDIEKEIDDLLPVEVKEQRVSNLLQSTMVGGCVAAMPILKMIPT
Oryza sativa 437 GNIDAPVDETVFDIEKEIDDLLPIEVKEQRLSNLLQATMVGGCVAAMPLLKKIPT
Zea mays 438 GNIDAPVDETVFDIEKEIDDLLPIEVKEQRLSNLLQASMVGGCVAAMPLLKKIPT
Vitis vinifera 437 GFIDAPVDETVFDVDKDVDDLLPVEVKEQRLSNLLQAMMVGACVAAMPVLKKIPT
Brachypodium distachyon 434 EGDEEGKLAGEFDPRKHIEAHLPVRVNEQRLSNLLQSLLVGGCVGAMPVIKMIPT
Hordeum vulgare 431 SDDEED-AKKKFDPEKHIDAYLPVRVNEQRMSNLIQSILVGVSMCALPLIKRIPT
Populus trichocarpa 434 EGDEDGKLAGEFDPRKHIEAHLPVRVNEQRLSNLLQSLLVGGCVGAMPVIKMIPT
cytosolic large loop region
B
ep
Y414A
Y373A/
Y398A/Y405A
out
in
C
5
10
15
20
25
30 (min)
WT
Y414A
Y373A/
Y398A/Y405A


low B
high B
high B
slow diffusion
slow diffusion
Golgi
Golgi
TGN/EE
TGN/EE
BOR1
BOR1
ubiquitin
ubiquitin
MVB/LE
MVB/LE
vacuole
vacuole
outer
inner
outer
inner
TGN/EE
BOR1
MVB/LE
Tyrosine-based signal-dependent trafficking pathway