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| Title | Polar localization and degradation of Arabidopsis boron transporters through distinct trafficking pathways |
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| Citation | Proceedings of the National Academy of Sciences of the United States of America, 107(11), 5220-5225 https://doi.org/10.1073/pnas.0910744107 |
| Issue Date | 2010-03-16 |
| Doc URL | https://hdl.handle.net/2115/49101 |
| Type | journal article |
| File Information | Supporting_Information.pdf, Supporting Information |



Supplemental Methods

Plasmid construction

To construct *NIP5;1 promoter::GFP-NIP5;1*, first a 2.5-kb DNA fragment upstream of the translation initiation codon of *NIP5;1* (*PNIP5;1*) was amplified from BAC clone F24G24 (obtained from the ABRC) by PCR with the primers listed in Supplemental Table 1. Using *Bam*HI and *Nco*I, the amplified fragment was subcloned into pTF441, which was generated from pBI221 (39) by substituting the β -glucuronidase (GUS) fragment with sGFP. The 2.5-kb *NIP5;1* upstream region was then excised using *Sph*I and *Nco*I, and was used to replace the cauliflower mosaic virus 35S RNA promoter (35S) in the *35S::sGFP-NIP5;1* construct (8). The resultant plasmid was named pMUT9. The *Bam*HI-*Eco*RI fragment containing *PNIP5;1-GFP-NIP5;1* and the terminator of the nopaline synthase gene was subcloned from pMUT9 into the *Bam*HI-*Eco*RI sites of the binary vector pMDC99 (40).

To express GFP-tagged BOR1 and its mutants under the control of the *BOR1* promoter, we constructed a Gateway (Invitrogen) compatible expression vector harboring the *BOR1* promoter. The approximately 3-kb *BOR1* upstream region was amplified by PCR using the primers F-pBOR1-*Bam*HI and R-pBOR1-*Kpn*I (see Supplemental Table 1 for sequences) with the BAC clone T3D7 as a template. Then the *BOR1* promoter fragment was replaced with the dual 35S promoter in pMDC32 (40) to obtain pAT100. GFP-tagged BOR1 and its mutants were generated as described below. The entire *BOR1-GFP* was amplified by PCR using the *BOR1-sGFP* (12) construct as a template. DNA fragments encoding GFP-tagged mutant BOR1 were generated by

fusion PCR using the primers listed in Supplemental Table 1. The fusion proteins have a linker of five amino acids (Gly-Gly-Gly-Gly-Ala) between the transporter and sGFP. The transporter-GFP fragment was subcloned into pENTR D-TOPO vector (Invitrogen), sequenced, and then subcloned into pAT100 using Gateway technology (Invitrogen).

In planta transformation was performed using the *Agrobacterium*-mediated floral dip method (41).

GFP imaging

Laser scanning confocal microscopy was performed using a Leica SP2, Zeiss LSM510, or LSM510META with following excitation and detection wavelengths, respectively: 488 nm and 500-540 or 505-550 nm for GFP; 543 nm and >650 nm for propidium iodide; 488 nm and >650 nm for FM4-64; and 561 nm and >575 nm for mCherry. Dual-color images of GFP and mCherry were acquired by sequential line switching, allowing the separation of channels by both excitation and emission. The plants were grown on vertically placed solid medium containing 0.3 μM boric acid, 2% sucrose, and 1.5% Gellan Gum (Wako Pure Chemicals, Osaka) for 4-9 days unless otherwise indicated. For the shift to a high B condition, plants grown in low B (0.3 μM boric acid) were transferred onto solid medium containing 100 μM boric acid. For application of dyes or inhibitors, the roots of the plants were cut and transferred to low B (0.3 μM) or high B (100 μM) liquid medium containing the dye or inhibitor, followed by incubation at room temperature. Propidium iodide (Molecular Probes) was used at 10 $\mu\text{g ml}^{-1}$. FM4-64 (Molecular Probes) was prepared as a 10 mM stock solution in water and used

at 4 μM . Cycloheximide (CHX) was prepared at 25 mM in water and used at 50 μM . Brefeldin A (BFA, Sigma) was prepared as a 50 mM stock solution in DMSO and used at 10 or 50 μM . Control treatments with 0.1% DMSO performed in parallel did not affect the localization or degradation of GFP-tagged proteins. Each experiment was carried out multiple times with at least two independent transgenic lines, and representative pictures are shown. For colocalization analysis of BOR1-GFP and mCherry-RabF2a/Rha1, multiple F1 plants from the representative parental lines were used.

FRAP analysis

For FRAP analysis without inhibitors, plants were grown in a chambered coverglass (Iwaki, Tokyo) containing solid medium with 0.3 μM boric acid, 2% sucrose, and 0.2% Gellan Gum for 6–7 days. For CHX treatment, plants were grown on vertically placed solid medium containing 0.3 μM boric acid, 2% sucrose, and 1.5% Gellan Gum for 4–5 days. The plants were then transferred to a chambered coverglass, and their roots were covered with medium containing 0.3 μM boric acid, 50 μM CHX, and 0.2% Gellan Gum. FRAP analysis was performed with a Zeiss LSM510Meta equipped with an inverted microscope, using a water-immersed 40 \times objective and 488-nm diode laser excitation. The 2- μm PM regions were bleached for two or three iterations of 163.83- μs bleach periods per pixel with 100% laser transmittance at a 600 pinhole size. Pre- and post-bleach scans were performed with 1% laser transmission, and GFP emission was detected between 505–550 nm.

