Title:

Evolutionary Divergence of Plant Borate Exporters and Critical Amino Acid Residues for the Polar Localization and Boron-dependent Vacuolar Sorting of AtBOR1.

Running head:

Evolution of Borate Exporters in Vascular Plants

Corresponding author：

Dr. J. Takano

Graduate School of Agriculture, Hokkaido University, Kita-ku N9-W9, Sapporo 060-8589, Japan

TEL; +81-11-706-3888

FAX; +81-11-706-4932

jtakano@abs.agr.hokudai.ac.jp

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Authors: Shinji Wakuta[1], Katsuhiko Mineta[2], Taro Amano[1], Atsushi Toyoda[3], Toru Fujiwara[3], Satoshi Naito[1,4], Junpei Takano[1]

Author's Address:

[1] Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

[2] Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia

[3] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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

Abbreviations: AP, adaptor protein; B, boron; BFA, brefeldin A; CHX, cycloheximide, BOR, borate exporter; GFP, green fluorescent protein; RG-II, rhamnogalacturonan II; SG, synthetic-galactose; TGN/EE, trans-Golgi network/early endosome; MVB/LE, multivesicular body/late endosome, ORF; open reading frame

Footnotes: The nucleotide sequences reported in this paper have been submitted to DNA Data Bank of Japan under accession numbers LC003521 (SmBOR1), LC003522 (SmBOR3), and LC003523 (SmBOR4).

Abstract

Boron (B) is an essential micronutrient for plants but is toxic when accumulated in excess. The plant *BOR* family encodes plasma membrane-localized borate exporters (BORs) that control translocation and homeostasis of B under a wide range of conditions. In this study, we examined the evolutionary divergence of BORs among terrestrial plants and showed that the lycophyte *Selaginella moellendorffii* and angiosperms have evolved two types of BOR (clades I and II). Clade I includes AtBOR1 and homologs previously shown to be involved in efficient transport of B under conditions of limited B availability. AtBOR1 shows polar localization in the plasma membrane and high-B induced vacuolar sorting, important features for efficient B transport under low-B conditions, and rapid downregulation to avoid B toxicity. Clade II includes AtBOR4 and barley Bot1 involved in B exclusion for high-B tolerance. We showed that three genes in *S. moellendorffii*, *SmBOR1* in clade I and *SmBOR3* and *SmBOR4* in clade II, encode functional borate exporters using yeast complementation and B transport assays. Furthermore, amino acid sequence alignments identified an acidic di-leucine motif unique in clade I BORs. Mutational analysis of AtBOR1 revealed that the acidic di-leucine motif is required for the polarity and high-B-induced vacuolar sorting of AtBOR1. Our data clearly indicated that the common ancestor of vascular plants had already acquired two types of BOR for low- and high-B tolerance, and that the BOR family evolved to establish B tolerance in each lineage by adapting to their environments.

Key words: Boron, Evolution, Exporter, Membrane trafficking

Introduction

Boron (B) is an essential mineral for plants and is crucial for maintaining cell wall structure. When availability is limited, B predominantly accumulates in the cell wall and covalently cross-links two rhamnogalacturonan II (RG-II) polysaccharide regions of pectin (Ishii and Matsunaga 1996, Kobayashi et al. 1996, O'Neill et al. 1996). A number of studies demonstrated that the RG-II-B complex is essential for the cell wall structure in rapidly growing tissues. Under B-limited conditions, in which growth of pumpkin is inhibited, an increase in monomeric RG-II was accompanied by inappropriate swelling of the cell wall (Ishii et al. 2001). *Arabidopsis thaliana mur1* mutant defective in formation of GDP-L-fucose, one of the sugar residues in RG-II, showed a dwarf phenotype (O'Neill et al. 2001). Knockdown of the pectin glucuronyl transferase 1 gene, which is involved in the biosynthesis of RG-II sugar chains, showed defects in development of male and female tissues (Iwai et al. 2006). Characterization of *A. thaliana* CTP:3-deoxy-D-manno-2-octulosonate cytidylyltransferase, an enzyme that activates 3-deoxy-D-manno-2-octulosonic acid (KDO), a specific monosaccharide component of RG-II, showed that the *cks* mutation led to pollen infertility due to the inhibition of pollen tube elongation (Kobayashi et al. 2011). Other functions of B in the cytoskeleton and plasma membrane have been reported, but their physiological relevance remains unclear (Bassil et al. 2004, Wimmer et al. 2009, Voxeur and Fry 2014).

