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Establishment of Genetically Encoded Biosensors for Cytosolic Boric Acid in Plant Cells

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SUMMARY

Boron (B) is an essential micronutrient for plants. To maintain B concentration in tissues at appropriate levels, plants use boric acid channels belonging to the NIP subfamily of aquaporins and BOR borate exporters. To regulate B transport, these transporters exhibit different cell-type specific expression, polar localization, and B-dependent post-transcriptional regulation. Here, we describe the development of genetically encoded biosensors for cytosolic boric acid to visualize the spatial distribution and temporal dynamics of B in plant tissues. The biosensors were designed based on the function of the *NIP5;1* 5'-untranslated region (UTR), which promotes mRNA degradation in response to an elevated cytosolic boric acid concentration. The signal intensities of the biosensor coupled with Venus fluorescent protein and a nuclear localization signal (*uNIP5;1-Venus*) showed a negative correlation with intracellular B concentrations in cultured tobacco BY-2 cells. When expressed in *Arabidopsis thaliana*, *uNIP5;1-Venus* enabled quantification of the B distribution in roots at single-cell resolution. In mature roots, cytosolic B levels in stele were maintained under low-B supply, while those in epidermal, cortical, and endodermal cells were influenced by external B concentrations. Another biosensor coupled with a luciferase protein fused to a destabilization PEST sequence (*uNIP5;1-Luc*) was used to visualize changes in cytosolic boric acid concentrations. Thus, *uNIP5;1-Venus*/*Luc* enables visualization of B transport in various plant cells/tissues.

INTRODUCTION

Boron (B) is one of the 17 essential elements of higher plants. In soil solution, B exists mainly as boric acid, a weak Lewis acid that forms the borate anion: $B(OH)_3 + H_2O \leftrightarrows B(OH)_4^- + H^+$ (p*K*a = 9.24). B is a structural component of the cell wall: borate crosslinks the pectic polysaccharide, rhamnogalacturonan-II (Funakawa and Miwa, 2015). Consistent with its physiological function in the cell wall, the first symptoms of B deficiency are cessation of root elongation, reduced leaf expansion, and loss of fertility (Shorrocks, 1997). In contrast, a high concentration of B leads to cell death by inducing DNA double-strand breaks and various disturbances of metabolism, and negatively affects plant growth (Sakamoto *et al.*, 2011; Reid *et al.*, 2004). Boric acid is a small, neutral molecule and thus is relatively permeable to biological membranes (Dordas and Brown, 2000; Stangoulis *et al*., 2001). Therefore, passive diffusion of boric acid represents an important mode of B transport in plants. However, to maintain B at the appropriate level in tissues under both limited and excess B conditions, plants utilize several boric acid/borate transporters (Yoshinari and Takano, 2017). In roots of *Arabidopsis thaliana*, a boric acid channel, NIP5;1, and a borate exporter, BOR1, are required for efficient B uptake and subsequent translocation into the xylem under limited B conditions (Takano *et al*., 2002; 2006). NIP5;1 and BOR1 localize preferentially in the outer (soil-side) and inner (stele-side) plasma membrane domains, respectively, of various root cells (Takano *et al*., 2010). The polar localization of the boric acid channel and borate exporter indicates the radial transport route of B toward the stele (Wang *et al*., 2017) and plays a role in the B distribution in roots (Shimotohno and Sotta *et al*., 2015). In contrast, under excess B conditions, the BOR4 transporter functions in B exclusion from tissues and cells (Miwa *et al*., 2007; 2014).

Importantly, accumulation of NIP5;1 and BOR1 is downregulated by post-transcriptional mechanisms under high-B conditions. Upon high-B supply, BOR1 is ubiquitinated, internalized from the plasma membrane by endocytosis, and transported to the vacuole for degradation (Takano *et al*., 2005; Kasai *et al.*, 2011). In contrast, NIP5;1 activity is mainly controlled at the level of *NIP5;1* mRNA accumulation, which is dependent on its 5' untranslated region (UTR) (Tanaka *et al*., 2011). High-B conditions enhance ribosome stalling at the minimum open reading frame, AUG-stop, in the 5'UTR, while suppressing the translation and inducing the degradation of *NIP5;1* mRNA (Tanaka *et al.*, 2016). This response is reportedly important for plant acclimation to high-B conditions (Tanaka *et al.*, 2011; 2016).

To understand the B-transport mechanisms in plant tissues, it is essential to determine the B levels in tissues. Traditionally, tissue sampling and measurement of B content has been applied. For this purpose, inductively coupled plasma mass spectrometry (ICP-MS) provides parts-per-billion (ppb)-level sensitivity in solution. Measurement of the stable isotopes ^{10}B and ^{11}B enables tracer analysis of B in plant tissues (Dannel *et al.*, 2000). Recently, application of laser ablation-ICP-MS allowed analysis of the B distribution along the roots of *A. thaliana* at a resolution of tens of micrometers (Shimotohno and Sotta *et al.*, 2015). However, it is theoretically difficult to achieve single-cell-level resolution and follow the distribution of B over time in complex tissues. To evaluate the B distribution and dynamics in plant tissues, a technique that enables B measurement at high spatio-temporal resolution in living cells is needed.

