Cell-Type Specificity of the Expression of Os BOR1, a Rice Efflux Boron Transporter Gene, Is Regulated in Response to Boron Availability for Efficient Boron Uptake and Xylem Loading

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We describe a boron (B) transporter, Os BOR1, in rice (Oryza sativa). Os BOR1 is a plasma membrane–localized efflux transporter of B and is required for normal growth of rice plants under conditions of limited B supply (referred to as -B). Disruption of Os BOR1 reduced B uptake and xylem loading of B. The accumulation of Os BOR1 transcripts was higher in roots than that in shoots and was not affected by B deprivation; however, Os BOR1 was detected in the roots of wild-type plants under -B conditions, but not under normal conditions, suggesting regulation of protein accumulation in response to B nutrition. Interestingly, tissue specificity of Os BOR1 expression is affected by B treatment. Transgenic rice plants containing an Os BOR1 promoter–β-glucuronidase (GUS) fusion construct grown with a normal B supply showed the strongest GUS activity in the steles, whereas after 3 d of -B treatment, GUS activity was elevated in the exodermis. After 6 d of -B treatment, GUS activity was again strong in the stele. Our results demonstrate that Os BOR1 is required both for efficient B uptake and for xylem loading of B. Possible roles of the temporal changes in tissue-specific patterns of Os BOR1 expression in response to B condition are discussed.

INTRODUCTION

Boron (B) is an essential nutrient for plants, and B deficiency has been reported in 132 crops in >80 countries (Shorrocks, 1997). B deficiency generally affects the growing portion of the plant. B cross-links rhamnogalacturonan-II (RG-II) in the cell wall (Matoh et al., 1993), and borate-RG-II complexes have been detected in a wide range of plant species (Matoh et al., 1996; Matsunaga et al., 2004). The cross-linking of RG-II by B is required for the normal expansion of Arabidopsis thaliana rosette leaves (O’Neill et al., 2001). This requirement is probably one basis for the symptoms that appear in young portions of B-deficient plants. B deficiency also affects membrane functioning and metabolic activities (for review, see Bolanos et al., 2004), but it is likely that these effects are indirect consequences of the deficiency.

The B content in the cell walls of shoots of well-fertilized rice (Oryza sativa) plants is in the range of 5 mg B kg⁻¹ of cell wall (Matoh et al., 1996). This value is 5- to 10-fold lower than the levels in dicotyledonous plants, the cell walls of which typically contain ~25 to 45 mg B kg⁻¹ of cell wall (Matoh et al., 1996). The B contents in leaf tissues of different plant species, grown in the same location, can differ considerably. Graminaceous species generally contain lower B content than dicotyledonous species (Marschner, 1995). The B requirements of graminaceous species are also lower than those of dicotyledonous species and correlate positively with the pectin contents of the cell walls (reviewed in Rerkasem and Jamjod, 2004). For example, symptoms of B deficiency appear in rice plants when the boric acid concentration in the hydroponic solution supporting these plants is <0.2 μM (Yu and Bell, 1998). In Arabidopsis, B deficiency symptoms are evident in wild-type plants grown in hydroponic solution containing 0.5 μM boric acid (Miwa et al., 2006). These differences between rice and dicotyledonous plants with respect to the B concentrations in the shoot tissues and the susceptibilities to B deficiency suggest differences in the B transport capacities of these plants.

It has long been believed that the passive transport of boric acid supplied sufficient B to the plant (Marschner, 1995). Recently, the active transport of B was reported in sunflower (Helianthus annuus) (Dannel et al., 1998). Soon thereafter, the Arabidopsis gene At BOR1 was identified as the first B transporter in a living system (Takano et al., 2002). At BOR1 is an efflux-type B transporter that functions in xylem loading and is essential for preventing B deficiency in shoots. Six BOR1-like genes are present in Arabidopsis. BOR1-like genes have also been found in a number of other plant species, including rice, suggesting the importance of BOR1 and similar genes in plants.

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In excess, B is toxic. Therefore, it is important to regulate B transport in response to B conditions in the environment, as with other essential nutrients. For example, major transporters in plants, such as the ammonium transporters (AMTs) (Loque and von Wirén, 2004) and iron transporters (Ishimaru et al., 2006) are regulated at the transcriptional level and respond to the status of the corresponding nutrient. The ion transporter gene At \textit{IRRT} is regulated at both transcriptional and posttranscriptional levels (Connolly et al., 2002). \textit{Arabidopsis} \textit{NIP5;1}, a B channel crucial for B uptake under low B conditions, is regulated at the mRNA level by the B status (Takano et al., 2006). It has also been demonstrated that At \textit{BOR1} is regulated posttranscriptionally in response to B availability (Takano et al., 2005). This regulation is likely to be important in preventing the overaccumulation of B in shoots under high B conditions.

To understand the molecular mechanisms involved in B transport in rice, we focused on Os \textit{BOR1}, the rice gene most similar to At \textit{BOR1}. The roles of Os \textit{BOR1} in B transport and the regulation of Os \textit{BOR1} are described.