B is required for growth of pteridophytes, lycophytes, and angiosperms (Bowen and Gauch 1965, Brown et al. 2002). Terrestrial plants are considered to have markedly increased usage of RG-II-B complex after the origin of tracheophytes in the Early Silurian, ~400 million years ago, to develop their complex structure for upright growth. The structure of RG-II is conserved in pteridophytes, lycophytes, and angiosperms, and the levels of RG-II-B in lycophytes and pteridophytes are 50 – 70-fold higher and those in dicotyledons and

monocotyledons are 80 – 150-fold higher than in bryophytes (Matsunaga et al. 2004). Therefore, tracheophytes are thought to have acquired the ability for massive synthesis of RG-II and efficient translocation of B to fulfill the demand in rapidly growing tissues.

On the other hand, excess B is toxic to living organisms. It has been proposed that B inhibits the functions of *cis*-diol-containing compounds, such as ATP, NAD^+, and RNA, by binding to the *cis*-diol (Reid et al. 2004). In the yeast, *Saccharomyces cerevisiae*, and angiosperms, B export from cells has been shown as a primary mechanism involved in conferring high-B tolerance (Hayes and Reid 2004, Miwa et al. 2007, Sutton et al. 2007, Takano et al. 2007).

These observations raise questions regarding how B is transported across biological membranes. B is present mainly as boric acid in solution at physiological pH in the absence of interaction with biomolecules. Boric acid is a weak Lewis acid with a pKa of 9.24 [$B(OH)_3 + H_2O = B(OH)_4^- + H^+$]. As a small neutral molecule, boric acid can be transported relatively easily across biological membranes by passive diffusion (Dordas et al. 2000). In addition to the passive diffusion of boric acid, two groups of transport protein for boric acid/borate have been identified in *A. thaliana*—the boric acid channel, which belongs to the major intrinsic protein family, and the borate exporters (BORs) that show homology to the mammalian Slc4 family bicarbonate (HCO_3^-) transporters (Takano et al. 2008, Parker and Boron 2013). Among the mammalian Slc4 family, a close BOR homolog, NaBC1, was characterized as a Na^+-coupled $B(OH)_4^-$ transporter by electrophysiology experiments (Park et al. 2004). This suggests that plant BORs also transport borate rather than boric acid. In addition, *S. cerevisiae* has a BOR homolog, Bor1p, and its B export function was demonstrated using a *bor1* deletion mutant (Takano et al. 2002, 2007).

AtBOR1 is required for efficient translocation of B from the roots to the shoots under low-B conditions (Takano et al. 2002). AtBOR1 is localized to the plasma membrane

and shows polarity toward the stele side (Takano et al. 2010). The polarity of AtBOR1 is assumed to direct transport of B to the stele side, which enhances efficiency of radial transport of B under B limited conditions. However, when plants are supplied with higher concentrations of boric acid, AtBOR1 is internalized into the trans-Golgi network/early endosome (TGN/EE) and transferred into the vacuole via the multivesicular body/late endosomes (MVB/LE) (Takano et al. 2005, 2010, Viotti et al. 2010). The vacuolar sorting of AtBOR1 should rapidly inactivate radial transport of B to avoid over-translocation of B to shoots. AtBOR2, the closest paralog of AtBOR1, also shows the same polarity and B-dependent vacuolar sorting, but has a different physiological function from that of AtBOR1 (Miwa et al. 2013). An *AtBOR2* mutant showed reduced root cell elongation under conditions of low B supply. The total B concentrations in roots were not different from the wild-type, while the proportion of cross-linked RG-II was reduced, suggesting that AtBOR2 mediates transport of borate for cross-linking of RG-II under low-B conditions. In contrast, AtBOR4 is considered to be involved in high-B tolerance. The overexpression of AtBOR4 confers high-B tolerance in *A. thaliana* (Miwa et al. 2007). AtBOR4 is localized on the plasma membrane with weak polarity toward the soil side and is not degraded in response to high-B (Miwa et al. 2007, Łangowski et al. 2010). Therefore, AtBOR4 can direct exclusion of B from the roots under high-B conditions. These findings suggest that the polarity and B-dependent degradation are crucial mechanisms determining the physiological function of BORs for low- or high-B tolerance.