Genetically encoded biosensors enable quantification of analytes at the single-cell level. A pioneering example is the auxin sensor, DII-VENUS, which uses the function of the Aux/IAA auxin-interaction domain. The DII domain in Aux/IAA is essential and sufficient for binding to SCF^{TIR1} protein-ubiquitin ligase. This interaction is directly stimulated by auxin, resulting in rapid degradation of Aux/IAA (Gray *et al.*, 2001). Fusing the DII domain to VENUS-NLS enabled the generation of maps of relative auxin levels at cellular resolution in various *A. thaliana* tissues (Brunoud *et al.*, 2012). Biosensors for other phytohormones, such as abscisic acid or jasmonic acid, were subsequently developed using a similar strategy (Jones *et al.*, 2014; Waadt *et al.*, 2014; Larrieu *et al.*, 2015). Another widely used biosensor, the Förster resonance energy transfer (FRET)-based calcium (Ca) sensor, changes in color upon binding of the second messenger Ca^{2+} (Miyawaki *et al*., 1997). The cytoplasmic Ca^{2+} sensor Yellow Cameleon-Neo was used to detect salt stress-induced Ca^{2+} waves for long-distance root-to-shoot signaling (Choi *et al.*, 2014). Several biosensors for plant essential minerals have also been developed (Goron and Raizada, 2014). For example, genetically encoded FRET sensors for intracellular Zn^{2+} were established to measure changes in the cytosolic Zn concentration in *A. thaliana* root in response to extracellular Zn (Vinkenborg *et al.*, 2009; Lanquar *et al.*, 2014). A FRET-based biosensor for inorganic phosphate (Pi) was used to monitor the Pi dynamics in cytosol and in plastids (Mukherjee *et al.*, 2015). Further, a Ca^{2+} sensor using a nested cassette of green- and orange-emitting fluorescent proteins has been reported (Ast *et al.*, 2017).

Here, we describe the development of genetically encoded biosensors for cytosolic boric acid based on the *NIP5;1* 5'UTR. The *NIP5;1* 5'UTR contains a minimum open reading frame, AUG-stop, responsible for B-dependent repression of translation and mRNA degradation (Tanaka *et al.*, 2011; 2016). In a wheat germ extract *in vitro* translation system, the response dependent on the *NIP5;1* 5'UTR was observed at > 10 µM boric acid, but not under supply of 500 µM NaCl, KNO_3, or KH_2PO_4, indicative of a boric acid-specific response

(Tanaka *et al.*, 2016). Since the major form of B in the cytosol is boric acid, the *NIP5;1* 5'UTR was regarded as an ideal tool to monitor B levels in various plant cell types. To visualize the B distribution at the single-cell level, we designed a biosensor that comprised a fluorescent reporter fused to a nuclear localization signal. The relationship between the fluorescence intensity and intracellular B level was analyzed in cultured transgenic tobacco cells, and the dynamic range was confirmed to be physiologically relevant. By introducing this biosensor to *A. thaliana* plants, we performed a quantitative analysis of intracellular B levels under various B conditions. Additionally, to visualize time-dependent changes in B dynamics, we designed a biosensor that comprised a luciferase protein with a short life. Our findings provide insight into the condition-dependent changes in B transport in plant tissues.

RESULTS AND DISCUSSION

Determination of the dynamic range of cytosolic B for *uNIP5;1-Venus* using tobacco BY-2 cells

To analyze the B distribution in intact seedlings, we developed a genetically encoded biosensor for cytosolic boric acid using the function of the *NIP5;1* 5'UTR, which induces ribosome stalling followed by mRNA degradation, depending on the boric acid concentration (Tanaka *et al.*, 2011; 2016). Therefore, we hypothesized that *NIP5;1* 5'UTR would respond to B concentrations in various cell types. We fused the DNA sequence encoding the 3×mVenus fluorescent protein, a fast-maturating variant of yellow fluorescent protein, to the *NIP5;1* 5'UTR sequence. For visualization of signals at single-cell resolution, a nuclear localization signal (NLS) sequence was added and the *UBQ10* promoter was utilized for ubiquitous expression in various cell types (*proUBQ10:NIP5;1 5'UTR:NLS-3×mVenus*) (Figure S1a and Appendix S1). NLS-3×mVenus was used to avoid protein diffusion into neighboring cells according to a study showing the relationship between protein size and protein diffusion via plasmodesmata (Crawford and Zambryski, 2000). Therefore, sensing occurs in the cytosol and the reporter signal appears in the nucleus in each single cell. A construct lacking *NIP5;1* 5'UTR (*proUBQ10:NLS-3×mVenus*) (Figure S1b and Appendix S1) was prepared as a control.

The biosensor and its control, henceforth called *uNIP5;1-Venus* and *cont-Venus*, respectively, were introduced into tobacco BY-2 cells to assess the B-dependent response. Two transgenic cell lines with *uNIP5;1-Venus* or with *cont-Venus* were generated and maintained as suspension culture in liquid MS medium containing 100 μM B. For analysis of B dependency, cells were washed twice with B-free medium and transferred into fresh medium containing 1 to 3000 μM B. After culture for 48 h, Venus fluorescence in *uNIP5;1-Venus* transformed cells was decreased in the presence of a high B concentration, while no such change was observed in *cont-Venus* transformed cells (Figure 1a, b and Figure S2a, b). These results showed that the *NIP5;1* 5'UTR is affected by the B concentration. To quantify the fluorescence intensity, total protein was extracted from the cells and subjected to spectrofluorometric analysis. At the same time, to measure the intracellular B concentration, the cell exudate was collected by a freeze-thaw method. Just before collecting cells, strontium (Sr), which is not readily taken up by cells, was added to the culture to estimate residual medium contamination (see Experimental Procedures for details). Comparison of the B concentrations in the medium and cell exudate revealed an almost linear correlation, and the relationship was not markedly different