RESULTS

At \textit{BOR1}-Like Genes in Rice

A database search of the rice genome for At \textit{BOR1}-like genes identified four genes. We obtained cDNAs corresponding to these genes, determined their nucleotide sequences, and identified their intron-exon structures and open reading frames (ORFs). Based on the predicted amino acid sequences, we named these genes Os \textit{BOR1}-4. The identifiers of these genes are shown in Table 1. The nucleotide sequences of the cDNAs corresponding to Os \textit{BOR1} and Os \textit{BOR3} are identical to sequences in the database (GenBank accession numbers AK070617 and AK072421, respectively). The nucleotide sequences of the Os \textit{BOR2} and Os \textit{BOR4} cDNAs were confirmed by direct sequencing of an RT-PCR product and three independently isolated RT-PCR products (accession numbers DQ421408 and DQ421409, respectively).

A phylogenetic analysis of the predicted amino acid sequences of the \textit{Arabidopsis} and rice \textit{BOR1}-like genes suggested that Os \textit{BOR1} is most similar to At \textit{BOR1}, and Os \textit{BOR2}, 3, and 4 are more distantly related to At \textit{BOR1} than Os \textit{BOR1} (Figure 1). Three of the four rice At \textit{BOR1}-like genes, Os \textit{BOR2}, 3, and 4, form a branch distinct from the \textit{Arabidopsis} genes (Figure 1). Os \textit{BOR1} is predicted to encode a polypeptide of 711 amino acids. The Phobius program (Kall et al., 2004) predicted that Os \textit{BOR1} contains 10 transmembrane domains, as does At \textit{BOR1}.

Os \textit{BOR1} Reduces the B Concentration in Yeast Cells

To examine the B efflux activity of Os \textit{BOR1}, we expressed the gene in the \textit{Saccharomyces cerevisiae} strain \textit{scbort}. (Takano et al., 2002; Nozawa et al., 2006). \textit{scbort} cells lack an endogenous B efflux transporter. Cells in the mid-log phase cultured in liquid medium were exposed to 100 μM boric acid for 60 min, and the B concentration in the cells was determined. Transformants carrying the empty vector pYES2 and exposed to 100 μM B accumulated B to ~800 μmol/kg dry weight (Figure 2A), whereas the B concentrations in cells expressing At \textit{BOR1} (Takano et al., 2002) or Os \textit{BOR1} were ~270 and 540 μmol/kg dry weight, respectively (Figure 2A), reducing the B concentrations to 66 and 32% of the vector control, respectively. These results suggest that Os \textit{BOR1} is an efflux transporter of B, as is At \textit{BOR1}.

Os \textit{BOR1} Localizes to the Plasma Membrane

We investigated the subcellular localization of Os \textit{BOR1}. A cauliflower mosaic virus 35S RNA (referred to as 35S) promoter: GFP-Os \textit{BOR1} cDNA fusion construct was introduced into onion (\textit{Allium cepa}) epidermal cells by particle bombardment (Figure 2B). The sGFP-Os \textit{BOR1} fusion protein localized to the cell periphery, in contrast with the protein produced from Pro35S:sGFP (Figure 2B). We confirmed this plasma membrane localization of the sGFP-Os \textit{BOR1} fusion protein by plasmolyzing the cells with 1 M mannitol (Yamaguchi et al., 2005). The fluorescence signal was localized only at the thin layer of the cytosol and not at the cell wall (data not shown). Similar results were obtained with the bombardment of plasmids containing a Pro35S:Os \textit{BOR1}-sGFP fusion gene (data not shown). These results suggest that Os \textit{BOR1} localizes to the plasma membrane.

Table 1. Nomenclature of At \textit{BOR1}-Like Genes in Rice and \textit{Arabidopsis}

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<th>At \textit{BOR2}</th>
<th>At \textit{BOR3}</th>
<th>At \textit{BOR4}</th>
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<td>At1g74810</td>
<td>At5g25430</td>
<td>At4g35210</td>
</tr>
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</table>

GenBank accession numbers and locus identifiers are shown. Locus identifiers are the current version, except for Os \textit{BOR2}, which represents The \textit{Arabidopsis} Information Resource version 3.
Accumulation of Os BOR1 Transcripts in Rice and Their Detection in the Root Endodermis

We examined the accumulation of Os BOR1 transcripts in shoots and roots in response to the B status. Plants were grown in medium (Fukuda et al., 2004) containing 18 μM boric acid (referred to as normal medium in this manuscript) for 10 d and then exposed to -B medium for 3 d. The accumulation of Os BOR1 transcripts relative to the Ubi1 transcript levels in leaves and roots was determined by RT-PCR. The relative accumulation of Os BOR1 transcripts to Ubi1 transcripts in roots was 10 times higher than the relative value in leaves (Figure 3A). The accumulation of Os BOR1 transcripts in leaves and roots was not affected by 3 d of B deprivation (Figure 3A).

To analyze the cell-type specificity of Os BOR1 mRNA accumulation, we performed in situ hybridization using the Os BOR1 ORF region as a probe. In the roots of rice plants grown for 4 d in Murashige and Skoog medium, which contains 100 μM boric acid, Os BOR1 mRNA was detected in the endodermis and possibly the pericycle, but little Os BOR1 mRNA accumulated in root tips (Figure 3B).