The amino acid residue required for the degradation of AtBOR1 was identified by analysis of a series of chimeric proteins generated between AtBOR1 and AtBOR4, and AtBOR1 variants with amino acid substitutions. Y398 and Y405 residues in AtBOR1 are required for the polarity and vacuolar sorting, presumably as critical residues of tyrosine-based motifs involved in selective sorting into clathrin-coated vesicles (Takano et al.

2010). In addition, K590 was found to be the site of ubiquitination required for vacuolar sorting in response to high-B conditions (Kasai et al. 2011).

Recent studies have established the physiological function of BORs in rice and barley. OsBOR1 is required for uptake and xylem loading of B under low-B conditions (Nakagawa et al. 2007). Bot1 contributes to the high-B tolerance of Sahara, a barley landrace, by exclusion of B from the roots (Hayes and Reid 2004, Sutton et al. 2007). In addition, B transport activities of BORs identified from grape, citrus, and wheat were characterized in heterologous expression systems (Pérez-Castro et al. 2012, Cañon et al. 2013, Leaungthitikanchana et al. 2013).

This study examined the evolution process of BORs in plant species, including angiosperms, the bryophyte *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii*. Bryophytes, non-vascular plants, are the first plant groups to have colonized the land. The most primitive extant vascular plants, lycophytes, arose subsequently. Thus, comparative analysis of bryophytes and lycophytes provides a key to understand how vascular plants have evolved nutrient transport in association with the development of the vasculature. Inventories of ammonium and urea transporters (De Michele et al. 2011), sucrose and monosaccharide transporters (Lalonde and Frommer 2012), amino acid transporters (Wipf et al. 2012), and potassium ion transporters (Gomez-Porras et al. 2012) in *S. moellendorffii* implied the existence of similar nutrient transport systems in lycophytes and euphyllophytes. We demonstrated the boric acid/borate transport activity of BORs in *S. moellendorffii* and identified a conserved sorting motif in a subgroup of BORs consisting of AtBOR1 and two *S. moellendorffii* BORs. We further examined the involvement of this motif in the polarity and B-dependent vacuolar sorting of AtBOR1. The present study addresses the molecular basis for the differential functions of plant BORs; efficient B translocation for RG-II-B formation and B exclusion for high-B tolerance.

Results

Collection of BOR sequences

The *A. thaliana* genome harbors six AtBOR1 (At2g47160) paralogs, At3g62270, At3g06450, At1g15460, At1g74810, At5g25430, and At4g32510, which were designated as AtBOR2, AtBOR3, AtBOR4, AtBOR5, AtBOR6, and AtBOR7, respectively (Nakagawa et al. 2007). B transport activity of AtBOR1, AtBOR2, and AtBOR4 was demonstrated in yeast and *A. thaliana* (Takano et al. 2002, Miwa et al. 2007, Miwa et al. 2013).

To analyze the evolutionary divergence of BORs in land plants, amino acid sequences were screened from the bryophyte, *P. patens*, lycophyte, *S. moellendorffii*, and angiosperms, *Oryza sativa* and *Glycine max*. BOR candidate sequences were collected using AtBOR1, AtBOR2, AtBOR3, AtBOR4, AtBOR5, AtBOR6, and AtBOR7 as queries by PSI-BLAST (Position-specific iterated BLAST) search (Altschul et al., 1997). The *P. patens*, *S. moellendorffii*, *A. thaliana*, *O. sativa*, and *G. max* genomes were found to harbor 35, 19, 13, 11, and 12 corresponding sequences, respectively. Duplicated sequences derived from the same locus in *A. thaliana*, *O. sativa*, and *G. max* genomes were excluded. As AtBOR1, AtBOR2, AtBOR3, AtBOR4, AtBOR5, AtBOR6, and AtBOR7 contain at least eight putative transmembrane regions according to TMHMM Server v. 2.0 (Krogh et al., 2001), amino acid sequences containing less than seven transmembrane regions were also excluded. The *P. patens* genome then showed two BOR-like sequences (PpBOR1 and PpBOR2), the *S. moellendorffii* genome had four BOR-like sequences (SmBOR1, SmBOR2, SmBOR3, and SmBOR4), the rice genome had three BOR-like sequences that were identical to OsBOR1, OsBOR3, and OsBOR4 reported previously (Nakagawa et al. 2007), and the *G. max* genome had 11 BOR-like sequences. The proteins obtained from PSI-BLAST are listed in Supplemental Table 1.