among untransformed cells and cells transformed with *cont-Venus* or *uNIP5;1-Venus* (Figure 1c), indicating that our constructs did not have a negative influence on intracellular B nutrition. We next analyzed the correlation of Venus fluorescence with intracellular B concentration; the results confirmed that *uNIP5;1-Venus* responds to intracellular B in the range of 30~500 µM (Figure 1d and Figure S2c). It is important to note that the lower limit was slightly higher than that measured by *NIP5;1 5'UTR:LUC* in a wheat germ extract *in vitro* translation system (> 10 µM; Tanaka *et al*., 2016). For the treatment of BY-2 cells at a B concentration of 1–10 µM, cells maintained in media containing 100 µM B were used, because BY-2 cells cannot survive in media containing 1–10 µM B. Although the cellular B concentrations of BY-2 cells treated with 1–10 µM B for 48 h were lower than those under 30 µM B, it is likely that 1–10 µM B is not a physiologically relevant concentration for BY-2.

Observation of fluorescence derived from *uNIP5;1-Venus* in *Arabidopsis* seedlings

We next characterized the biosensor in *A. thaliana*. Two independent transgenic lines with *uNIP5;1-Venus* (lines #6 and #9) and one control line with *cont-Venus* were examined. Using those transgenic plants, we first tested the responsiveness of the biosensor to B at the whole-plant level. Plants were grown for 5 days on solid medium containing 1 to 1000 µM B and Venus fluorescence was visualized by epifluorescence microscopy. The fluorescence intensities in the roots and shoots of *uNIP5;1-Venus* transgenic plants were high under limited-B conditions (1 to 10 µM), and gradually declined with increasing B concentration (Figure 2a). A significant decline of the fluorescence intensity in the presence of 1000 µM B was also observed in another *uNIP5;1-Venus* transgenic line (Figure 2b), whereas there was no apparent reduction in fluorescence intensity with increasing B concentration in the control line (Figure 2c). Thus, the biosensor was functional in both roots and shoots. To quantitatively examine the relationship between B concentration and Venus fluorescence intensity, we performed immunoblot analysis of Venus protein level and ICP-MS analysis of B concentration in plant tissues. Total protein extract from transgenic plants harboring *uNIP5;1-Venus* was subjected to immunoblotting; the results showed that the Venus level was 10-fold higher in the presence of 1–10 µM B compared to 1000 µM B, and a significant decrease in protein levels was observed under 100 µM B in both the shoots and roots of *uNIP5;1-Venus* line #9 (Figure 2d and Figure S3a; $p < 0.01$, Dunnett's test). A similar tendency was observed in *uNIP5;1-Venus* line #6, although the Venus level in the shoot was higher in the presence of 30 µM B (Figure 2e and Figure S3b). In the control line, the Venus protein level was ~30% lower in roots in the presence of the highest B concentration, but the value did not differ significantly from that at 1 µM B (Figure 2f and Figure S3c; $p > 0.01$, Dunnett's test). Therefore, in the wild-type background, *uNIP5;1-Venus* responded to external B concentrations > 30 µM in roots and shoots.

Tissue B levels were determined by digesting tissues with nitric acid followed by ICP-MS analysis. The B concentrations in shoots and roots showed an overall positive correlation with those in the medium, although the tissue B concentration was maintained at > 40 µmol/kg fresh weight in the presence of < 30 µM B (Figure 2g). This phenomenon is consistent with previous reports of accumulation of B via B-transport proteins (Noguchi *et al*., 2000; Takano *et al*., 2002; Yoshinari and Takano, 2017). Importantly, a negative correlation was observed between the Venus protein level and the B concentration in the range of ~100 to 1000 µmol/kg fresh weight (Figure 2h).

Quantitative analysis of B distribution in *Arabidopsis* roots using *uNIP5;1-Venus*

Next, we used *uNIP5;1-Venus* to quantify the B distribution in roots at single-cell resolution. Confocal imaging of Venus in the mature portion of *Arabidopsis* roots showed that the signals were stronger under low-B supply, especially in the outer cell layers, in two independent *uNIP5;1-Venus* lines (Figure 3a and Figure S4). To quantify the signal, we obtained Z-stack images covering stele (1 μm × 10) and made maximum intensity projections (Figure 3a, Figure S4, and Methods S1). The signal intensities in the nuclei were measured and the mean and standard deviation signal intensity in each cell layer were calculated. Taking into consideration the reduction in light intensity during passage through tissue and possible differential expression in different cell layers, the intensity values from *uNIP5;1-Venus* were normalized to those of the two control lines in the presence of 3 μM B (Figure 3b; see Experimental Procedures, Methods S1, and Figure S5 for details). The relative fluorescence levels were higher in the presence of 1 μM B in epidermal, cortical, and endodermal cells than in stellar cells (Figure 3c). The relative fluorescence levels in epidermal, cortical, and endodermal cells significantly decreased at 3 and 10 μM B in *uNIP5;1-Venus* line #6 and at 10 μM B in line #9 (Figure 3c; $p < 0.05$, Dunnett's test). However, the relative fluorescence levels did not decrease in stellar cells. These results suggest that the B concentration in outer cell layers is influenced by the B concentration in the medium, but that in stele is maintained at a higher level under low-B supply. These findings are consistent with our previous observation that the tracer B concentration in root-cell sap, which corresponds to all unbound B in the root, increased in proportion to the tracer B concentration in the medium, while that in xylem exudate was more concentrated than that in the medium under low-B conditions (Takano *et al.*, 2002). Our results using *uNIP5;1-Venus* further suggest that the cellular B concentration is influenced by that in the apoplast because of the high membrane permeability of boric acid (Dordas and Brown, 2000; Stangoulis *et al.*, 2001), and the B concentration in stele, including xylem (regarded as apoplast), and vascular cells is maintained at a high level by the functions of the borate exporter BOR1 and the Casparian strip, which separates apoplast in stele from apoplast in cortical cells (Barberon and Geldner, 2014; see models in Figure S6a, b).