Preparation and immunoblot analysis of microsomal proteins

The transgenic plants were grown on vertically placed solid medium containing 0.3 μM boric acid (-B) or 100 μM boric acid (+B) for 16 days. All steps in the preparation of proteins were conducted at 4°C or on ice. Tissues were homogenized in buffer (250 mM Tris, pH 8.5, 290 mM sucrose, 25 mM EDTA) supplemented with 75 mM 2-mercaptoethanol, 1 mM PMSF, and protease inhibitors (Complete Mini; Roche), followed by centrifugation at $10,000 \times g$ for 15 min at 4°C. The resultant supernatant was transferred to a new tube (Beckman) and centrifuged at $100,000 \times g$ for 30 min at 4°C. The pellet, representing the microsomal fraction, was resuspended in storage buffer (50 mM potassium phosphate buffer, pH 6.3, 1 mM magnesium sulfate, 20% glycerol) supplemented with 1 mM PMSF and protease inhibitors. The protein concentration was measured using a protein assay system (Bio-Rad). NuPAGE LDS sample buffer (Invitrogen) and 2-mercaptoethanol (final concentration, 2.5%) were added to the samples for immunoblotting, and the samples were incubated at 70°C for 10 min. Microsomal proteins (5 μg) were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene fluoride membranes by electroblotting. The membrane was blocked by incubation in TBST containing 2% skim milk. Mouse anti-GFP monoclonal antibody (Nakalai Tesque, Kyoto) was used at 10,000-fold dilution in Can Get Signal Solution 1 (Toyobo); as a secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare) was used at 20,000-fold dilution in Can Get Signal Solution 2 (Toyobo). Detection was performed

using Immobilon Western chemiluminescent HRP substrate (Millipore). The membranes were stained with 0.25% Coomassie brilliant blue-R250 after detection.

Quantitative RT-PCR

Plants were grown on vertically placed solid medium containing 0.3 μ M boric acid for 12 days. Total RNA was extracted using a RNeasy mini kit according to the manufacturer's protocol (Qiagen). Genomic DNA contamination was eliminated by on-column digestion of DNA during RNA purification using a RNase-Free DNase set (Qiagen). RNA was reverse transcribed using SuperScript III (Invitrogen) and an oligo-d(T) primer. The cDNA was amplified by PCR in a LightCycler with FastStart DNA Master SYBR Green I as recommended by the manufacture (Roche). The primers used in real-time PCR are described previously (8). Primers were confirmed to be specific for the target genes by electrophoresis of PCR reactions and by melting curve analysis of PCR products using the LightCycler instrument.

39. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions— β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901-3907.

49. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol* 133:462-469.

41. Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735-743.

Supplemental Table 1. DNA oligonucleotides used in this study

| Name | Sequence* | Construct |
|--------------------------|---|---|
| F-PNIP5;1- <i>Bam</i> HI | GGT <u>GGATCC</u> GAAAGCAAGCATTCCCTG | NIP5;1pro:GFP-NIP5;1 |
| R-PNIP5;1- <i>Nco</i> I | GAG <u>CCATGG</u> CCAACGTTTTTTTTTTTGGT | NIP5;1pro:GFP-NIP5;1 |
| F-pBOR1- <i>Bam</i> HI | TTT <u>GGATCC</u> GGGAGATGACTAACAACGACA | BOR1promoter |
| R-pBOR1- <i>Kpn</i> I | TCT <u>GGTACCT</u> CTCAGTCAAATCGTCAAACAG | BOR1promoter |
| caccATG-BOR1 | CACC ATGGAAGAGACTTTTTGTGCCGTTTG | BOR1 –GFP etc. |
| sGFPstop | <i>TTACTTGTACAGCTCGTCCATGCC</i> | all BOR1(mutant)-GFP |
| BOR1(Y373A)-F | CAACT CTTAAAG CCC AGTTGCTTC | BOR1(Y373A)-GFP, BOR1(Y373A/Y398A/Y405A)-GFP |
| BOR1(Y373A)-R | GAAGCAACTG GGC TTTAAGAGTTG | BOR1(Y373A)-GFP, BOR1(Y373A/Y398A/Y405A)-GFP |
| BOR1(Y398A)-F | TGGGTCAACT CGCT GACAAT ATG | BOR1(Y398A)-GFP |
| BOR1(Y398A)-R | CATATTGTC AGCG AGTTGACCCA | BOR1(Y398A)-GFP |
| BOR1(Y405A)-F2 | ATGCAAGAAG CT GCCC CATCA CATGC | BOR1(Y405A)-GFP |
| BOR1(Y405A)-R2 | GCATGTGATG GGC AGCTTCTTGCAT | BOR1(Y405A)-GFP |
| BOR1(Y398AY405A)-F | GACAATATGCAAGAAGCT GCCC CATCACATG | BOR1(Y373A/Y398A/Y405A)-GFP |
| BOR1(Y398AY405A)-R | AGCTTCTTGCATATTGTC AGCG AGTTGACCCA | BOR1(Y373A/Y398A/Y405A)-GFP |

*Restriction enzyme recognition sites are underlined, introduced mutations are shown in bold, and transcription initiation and termination codons are shown in italics.