Os BOR1 Accumulation Increases under -B Conditions

The accumulation of Os BOR1 was examined using immunoblot analysis with an antiserum raised against a partial Os BOR1 peptide. Microsomal fractions were prepared from the roots of wild-type plants grown in normal or -B medium. Two bands with predicted molecular masses of 82 and 76 kD were detected in samples prepared from -B plants. No corresponding bands were detected in the wild type under normal B conditions (Figure 3C). The predicted molecular mass of Os BOR1 is 79,336 D, similar to the positions of the bands. These results suggest that the accumulation of Os BOR1 is enhanced under B deprivation, similar to At BOR1 (Takano et al., 2005). Two bands were detected at this position. It is possible that the upper band represents an ubiquitinated form of Os BOR1. Identical band patterns were observed for Os BOR1 expressed in yeast (data not shown), suggesting that Os BOR1 is likely to be ubiquitinated in yeast.

Molecular Characterization of Tos17 Insertion Mutants in Os BOR1

Two independent insertion lines of the retrotransposon Tos17 in the Os BOR1 gene were obtained from the Rice Genome

Figure 1. Phylogenetic Analysis of At BOR1 and At BOR1-Like Genes in Arabidopsis and Rice.

A phylogenetic analysis was performed with MEGA 3.1 (http://www.megasoftware.net) using the neighbor-joining method (Saitou and Nei, 1987). Aligned sequences corresponding to residues 65 to 412 of Os BOR1 were used to generate the phylogenetic tree. The accession numbers and gene identifiers of each gene are shown in Table 1.

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Molecular Characterization of Tos17 Insertion Mutants in Os BOR1

Two independent insertion lines of the retrotransposon Tos17 in the Os BOR1 gene were obtained from the Rice Genome

Figure 2. B Export Activity and Subcellular Localization of Os BOR1.

(A) B concentration in yeast cells expressing At BOR1 and Os BOR1. The B concentrations in scbor1 J mutant cells carrying pYES2 (black bar) or pYES2 with the At BOR1 cDNA (white bar) or the Os BOR1 cDNA (gray bar) are shown. Cells were exposed for 60 min to medium containing 100 μM boric acid (n = 3). DW, dry weight.

(B) Subcellular localization of Os BOR1-sGFP in onion epidermal cells. Free and Os BOR1 indicate cells bombarded with the Pro3SS:sGFP (pTH2; Chiu et al., 1996) or Pro3SS:Os BOR1-sGFP constructs, respectively. Left and right panels represent fluorescence and light microscopy, respectively. Bars = 100 μm.
Resource Center in Tsukuba, Japan (Miyao et al., 2003). DNA fragments of the sequences at both sides of the boundaries between Os BOR1 and the Tos17 insertions were amplified and the nucleotide sequences determined. In both lines, the Tos17 insertion had inserted in exon 12, but at different positions (Figure 4A). The Tos17 insertions are located 1941 and 2116 bp from the first ATG of the Os BOR1 cDNA, and these lines were named osbor1-1 and osbor1-2, respectively (Figure 4A).

The accumulation of Os BOR1 transcripts in these mutant lines was determined by RT-PCR using the primers 913 and 914 (Table 2), which anneal upstream and downstream of the Tos17 insertion sites in both osbor1-1 and osbor1-2 (Figure 4A). In both lines, a band of the expected size (270 bp) was detected in the wild type and the heterozygous mutants, but not in homozygous plants (Figure 4B). PCR using the primers 927 and 929, which anneal two positions upstream of the Tos17 insertions (Figure 4A), produced bands with the expected size (1.74 kb) from the wild type and osbor1-1 and osbor1-2 heterozygotes, but not from osbor1-1 homozygotes (Figure 4B). In osbor1-2 homozygotes, a weak band of the expected size was detected (Figure 4B). These results suggest that no detectable Os BOR1 transcript accumulates in osbor1-1, whereas in osbor1-2, a nearly full-length Os BOR1 transcript, likely to contain a portion of the Tos17 sequence, accumulates.

Disruption of Os BOR1 Causes Increased Sensitivity to B Deficiency

The growth of wild-type plants was slightly reduced under -B conditions (Figure 5A). Growth was obviously reduced in young leaves, similar to other plant species. The growth of osbor1-1 and osbor1-2 was indistinguishable from that of the wild type under conditions of normal B supply (Figure 5A), but under -B conditions, the growth of the mutants was reduced in both the aerial portions and the roots. The growth reduction in osbor1-1 mutant plants under -B conditions was more severe than that of osbor1-2 (Figure 5A). The reduced growth under -B conditions in two independent Tos17 insertion lines suggests that the defect was caused by the disruption of the Os BOR1 gene. Osbor1-1 plants were smaller than wild-type plants at maturity, and most of the seeds of osbor1-1 plants did not mature.