Observation of luminescence derived from *uNIP5;1-Luc* in *Arabidopsis* seedlings

The ability of *uNIP5;1-Venus* to analyze the temporal dynamics of B is limited by the stability of Venus protein (Snapp, 2009). Indeed, when plants with *uNIP5;1-Venus* were transferred from low-B (0.3 μM) to high-B (50 μM) conditions, the Venus fluorescence intensity gradually decreased in the first several hours and continued to decrease until 24 h after the change (Figure S7a, b). To overcome this limitation, we developed another B sensor that enabled detection of changes in cytosolic B concentrations with improved time resolution. We utilized the green-emitting luciferase from a Brazilian click beetle (Eluc) with a protein destabilization signal PEST sequence (Toyobo; Rogers *et al.*, 1986) to achieve a short half-life under the control of the *UBQ10* promoter and *NIP5;1* 5'UTR (*proUBQ10:NIP5;1 5'UTR:ElucPEST*) (Figure S1c and Appendix S1). A construct without *NIP5;1* 5'UTR (*proUBQ10:ElucPEST*) (Figure S1d and Appendix S1) was prepared as a control. This biosensor and its control, henceforth called *uNIP5;1-Luc* and *cont-Luc*, respectively, were introduced into *A. thaliana* wild type Col-0.

To assess the responses of *uNIP5;1-Luc* to different B conditions in plants, two independent transgenic *A. thaliana* lines were generated. Plants grown on medium containing 0.3 μM B and 100 μM D-luciferin were transferred to medium containing 0.3–1000 μM B and 100 μM D-luciferin, and incubated for 3 h. A significant decrease in Luc intensity was observed from 10 to 1000 μM B in *uNIP5;1-Luc* line #8, and from 3 to 100 μM B conditions in *uNIP5;1-Luc* line #28 (Figure 4b, c; $p < 0.05$, Steel-Dwass test), whereas there was no significant difference in the *cont-Luc* line among the B concentrations tested (Figure 4d; $p > 0.05$, Steel-Dwass test). Therefore, we concluded that *uNIP5;1-Luc* in the roots of the transgenic lines responded to B concentrations in medium in the range of 10 to 100 μM (Figure 4a–c), but *cont-Luc* did not (Figure 4d). To examine the time-dependent response of *uNIP5;1-Luc*, we visualized the luminescence signal after a shift to high-B conditions. *A. thaliana* plants with *uNIP5;1-Luc* or *cont-Luc* grown on medium containing 0.3 μM B were shifted to medium containing 0.3 or 50 μM B. Time-lapse imaging revealed that the luminescence signals from *uNIP5;1-Luc* lines began to decrease 30 min after transfer to the 50 μM B condition and reached a steady-state level within 120 min (Figure 4e, f, h, i). In contrast, the signal increased when shifted to 0.3 μM B medium (Figure 4e, f, h, i). The luminescence signal intensity of *cont-Venus* increased when shifted to medium containing 0.3 or 50 μM B (Figure 4g, j), likely due to a fresh supply of D-luciferin. Significant differences in the Luc signal following transfer to the 0.3 and 50 μM B conditions were observed after 30 and 20 min in *uNIP5;1-Luc* lines #8 and #28, respectively (Figure 4h, i; $p < 0.05$, paired *t*-test), but not in *cont-Luc* plants over the period of the analysis (Figure 4j; $p > 0.05$, paired *t*-test). Due to the time required for turnover of Eluc-PEST proteins, *uNIP5;1-Luc* does not report changes in B concentration in real time. However, our results show that *uNIP5;1-Luc* responds to increasing B concentrations within 1 h, representing an improvement of more than 10 times in temporal resolution compared to *uNIP5;1-Venus* (Figure S7) and providing temporal information on the B distribution in tissues.

Analysis of B uptake in *Arabidopsis* roots using *uNIP5;1-Luc*

We used *uNIP5;1-Luc* to visualize differences in B uptake into root cells in transgenic plants expressing BOR1 variants. BOR1 is a borate exporter that localizes in the stele-side domain of the plasma membrane in root cells (Takano *et al.*, 2010). The function of BOR1 is required for translocation of B toward the stele under low-B conditions but is repressed by ubiquitination at the K590 residue and subsequent degradation in the vacuole (Takano *et al.*, 2005; Kasai *et al.*, 2011). Previously, we showed that fusion of hygromycin phosphotransferase (HPT) to BOR1-GFP weakened the polar localization and that introduction of the K590A mutation stabilized accumulation in the plasma membrane under high-B conditions. Interestingly, *A. thaliana* plants ubiquitously expressing the weak-polarization and stabilized BOR1 variant under the control of the *UBQ10* promoter (*proUBQ10:BOR1(K590A)-GFP-HPT*) showed improved root and shoot growth under excess-B conditions compared to the wild-type Col-0 and BOR1-GFP-HPT transgenic plants (Wakuta *et al.*, 2016). In the BOR1(K590A)-GFP-HPT lines, the total B concentrations in roots were similar and those in shoots were higher than in the Col-0 and BOR1-GFP-HPT lines. We thus reasoned that tolerance to excess B conditions was induced by exclusion of B from the cytosol in various cell types. To investigate this, *uNIP5;1-Luc* was introduced into the BOR1-GFP-HPT and BOR1(K590A)-GFP-HPT lines. The changes in luminescence signals were not markedly different among plants after shifting from 0.3 μM B to 0.3 μM B (Figure 5a, b). The