To confirm that the growth defects were caused by the insertion in Os BOR1, we conducted three experiments. First, we grew 10 progeny of osbor1-2 heterozygous plants under both normal and -B conditions. Under normal conditions, all of the plants were indistinguishable from wild-type plants. Under -B conditions, 3 of the 10 plants grew less well than the others (data not shown). The three poorly growing plants were found to be

![Figure 3. Accumulation of Os BOR1 Transcripts and Os BOR1 in Roots in Response to B Conditions, and Localization of Os BOR1 Transcripts.](image)

(A) Quantitative RT-PCR determination of Os BOR1 mRNA accumulation in response to B conditions. Seventeen-day-old wild-type plants were grown in normal (white bars) or -B (gray bars) medium for 3 d. Averages ± sd of Os BOR1 mRNA accumulation relative to the Ubi1 transcript levels are shown for leaves and roots (n = 3).

(B) In situ hybridization analysis of Os BOR1 transcripts in rice roots. The top and bottom panels show longitudinal sections of the root elongation zone and root tips, respectively. The sections were hybridized with antisense or sense Os BOR1 probes. The magnifications of the four panels are identical. Bar = 10 μm.

(C) The roots of 17-d-old wild-type plants grown in normal or -B medium for 3 d were harvested. Five micrograms of microsomal proteins from roots were subjected to SDS-PAGE separation followed by immunoblot analysis using an anti-Os BOR1 antibody (top panel). The gel was stained with Coomassie blue (bottom panel). The numbers at right represent molecular masses (kilodaltons).
resistant (Hygr) plants of the T1 generation of the transgenic lines were obtained. Hygromycin-moter was introduced into homozygous Tos17 were observed in plants carrying the other seven plants were homozygous. A full-length Os BOR1 cDNA under the control of the 35S promoter was introduced into homozygous osbor1-1, osbor1-1 heterozygote for the Tos17 insertion; osbor1-1/osbor1-1, osbor1-1 homozygote for the Tos17 insertion; Os BOR1/osbor1-2, osbor1-2 heterozygote for the Tos17 insertion; osbor1-2/osbor1-2, osbor1-2 homozygote for the Tos17 insertion.

homzygous for the Tos17 insertion in Os BOR1, but none of the other seven plants were homozygous. Second, we crossed a heterozygous osbor1-1 plant (genotype Os BOR1/osbor1-1) and a homozygous osbor1-2 plant (osbor1-2/osbor1-2) and observed the growth of the F1 progeny in -B medium. Seven of the 14 F1 progeny showed reduced growth under -B conditions. A typical F1 plant grown under these conditions was reduced to 10 and 70% of that of the wild type, respectively. The results suggest that Os BOR1 is required for the maintenance of growth under -B conditions.

B Transport Properties of osbor1 Mutant Plants

Next, we measured the B concentrations in wild-type and osbor1-1 and osbor1-2 plants that had been grown in normal or -B medium for 2 weeks (Figure 5D). The B concentration in the shoots of osbor1-1 and osbor1-2 plants grown under the normal conditions was reduced to ~50 and 70% that of wild-type

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Table 2. Primer Sequences
Figure 5. Physiological Analysis of osbor1 Mutant Plants and Complementation of osbor1 with the Pro35S:Os BOR1 cDNA Construct.

(A) One-week-old wild-type and mutant plants were grown for 4 weeks in normal or -B medium before the representative photos shown were taken. 1-1, osbor1-1; 1-2, osbor1-2; 1-1 × 1-2 F1, F1 progeny of a cross of osbor1-1 and osbor1-2. Bars = 10 cm.

(B) Growth of osbor1-2 containing (+) or lacking (−) the Pro35S:Os BOR1 transgene after 4 weeks of -B treatment. Bar = 10 cm.

(C) Shoot growth of the wild type (W) and osbor1-1 (1) and osbor1-2 (2) grown for 2 weeks in normal or -B medium. Averages and SE (n = 3 to 5) are shown. An asterisk above a bar indicates a significant difference (P < 0.05) between the wild type and the mutant by the Student’s t test.

(D) B concentration on a fresh weight basis in shoots and roots of wild type (W) and osbor1-1 (1) and osbor1-2 (2) grown for 2 weeks (the same materials as [C]). Averages and SE (n = 3 to 5) are shown. An asterisk above a bar indicates a significant difference (P < 0.05) between the wild type and the mutant by the Student’s t test.

(E) B concentrations in the cell sap of roots of wild type (W) and osbor1-2 (2) grown for 3 d in normal medium or medium containing 0.03 μM B. Seven-day-old plants germinated with deionized water were grown for 10 d in normal medium before the treatment. Averages and SE (n = 3) are shown. The difference between the wild type and the mutant was not significant.

(F) Concentration of B in the xylem sap of wild-type (W) and osbor1-2 (2) plants grown for 3 d in normal medium or medium containing 0.03 μM B. The plants were precultured as in (E). Averages and SE (n = 3) are shown. An asterisk above a bar indicates a significant difference (P < 0.05) between the wild type and the mutant by the Student’s t test.
plants, respectively (Figure 5D). In the roots of these plants, there was a trend that the concentration was less in the mutants than the wild-type plants. By contrast, in the shoots and roots of plants grown under the -B conditions, no significant reduction of B concentration between the two mutants and the wild type was observed. From these results, we concluded that Os BOR1 is likely to play a role in the uptake and xylem loading of B from roots to shoots.