luminescence signal intensity in roots was decreased after shifting to 50 µM B medium in all plants, but increased after 80 min in the plants expressing BOR1(K590A)-GFP-HPT (Figure 5c, d). The intensities in BOR1(K590A)-GFP-HPT lines #4 and #8 were significantly higher than in plants with a wild-type background after 30 and 70 min, respectively (Figure 5c, d; $p < 0.05$, Dunnett's test). This suggests that intracellular B concentration in the BOR1(K590A)-GFP-HPT lines increased after transfer to a higher B concentration and decreased thereafter. The NIP5;1 boric acid channel may have downregulated upon transfer to high-B conditions, and so the rate of efflux of boric acid/borate by BOR1(K590A)-GFP-HPT exceeded that of influx of boric acid by NIP5;1 and passive diffusion across the plasma membrane in root cells. Therefore, using *uNIP5;1-Luc*, we demonstrated that BOR1(K590A)-GFP-HPT exported B to reduce the intracellular B concentration.

Conclusion

We established and characterized genetically encoded biosensors for cytosolic boric acid. Our biosensors were designed based on the function of the *NIP5;1* 5'UTR sequence in degrading mRNA in response to cytosolic accumulation of boric acid. The biosensors enable visualization of B distribution/dynamics at physiologically relevant levels in various tissue and cell types. Characterization of *uNIP5;1-Venus* in tobacco BY-2 cells confirmed its ability as an indicator of cytosolic boric acid. In *A. thaliana* roots, *uNIP5;1-Venus* enabled visualization of changes in B concentration in epidermal, cortical, and endodermal cells and maintained B accumulation in stellar cells under physiologically low-B conditions, where facilitated transport of B is required for plant growth (see models in Figure S6). To increase the temporal resolution, we also established *uNIP5;1-Luc* using a luciferase with a short life. *uNIP5;1-Luc* facilitated visualization of the response of cytosolic B to external B when plants grown under a physiologically low-B condition were supplied with an adequate B concentration. We expect that these biosensors will be applied to reveal various aspects of B homeostasis in different plant organs at different growth stages.

EXPERIMENTAL PROCEDURES

Construction of biosensors for boric acid

A fragment of the *UBQ10* promoter with *Hin*dIII and *Nhe*I linker sequences, a fragment of *NIP5;1* 5'UTR with *Xba*I and *Kpn*I linker sequences, and a fragment of *ElucPEST* with *Kpn*I and *Eco*RI linker sequences were subcloned into the *Hin*dIII and *Eco*RI sites of pMDC99 (Curtis and Grossniklaus, 2003), resulting in pSW52 (*proUBQ10:NIP5;1 5'UTR:ElucPEST*: *uNIP5;1-Luc*). Fragments of the *proUBQ10:NIP5;1* 5'UTR were digested from pSW52 by *Hin*dIII and *Kpn*I. Fragments of *NLS-3×mVenus* with *Kpn*I and *Sac*I linker sequences were amplified by PCR using *pGreen229-NLS-3×mVenus* (Vermeer *et al*., 2014) as a template. The fragments containing *proUBQ10:NIP5;1 5'UTR* and *NLS-3×mVenus* were cloned into the *Hin*dIII and *Sac*I sites of pSW52, resulting in pSW54 (*proUBQ10:NIP5;1 5'UTR:NLS-3×mVenus*: *uNIP5;1-Venus*). The recognition sequence of *Kpn*I was introduced between *proUBQ10* and *NIP5;1 5'UTR* in pSW52 and pSW54 using the

following primers: 5'-gccgggcccggtacccataagctcaaagactaacc-3' and 5'-ggtaccgggcccggctgttaatcagaaaaactcag-3'. The fragments containing *NIP5;1* 5'UTR with two *Kpn*I sites were removed from pSW52 and pSW54 and ligated, resulting in pSW77 (*proUBQ10:ElucPEST*: *cont-Luc*) and pSW78 (*proUBQ10:NLS-3×mVenus*:*cont-Venus*), respectively. The inserted DNA fragments were confirmed by sequencing. The whole sequences of the inserts in pSW52, pSW54, pSW77, and pSW78 are shown in Appendix S1.

Maintenance and transformation of tobacco BY-2 Cells

The tobacco BY-2 cell line was maintained in modified Linsmaier and Skoog medium (Nagata *et al*., 1992) and the culture was diluted with fresh medium weekly (1:100 (v/v)]. For transformation, a 4 mL aliquot of a 4-day-old BY-2 culture was co-cultivated with 100 µL of an overnight-grown *Agrobacterium* culture (optical density at 600 nm [OD_{600}] = 1 – 3) in a 10 cm Petri dish for 2 days at 26°C in the dark. Cells were washed three times with 10 mL of fresh medium and grown on solid medium containing 500 µg/mL Claforan and 20 µg/mL hygromycin for 3 weeks at 26°C in the dark. A single colony was isolated and expanded in liquid culture for further characterization.