To further characterize the transport properties in these mutants, we determined the B concentrations in root cell and xylem saps. osbor1-2 mutant plants were grown hydroponically for 18 d in normal B medium and then grown in normal B or -B (0.03 μM in this particular experiment) medium for 3 d before the collection of xylem and root cell saps. The B concentrations in root cell sap were similar in the wild type and the mutant after both normal and -B treatments (Figure 5E). In wild-type and osbor1-2 root cell saps, the B concentrations were in the range of 6 to 7 μM and ~1 μM after 3 d of exposure to normal or 0.03 μM B medium, respectively. The B concentrations in root cell saps under normal conditions (6 to 7 μM) were less than half the B concentrations in standard nutrient solution (18 μM), suggesting that rice plants do not concentrate B upon its uptake from the medium. Under -B conditions, the B concentrations in root cell saps in both the wild type and the osbor1-2 mutant (~1 μM) were ~30-fold higher than those from plants grown in low B medium (0.03 μM), presumably representing the residual B from the preculture in normal medium.

By contrast, the B concentration in xylem sap of wild-type plants grown in normal medium containing 18 μM boric acid was ~45 μM, suggesting that rice is able to concentrate B in the process of xylem loading. The B concentration in xylem sap collected from osbor1-2 plants grown in normal medium was 60% of that in the wild type (Figure 5F), suggesting that Os BOR1 is involved in the xylem loading of B. Moreover, in plants grown in -B medium for 3 d, the B concentration in the mutant was <20% that in wild-type plants (Figure 5F), suggesting a greater role for Os BOR1 under -B conditions than under normal conditions.

**Tissue Specificity of Os BOR1 Promoter Activity in Roots Affected by B Status**

Twenty-nine independent transgenic rice lines carrying the ProOsBOR1:β-glucuronidase (GUS) construct were generated, and three lines (lines 1, 8, and 18) with relatively high GUS expression were selected for detailed analysis of GUS activities. After growth in normal medium, the plants were grown in -B medium for 3 or 6 d. Some of the -B-treated plants were then transferred back to normal medium for 3 d. We observed the cell-type specificities of the GUS activities in the roots ~2 cm from the root tips, corresponding to the elongation zone. In this zone, root differentiation is completed and cell types can easily be recognized. We also used root tips containing meristematic tissue for GUS staining. The differential GUS patterns in response to B conditions were not evident in the root tips (data not shown).

To ensure that these differences were not due to artifacts from the process of GUS staining, Pro35S:GUS transgenic rice plants were similarly stained. In these plants, GUS staining was observed throughout cross sections of roots (Figure 6E) and did not change with changing B conditions (data not shown). These results have established that the cell-type specificity of GUS activity in transgenic rice plants carrying the ProOsBOR1:GUS construct changes in response to the B content of the medium.

**Os BOR1 Promoter Activity Does Not Strongly Respond to B Conditions**

We also performed a fluorometric GUS assay using the same three transgenic lines (1, 8, and 18). The GUS activities in roots of the treated transgenic plants were not significantly altered, except for transgenic line 8 at 6 d of -B treatment (Figure 6G), in which it was upregulated by 20%. It is likely that the overall Os BOR1 promoter activity is regulated little, if at all, in response to the B conditions in the medium.

**DISCUSSION**

**Os BOR1 Is an Ortholog of At BOR1**

Four At BOR1-like genes exist in the rice genome and seven At BOR1-like genes, including At BOR1, are present in Arabidopsis (Figure 1). Among the four genes, Os BOR1 shows the strongest similarity to At BOR1 (Figure 1). A phylogenetic analysis suggested that three of the four genes are distinct from Arabidopsis genes. These differences in the number of genes and their phylogenetic relationships are likely to be a reflection of different B transport systems, requirements, and regulation in rice and Arabidopsis. The B requirement of graminaceous plants is generally lower than that of dicotyledonous plants, and the demand for B transport activity may also be lower in graminaceous plants than in dicotyledonous plants.

We previously demonstrated a role for At BOR1 in xylem loading (Takano et al., 2002) and its regulation by B nutrition (Takano et al., 2005). As reported here, the functions and regulation of Os BOR1 are generally similar to those of At BOR1:
both are B efflux transporters (Figure 2A) that localize to the plasma membrane (Figure 2B), are expressed in pericycles (Figure 3B), are involved in the xylem loading of B (Figure 5F), and are required for normal growth especially under B-limited conditions (Figure 5A). The accumulation of both proteins is elevated under low B conditions (Takano et al., 2005; Figure 3C). Together with the sequence similarities, these functional similarities establish that OsBOR1 is an ortholog of AtBOR1.

**OsBOR1 Is Involved in the Entry of B into Roots**

OsBOR1 has several unique features that distinguish it from AtBOR1. One is its involvement in the uptake of B into roots. In plants grown for long periods under -B conditions, the B concentration in osbor1-1 roots was less than that in wild-type roots (data not shown). We have repeated similar experiments several times. Although in this particular experiment (short -B treatment) shown in Figure 5D, reduction in B concentration in roots of the mutants was not statistically significant, in several independently conducted experiments, and the reduction was evident and statistically significant after treatments with -B for a long period of time (data not shown). Based on the data obtained from similar experiments, we are confident that OsBOR1 is necessary for B uptake especially after exposure to -B for long periods. This is in clear contrast with AtBOR1, which is involved in xylem loading but not in the uptake of B into roots (Takano et al., 2002).

This unique feature of OsBOR1 that distinguishes it from AtBOR1 is likely to be due to the different cell-type specificities of expression in roots. In rice roots, Casparian strips, barriers that prevent ions and solutes from freely entering the stele, are found in both the exodermis and endodermis (Ma et al., 2006). The expression of OsBOR1 was observed in these tissues (Figures 6A to 6D). It is likely that the expression of OsBOR1 at these sites is important for the uptake of boric acid into the roots and its loading into the xylem. In the case of Si, the polar localization of Lsi1 is likely to be important for the uptake of Si from the soil into the roots (Ma et al., 2006). Similarly, OsBOR1 may be critical for the transport of boric acid into the root stele. Determining the polar localization of OsBOR1 may clarify these issues.

**Roles of OsBOR1 in the Long-Distance Transport of B**

The B concentration in the xylem sap of osbor1-2 mutant plants was lower than that in the wild type after 3 d of exposure to either normal or low B conditions (Figure 5F), suggesting that OsBOR1 is involved in the loading of B into the xylem, irrespective of the B concentration in medium. This is in clear contrast with AtBOR1, which is important in the efficient xylem loading of B, mostly under long-term -B conditions, but less important under conditions of normal B supply, presumably due the degradation of the different B regimes. +, 10 d of normal B treatment; +/-, 10 d of normal B treatment followed by 3 d of -B treatment; +/-/-, 10 d of normal B treatment followed by 6 d of -B treatment; +/-/-/-, 10 d of normal B treatment followed by 3 d of -B treatment and then 3 d of normal B treatment.
At BOR1 protein (Takano et al., 2005). This is also apparently in contrast with our findings that no accumulation of Os BOR1 was detected after 3 d of exposure to normal medium (Figure 3C). It is likely that Os BOR1 accumulates to a level below the detection limit of our immunoblot analysis and that level may be sufficient for enhancing the xylem loading of B. As shown in Figure 5D, B concentrations in the aerial portion of the mutants were reduced under the normal condition, suggesting that Os BOR1 functions under normal B condition. It is possible that under the control condition, Os BOR1 accumulates only to a low level, which is likely to be below the detection limit of our immunoblot analysis.

Although the concentration of B in shoots under -B was not significantly different from the wild type (Figure 5D), the shoot growth of the mutants under -B was severely reduced (Figure 5C). Thus, the total amount of B transported to shoots was much less in the mutant than the wild type, especially in the case of osbor1-1. It also indicated that Os BOR1 is important for xylem B transport both under -B and normal conditions.

The Accumulation of B in the Root Cell Walls of Rice Plants

Arabidopsis has no apparent B-concentrating mechanism that acts following the entry of B into roots. However, in wild-type rice plants, the total B concentration in roots grown under -B conditions for 2 weeks was 14 μM (Figure 5D), much higher than the B concentration in the medium. It is likely that rice plants have a high capacity to concentrate B upon its entry into roots. This process does not seem to be mediated by Os BOR1 because osbor1-1 and osbor1-2 also showed the same or more concentrated B contents than that in the medium (Figure 5D). B concentrations in roots (Figure 5D) were much higher than that in root cell sap (Figure 5E), suggesting that rice plants have mechanisms to deliver and concentrate B to the insoluble fraction of root cells, which is most likely to be the cell wall. This is a distinct feature of rice plants that contrasts with Arabidopsis in terms of B distribution within the roots.

It is worth mentioning that in roots of osbor1-1 grown under -B, B concentration was higher than the wild type (Figure 5D). It is possible that the reduced transport of B to shoots resulted in high accumulation of B.

Changes in the Cell-Type Specificity of Os BOR1 Expression in Roots Are Affected by B Conditions

We found that the cell-type specificity of Os BOR1 expression is affected temporally by the B content of the medium (Figures 6A to 6D). This represents a major difference between Os BOR1 and At BOR1. There are several reports of nutrient transporter genes that are induced by the nutrient conditions, including the rice genes AMT1;2 (Sonoda et al., 2003) and IRT1 (Ishimaru et al., 2006) and the Arabidopsis genes YSL2 (Schaaf et al., 2005) and the AMTs (for review, see Loque and von Wirén, 2004), but in these cases, no changes in cell-type specific patterns of expression were reported.

As discussed above, Os BOR1 is required for both the efficient uptake of B into the roots and the xylem loading of B. The effect of long-term -B treatment is most likely to be represented by the experiment shown in Figure 6C, in which plants were exposed to -B for 6 d. Os BOR1 expression was observed in both the exodermis and the stele, suggesting that the Os BOR1 in these cell types is responsible for B uptake and xylem loading, respectively.