Spectrophotometric analysis of fluorescence in BY-2

After incubation with various concentration of boric acid for 48 h, cells were pelleted (100 × *g* for 2 min at 4°C), chilled in liquid nitrogen, and homogenized by sonication in lysis buffer (100 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid [EDTA], 2 mM dithiothreitol [DTT], and 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). The extract was purified by ultracentrifugation (100,000 × *g*, 30 min), and the resulting supernatant was used for fluorometric analysis. For each sample, protein concentration was adjusted to 2 mg/mL using the Bradford method (Quick Start Protein Assay Kit I; Bio-Rad). Fluorescence measurements were performed on a JASCO-FP6500 spectrofluorometer using an excitation wavelength of 515 nm; emission spectra were recorded from 250 nm to 750 nm at 1 nm intervals in a microcell at room temperature and the results of three scans were averaged. Sensitivity was set to high for measuring extracts of untransformed or *uNIP5;1-Venus* transformed cells, and medium for *cont-Venus* cells. Venus fluorescence intensity was quantified at 528 nm. To correct for the background fluorescence of the solution components, a blank sample without cell extract and a control sample prepared from untransformed BY-2 cell extract were analyzed identically to biosensor-transformed cells. We first subtracted the mean blank fluorescence from the data. We next estimated the background fluorescence due to the turbidity of cell extract by calculating the average ratio of the fluorescence intensity of a test sample (S) to a control sample (C) from 490 nm to 510 nm at 1 nm intervals as follows:

$$\alpha = \frac{\sum_{\lambda=490}^{\lambda=510} I_s(\lambda)/I_c(\lambda)}{21}$$

where $I_s(\lambda)$ and $I_c(\lambda)$ refer to the fluorescence intensities at emission wavelength λ of a test sample (S) and a control sample (C) prepared from untransformed BY-2 cells, respectively. The α value was used to calculate the Venus fluorescence intensity (I_v) as follows:

$$I_V = I_S(528) - I_C(528) \cdot \alpha$$

Measurement and calculation of boron concentration in the medium and the cell

The cell culture was chilled on ice and $SrCO_3$ (Wako Pure Chemicals) was added to a final concentration of ~200 ppb. The cells were pelleted by centrifugation (100 × *g* for 2 min at 4°C) and the resulting supernatant was sampled as the medium fraction, whereas the cell pellet was frozen in liquid nitrogen and thawed at room temperature. The freeze-thawed cells were centrifuged (100,000 × *g* for 5 min) and the resulting supernatant was collected as the cell-exudate fraction. Samples (0.3 mL) were evaporated in a heat block set at 90°C. Next, two 0.3 mL volumes of concentrated HNO_3 followed by 0.2 mL of H_2O_2 were added to digest the sample at 120°C. The residues were dissolved in 0.08 M HNO_3 and subjected to measurement of B and Sr by ICP-MS (Agilent 7500, Agilent). With the assumption that Sr was not taken up into the cells, the cellular B concentration was calculated as follows:

$$B_C = (B_E - B_M \cdot (1 - \alpha))/\alpha$$

where α is the ratio of the cell volume to the total volume of cell exudate:

$$\alpha = 1 - Sr_E/Sr_M$$

The subscripts C, M, and E refer to the atomic percentages of B in the cell, medium, and cell exudate.

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. accession Col-0 was from our laboratory stock. Seeds were surface-sterilized and sown on medium (Takano *et al*., 2005) supplemented with 1% sucrose and various concentrations of boric acid, and solidified with 1.5% (w/v) gellan gum (Wako Pure Chemical). The plates were incubated at 4°C for 1–4 days and placed vertically in a growth chamber at 22°C with a 16 h light / 8 h dark cycle.

Generation of transgenic plants

A. thaliana Col-0 ecotype was used for transformation by the *Agrobacterium*-mediated floral-dip method (Clough and Bent, 1998). For selection of T1 transformants, solid medium containing half-strength Murashige Skoog salts, 1% (w/v) sucrose, 0.2% (w/v) gellan gum, and 20 mg/L hygromycin B was used (chemicals were obtained from Wako Pure Chemicals). The hygromycin-resistant T1 plants were transferred onto solid medium supplemented with 0.3 μM boric acid and grown for 4–6 days. The Venus signals in roots and shoots of *uNIP5;1-Venus* were visualized by epifluorescence microscopy, and we selected plants with strong Venus

fluorescence intensities. For *uNIP5;1-Luc*, the plants were supplied with 0.1 mM D-luciferin in solid medium for 2 h and plants with strong luminescence signals were selected using a LAS-3000 mini luminescence image analyzer (Fujifilm). In the T2 progeny, segregation of hygromycin resistance was examined and lines that theoretically carried a single T-DNA insertion were selected (11 lines for *uNIP5;1-Venus*, 22 lines for *cont-Venus*, 3 lines for *uNIP5;1-Luc*, and 8 lines for *cont-Luc*). Next, we selected plants with strong Venus and Eluc fluorescence intensities. In the T3 progenies of the T2 plants, segregation of hygromycin resistance was examined and three *uNIP5;1-Venus* lines, four *cont-Venus* lines, three *uNIP5;1-Luc* lines, and eight *cont-Luc* lines were established as homozygous plants. Lines #6 and #9 for *uNIP5;1-Venus*, lines #1, #15, and #16 for *cont-Venus*, lines #8 and #28 for *uNIP5;1-Luc*, and line #10 for *cont-Luc* were used in the analysis. To generate plants harboring *proUBQ10:BOR1-GFP-HPT* or *proUBQ10:BOR1(K590A)-GFP-HTP* with *uNIP5;1-Luc*, T3 plants harboring *proUBQ10:BOR1-GFP-HPT* and *proUBQ10:BOR1(K590A)-GFP-HTP* (Wakuta *et al*., 2016) and T3 plants expressing *uNIP5;1-Luc* were crossed. As a control, Col-0 and T3 plants expressing *uNIP5;1-Luc* were crossed. The F1 plants were used for the time-course analysis.