When roots were exposed to -B conditions for 3 d, Os BOR1 was strongly expressed in the exodermis (Figure 6B), suggesting that under such conditions Os BOR1 is more likely to be involved in B uptake from the medium into root steles. This cell type-specific pattern of expression is transient because after 6 d of -B treatment, the Os BOR1 expression in the stele returned back to high levels (Figure 6C). After several days of -B treatment, a steady flow of B from the soil to the stele may be established, which requires both the efficient uptake and the xylem loading of B. Although we did not examine the physiological consequences of the changes in the tissue specificity of Os BOR1 expression, these transient changes in the cell type-specific pattern of Os BOR1 expression are likely to reflect a transient shift in the B transport requirement of root tissues.

Figure 7. Model of the Roles of Os BOR1 and At BOR1 in Roots under Normal and -B Conditions.

The diagram shows the cell layers in roots, with the epidermis at the left side and xylem vessels to the right. Gray boxes indicate the positions of the Casparian strips. In rice, Casparian strips are present in both the exodermis and the endodermis, whereas in Arabidopsis, the strips are found only in the endodermis. Under normal B conditions, Os BOR1 is not abundantly present, and considering the strong Os BOR1 promoter activity in the endodermis, it is likely to be present in endodermis at a high abundance. After 3 d of -B treatment, Os BOR1 accumulation is higher overall, particularly in the endodermis. The expression pattern in rice corresponds well with the rice root structure, given the presence of two Casparian strip layers and the cell death that occurs in the cortex under submerged conditions (represented in cells shown with dotted lines). In Arabidopsis, At BOR1 is responsible for xylem loading but not for uptake under -B conditions and is not present under normal conditions. CS, Casparian strip; EN, endodermis; CO, cortex; SC, sclerenchyma cells; EX, exodermis; EP, epidermis; PC, pericycle; XY, xylem.
Based on these findings, we propose a model for the roles of Os BOR1 in B transport in rice, in contrast with those of At BOR1 in Arabidopsis (Figure 7). At BOR1 is expressed mainly in steles under -B conditions and is essential for the efficient xylem loading of B; under normal conditions, however, its accumulation is greatly reduced and xylem loading is likely to be a passive process. In rice, Os BOR1 expression was detected in both the exodermis and endodermis under -B conditions, and the gene may function in both B uptake and xylem loading. Expression in the exodermis may be important for the element to cross the outer Casparian strips, as rice submerged in water develops air spaces in cortical cells, which is likely to limit the symplastic transport of B to the steles. B is probably transported through the apoplasts in the cortical cells, in the roots of paddy rice. Then, B must cross the inner Casparian strip at the endodermis. Os BOR1 in the endodermis is likely to be important for the entry of B into the stele for xylem loading to take place. Changes in the cell-type specificity of the promoter activity are likely to reflect the roles of Os BOR1 under these conditions.

In summary, we demonstrated that Os BOR1 is a plasma membrane-localized B transporter that plays a role in both the xylem loading of B and its uptake into roots. At BOR1 is required for the efficient xylem loading of B but has not been demonstrated to be involved in B uptake. The cell-type specificity of Os BOR1 expression changes with the B status of the medium, providing an example of a mineral-nutrient transporter whose tissue-specific expression pattern changes depending on the nutritional conditions.

METHODS

Plant Materials and Culture

Rice plants (Oryza sativa cv Nipponbare) were grown hydroponically in a temperature-controlled (30/25°C day/night) greenhouse under natural light. Seeds were germinated in ion-exchanged water and grown for a week before transfer to hydroponic culture (Fukuda et al., 2004). For B deficiency treatment, hydroponic solutions with no added boric acid, or containing 0.03 μM boric acid, were used. Fifteen to twenty plants were grown in 1.5-liter containers. The pH of the hydroponic solutions was adjusted to 5.4 to 5.6 every other day, and the nutrient solutions were renewed weekly.

Phylogenetic Analysis

A similarity search of the entire rice genome was performed with the At BOR1 amino acid and cDNA sequences using the BLASTP and TBLASTN programs at the National Institute of Agrobiological Sciences (http://riceblast.dna.affrc.go.jp/), the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/Genome/PlantBlast.shtml), and the Knowledge-Based Oryza Molecular Biological Encyclopedia (http://cdna01.dna.affrc.go.jp/cDNA/). Locus numbers were obtained from The Institute for Genomic Research. Phylogenetic analysis was performed with MEGA 3.1 (http://www.megasoftware.net) using the neighbor-joining method (Saitou and Nei, 1987). Aligned sequences (residues 65 to 412 of Os BOR1) were used to generate the phylogenetic tree. The accession number and/or gene identifier of each gene is shown in Table 1.

Plasmid Construction

The sequences of all primers used in this study are shown in Table 2.
for each RNA sample, and three RNA samples were independently prepared for each treatment. The average value of each RNA sample was calculated, and three average values were used to calculate the average and standard deviation for each treatment. The primers 913 and 914 were used as specific primers for Os BOR1, and the Ubil-F and R primers were used as a control. All primer sequences are shown in Table 2.