Immunoblot analysis

Transgenic *A. thaliana* plants were grown on solid medium containing various concentration of B for 2 weeks, and roots and shoots were collected separately from about ten plants per condition. Tissues were rapidly frozen and crushed to produce fine powder. Total protein was extracted by sonication in ice-cold lysis buffer (20 mM Tris-HCl [pH 8.0], 20 mM KCl, 2.5 mM $MgCl_2$, 250 mM sucrose, 0.2% [w/v] Nonidet P-40, 6.25% [w/v] glycerol, 1 mM DTT, 2 mM EDTA, 1 mM PMSF, and Complete Protease Inhibitor Cocktail [Roche]), and centrifuged at 10,000 × *g* for 20 min at 4°C. The resultant supernatant was collected and its protein concentration was estimated by protein assay (Bio-Rad). Samples were denatured in Laemmli buffer, applied to 10% (w/v) polyacrylamide mini-gels, and transferred to polyvinylidene fluoride membranes (Merck Millipore) by electroblotting. Protein bands were detected using an ECL advance Western blotting detection kit (Amersham) according to the manufacturer's protocol. An anti-GFP mouse monoclonal antibody (Nacalai Tesque, clone GF200) was used at 1:2500 dilution. After immunoblotting, membranes were stained with Coomassie Brilliant Blue G-250 to visualize proteins.

Imaging and quantification of Venus signals in root cells

After 3 days of growth on solid medium containing various concentrations of B in a growth chamber at 22°C under a cycle of 16 h light (4:00–20:00) and 8 h dark, the roots were covered with a block of medium to allow nutrient uptake from the entire surface. The plates were placed vertically in the growth chamber and incubated for a further 2 days.

Imaging was performed between 11:00 and 15:00. The roots of plants were incubated in aqueous medium containing various concentrations of boric acid (w/o sucrose) and 20 μg/mL propidium iodide (Wako Pure Chemicals). After 10 min, the roots were washed with aqueous medium containing the same

concentrations of boric acid. The roots were placed on a cover glass and covered with a medium block and the aqueous medium. The middle section of roots was visualized using a 20× water-immersion lens at 12-bit depth using a TCS-SP8 confocal laser scanning microscope equipped with hybrid detectors (Leica). Excitation was performed using a 488 nm diode laser and detection was at 505–530 nm for Venus and at 650–750 nm for propidium iodide. Two Z-stack image sets (>20 each) were acquired at 1 μm intervals from the middle sections of roots.

Maximum intensity projection and signal quantification are described in the Supplemental Methods. Briefly, maximum intensity projection was performed using ImageJ software (NIH) from 10 Z-stack images covering the central cylinder of roots. Cell layers were separated into different images using MetaMorph software (Molecular Devices). Nuclei in each cell layer were identified by Bernsen's local thresholding method using ImageJ software. The integrated density of signals from each nucleus was analyzed using the Analyze Particle tool of ImageJ (Supplemental Figure 5 and Supplemental Methods). To control for the effects of laser and fluorescence transmission rates according to depth and variation in the transcription activity of *proUBQ10*, the cell-layer coefficients were determined using the values from the control lines to be as follows: 1.1, epidermis; 1.0, cortex; 0.39, endodermis; and 0.25, stele (Supplemental Figure 5a).

Measurement of luciferase activity

After growth for 3–5 days on solid medium, seedlings were transferred to fresh medium supplemented with 0.3 μM boric acid and 100 μM D-luciferin (Wako Pure Chemicals) and solidified with 0.5% (w/v) gellan gum. The plates were incubated for 18–21 h in a growth chamber to allow absorption of luciferin. The plants were transferred to solid medium containing the indicated concentrations of boric acid and 100 μM D-luciferin. Luminescence was detected using a LAS-3000 mini (Fujifilm) or LAS-4000 luminescence image analyzer (GE Healthcare).

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SHORT SUPPORTING INFORMATION LEGENDS

Figure S1. Schematic representation of the constructs used in this study.

Figure S2. Reponses of *uNIP5;1-Venus* in tobacco BY-2 cells related to Figure 1.

Figure S3. Immunoblot analysis of Venus protein in *Arabidopsis* tissues related to Figure 2d–f.

Figure S4. B distribution in the mature portion of roots related to Figure 3a.

Figure S5. Quantification of the fluorescence intensities in nuclei in different cell layers of *uNIP5;1-Venus*- and *cont-Venus*-expressing plants related to Figure 3c.

Figure S6. Models of B transport mechanisms and B distribution in the mature portion of *Arabidopsis* roots.

Figure S7. Time course analysis of the *uNIP5;1-Venus* response in roots.

Methods S1. Quantification of fluorescence signals in nuclei in cell layers of the mature portion of roots.

Appendix S1. DNA sequences of *proUBQ10:NIP5;1 5'UTR:NLS-3×mVenus*, *proUBQ10:NLS-3×mVenus*, *proUBQ10:NIP5;1 5'UTR:ElucPEST*, and *proUBQ10:ElucPEST*.

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FIGURE LEGENDS

Figure 1. Responses of *uNIP5;1-Venus* in tobacco BY-2 cells. (a, b) Venus fluorescence in cells transformed with *uNIP5;1-Venus* (a) or *cont-Venus* (b) was measured using a spectrofluorometer. Protein extract (2 mg/mL) was analyzed in three independent experiments (mean ± SD, n = 3, $p < 0.05$, Steel-Dwass test; different letters above each bar indicate a significant difference among the B conditions). (c) Relationship between B concentration in medium and that in cells. (d) Changes in Venus fluorescence intensity in response to intracellular B concentration on a logarithmic scale. The shaded area in (d) shows the predicted working range of *uNIP5;1-Venus* (30–500 µM B).

Figure 2. Responses of *uNIP5;1-Venus* in *Arabidopsis thaliana* tissue. (a–c) Fluorescence in the independent *uNIP5;1-Venus* (*proUBQ10:NIP5;1 5'UTR:NLS-3×mVenus*) lines #9 (a) and #6 (b) and the *cont-Venus* line #1 (*proUBQ10:NLS-3×mVenus*) (c) cultured for 4–6 days on medium containing various B concentrations was observed under a fluorescence stereomicroscope. Bars, 5 mm. Enlarged images of *uNIP5;1-Venus* line #6 at 1 and 100 µM B are shown in Figure S4. (d–f) Venus protein levels in shoots (gray) and roots (black) of *uNIP5;1-Venus* lines #9 (d) and #6 (e) and a *cont-Venus* line (f) grown under various B concentrations were quantified by immunoblot analysis, as shown in Figure S3. The band intensity was quantified using the Gels function of ImageJ software and is shown as the ratio to band intensity under the 1 µM B condition (mean ± SD, n = 3). *, statistically different from the 1 µM B condition ($p < 0.01$, Dunnett's test). (g) Correlation between the B concentration in medium and that in shoots (gray) or roots (black) of *uNIP5;1-Venus* transgenic line #9. Tissues were collected from about ten plants per condition and digested with concentrated nitric acid, and the B level was measured by ICP-MS (mean ± SD, n = 5). (h) Correlation between the Venus fluorescence intensity with the B concentration in shoots (gray) or roots (black).

Figure 3. Quantification of the distribution of B in the mature portion of roots. (a, b) Maximum intensity projection of confocal images (ten images at 1-µm intervals) of Venus fluorescence (Green Fire Blue pseudocolor; lookup table is shown on the right side) merged with an image of propidium iodide fluorescence (red) for the *uNIP5;1-Venus* (a) and *cont-Venus* lines (b). The plants were grown for 5 days on medium containing various B concentrations and the middle sections of the roots were imaged. Bar, 200 µm. (c) Relative fluorescence levels of nuclei in different cell layers. The fluorescence intensities in independent nuclei were measured as described in the Supplemental Methods in *uNIP5;1-Venus* lines grown in the presence of 1, 3, or 10 µM B and *cont-Venus* lines grown in the presence of 3 µM B (Figure S5). To control for the effects of the laser and fluorescence transmission rates according to the depth and variation in the transcription efficiency of *proUBQ10*, the cell layer coefficient was determined using values obtained from the *cont-Venus* control lines (Figure S5a). The average *uNIP5;1-Venus* fluorescence values (Figure S5b, c) were normalized to the cell-layer coefficients and are shown here (mean ± SD, n = 4–5 plants). *, statistically different from the 1 µM B condition ($p < 0.05$, Dunnett's test).

Figure 4. Responses of *uNIP5;1-Luc* to different B concentrations. (a) Luminescence of transgenic plants expressing *uNIP5;1-Luc* (lines #8 and #28) or *cont-Luc*. Plants grown on medium containing 0.3 µM B for 5 days were pre-incubated with D-luciferin for 21 h. Next, plants were transferred to medium containing the indicated concentration of B and incubated for 3 h. Scale, 1 cm. (b–d) The mean luminescence intensity per root of *uNIP5;1-Luc* lines #8 (b) and #28 (c) and *cont-Luc* (d) was measured using ImageJ (mean ± SD, n = 7–8, $p < 0.05$, Steel-Dwass test; different letters above each bar indicate a significant difference among the B conditions). (e–g) Changes in Eluc luminescence in the root tips of *uNIP5;1-Luc* lines #8 (e) and #28 (f) and *cont-Luc* (g) after transferring to fresh medium containing 0.3 or 50 µM B. Luminescence was detected over 160 min at 10-min intervals. Luminescence images of representative plants are shown. (h–j) Quantification of luminescence after transferring to fresh medium containing 0.3 (white) or 50 (black) µM B. Values are relative to that measured at 10 min after the transfer (mean ± SD, n = 4–6).

Figure 5. Time course of B uptake into the roots of transgenic plants expressing BOR1 variants. (a–d) Changes in the luminescence intensity of transgenic plants harboring *uNIP5;1-Luc* and *proUBQ10:BOR1-GFP-HPT* or *proUBQ10:BOR1(K590A)-GFP-HPT* constructs. Plants were grown on medium containing 0.3 µM B for 3 days, transferred to medium with 0.3 µM B and 100 µM D-luciferin, and incubated for 18 h. Then, plants were transferred to solid medium containing 0.3 (a, b) or 50 (c, d) µM B supplemented with 100 µM D-luciferin. Luminescence was detected over 160 min at 10-min intervals. (a, c) Images of representative roots. Scale, 1 cm. (b, d) Luminescence intensity per root measured using ImageJ; values relative to that at 0 min are shown (mean ± SD, n = 3).