**In Situ Hybridization**

Wild-type rice plants were germinated in liquid Murashige and Skoog medium, and 4 d later, roots were excised and fixed as described by Itch et al. (2000). Microtome sections of 9 μm in thickness were placed on glass slides coated with Vectabond (Vector Laboratories). The Os BOR1 ORF was amplified by PCR with the primers 927 and 1019 (Table 2) and fused into pGEM-T Easy (Promega). After determination of the direction of the insert, digoxigenin-labeled antisense and sense probes were prepared using SP6 and T7 RNA polymerase (Roche), respectively. In situ hybridization and immunological detection of signals were performed using a TSA Plus DNP (AP) System (Perkin-Elmer). The detection was performed as described by Kouchi and Hata (1993).

**Immunoblot Analysis**

Preparation of microsomal proteins from bulk roots was performed as described by Takano et al. (2005). Samples containing 5 μg of protein, determined using the Bio-Rad Quick Start Bradford Dye Reagent, were mixed with an equal volume of 2% SDS-PAGE sample buffer (Takano et al., 2005) and incubated on ice for 10 to 20 min before loading. The rabbit anti-Os BOR1 antibody was raised against an Escherichia coli-expressed partial peptide of Os BOR1 (N355-E464) and used at 1000-fold dilution.

**Establishment and Analysis of Transposon-Tagged Os BOR1 Lines**

Two rice lines, NC0170 and NC0255, which carry insertions of the retrotransposon Tos17 in the Os BOR1 gene, were kindly provided by Hirohiko Hirochika and Akio Miyao (Rice Genome Resource Center; Establishment and Analysis of Transposon-Tagged Os BOR1 Lines). The positions of the Tos17 insertions in Os BOR1 were confirmed by PCR and sequencing. For PCR amplification of the boundary sequences between Tos17 and the rice genome, the primers SP6 and T7 RNA polymerase (Roche) were used for both mutants. The Os BOR1-specific primers 928 and 930 (Table 2) were used for Osbor1-specific primers 928 and 930 (Table 2) were used for osbor1-2. To detect the corresponding PCR product from Os BOR1, the primers 913 and 914 were used.

**Rice Transformation**

Rice transformation plasmids were introduced into Agrobacterium tumefaciens (EHA101 or GV3101). Agrobacterium-mediated transformation of rice plants was performed as described by Toki (1997), except that rice seeds were precultured for 5 d on medium containing 2 mg/L 2,4-D. Three independent transgenic plants that carried the osbor1-2 mutation were regenerated from the transformation with pYN22 (containing Pro35S Os BOR1 cDNA), and 29 independent transgenic plants were regenerated from the transformation with pYN35 (containing ProOsBOR1:GUS).

**Preparation of Plant Samples and Determination of B Concentrations**

Appropriate portions of plants were harvested and their fresh weights determined. For sample collection, a region of ~1 to 1.5 cm of the boundary between the aerial portions and roots was excluded. The B concentrations in the samples were determined as described by Takano et al. (2002).

To collect xylem fluid, plants were germinated and grown in normal medium for 18 d. When the plants were at the five-leaf stage, they were transferred to either normal medium or medium containing 0.03 μM B and grown for 3 d. Then, the shoots of the plants were removed with razor blades 1 to 2 cm above the junction between the shoot and the root. The xylem fluid (20 to 100 μL) that exuded through the cut ends of the stems and leaves was collected over several hours from the late afternoon into the evening.

To obtain root cell sap, roots of the same plants that were used for xylem fluid collection were harvested and stored at −80°C. After thawing at room temperature, fresh weights were determined and the samples were placed in 0.22-μm filter units (Ultrafree-MC, Millipore). The tissues were reeled off at −20°C and thawed at room temperature. Four to five freeze-thaw cycles were conducted, and the cell saps were collected by centrifugation at 1000g for 5 min at room temperature (Noguchi et al., 2000).

**Histochemical Analysis of GUS Activities**

Root sections of 1.5 to 2 cm from the root tips were washed with ion-exchanged water prior to harvest. Histochemical staining was performed according to Inoue et al. (2003), with the following modifications. The sections were incubated at 37°C for 60 min in GUS reaction buffer (Inoue et al., 2003). After staining, the sections were washed in 70% ethanol and held in 70% ethanol until observation. The sections were embedded in 4% agar and then cut into 130-μm sections using a DTK-100 microslicer (Dosaka EM). GUS staining was observed using an Axioscope microscope (Carl Zeiss). For a positive control for GUS staining, transgenic rice plants containing the pG121Hm vector were used (Hiei et al., 1994).

**Determination of GUS Activities**

Root tips (2 cm from the tip) of hydroponically grown plants were harvested and subjected to GUS assays (Jefferson, 1987). Protein concentrations were determined using Quick Start Bradford Dye Reagent, and the Ubi1-F and R primers were used as a control. All primer sequences are shown in Table 2.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AK070617, DQ421408, AK072421, and DQ421409.

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