cDNA cloning of BORs from *Physcomitrella patens* and *Selaginella moellendorffii*

The plant BORs experimentally demonstrated to act as borate exporters have been limited to those of angiosperms. To identify functional B exporter genes, RT-PCR was performed and three open reading frames (ORFs) in cDNAs were isolated from *S. moellendorffii*. The ORFs of SmBOR1, SmBOR3, and SmBOR4 were 2100, 1737, and 1743 bp in length and encoded proteins of 699, 578, and 680 amino acids, respectively (Supplemental Fig. 1). Although SmBOR2 cDNA could not be amplified from the samples used in this study, the SmBOR2 ORF was predicted to be 1923 bp in length and encode a 640-amino-acid protein.

B transport activities of BORs from *Selaginella moellendorffii* in yeast

To test complementation of the growth of an *S. cerevisiae* mutant lacking Bor1p under high-B conditions, SmBOR1, SmBOR3, and SmBOR4 were expressed under the control of the *GAL1* promoter using multi-copy 2 μm plasmids. As the *S. cerevisiae bor1* deletion mutant lacks B export activity, the growth of the mutant is more sensitive to high-B conditions than the wild-type (Takano et al. 2007). Yeast cell cultures in the stationary phase were used for spotting assay on synthetic galactose (SG) medium supplemented with 0, 15, 20, or 30 mM boric acid. Colonies expressing SmBOR1 grew better than those carrying the empty vector on SG medium supplemented with 15 and 20 mM boric acid (Fig. 1A). Colonies expressing SmBOR3 and SmBOR4 grew better than those carrying the empty vector on SG medium supplemented with 15, 20, and 30 mM boric acid (Fig. 1A). There were no differences when yeast cells were grown on SG medium without addition of boric acid.

We then directly measured B transport activities of SmBOR1, SmBOR3, and SmBOR4. It was reported previously that the concentrations of B were decreased in yeast cells expressing AtBOR1, AtBOR2, AtBOR4, OsBOR1, CmBOR1, and VvBOR1 (Takano et al. 2002, Miwa et al. 2007, Nakagawa et al. 2007, Pérez-Castro et al. 2012, Cañon et al. 2013,

Miwa et al. 2013). Yeast cells expressing SmBOR1, SmBOR3, and SmBOR4 were incubated in the presence of 0.5 mM boric acid for 1 h, and the soluble B concentrations in yeast cells were determined by inductively coupled plasma mass spectrometry. The B concentrations in yeast cells expressing SmBOR1, SmBOR3, and SmBOR4 were 17%, 87%, and 89% lower than that in cells carrying the empty vector, respectively (Fig. 1B). The decreases in B concentration were significant for SmBOR1 ($P < 0.05$), SmBOR3 ($P < 0.01$), and SmBOR4 ($P < 0.01$) compared with controls, as determined by Student's *t*-test. Therefore, we concluded that SmBOR1, SmBOR3, and SmBOR4 are functional borate exporters.

Construction of phylogenetic tree of plant BORs

For multiple alignment and phylogenetic analysis of plant BORs, 32 amino acid sequences were selected from the bryophyte, *P. patens*, lycophyte, *S. moellendorffii*, and angiosperms, *A. thaliana*, *Oryza sativa*, and *Glycine max* (Supplementary Table 1). In addition to these sequences, plant BORs experimentally shown to function as borate exporters and OsBOR2 (Nakagawa et al. 2007) were used for construction of a phylogenetic tree. The phylogenetic tree identified three clades (Fig. 2A). Clade I contained AtBOR1, AtBOR2, and OsBOR1, which are functional under conditions of B limitation (Takano et al. 2002, Nakagawa et al. 2007, Miwa et al. 2013), while clade II contained AtBOR4 and barley Bot1, which are responsible for high-B tolerance (Miwa et al. 2007, Sutton et al. 2007). Clade II also contained OsBOR4, which is specifically expressed in pollen and is required for normal pollen germination and/or tube elongation (Tanaka et al. 2013). Clade III was composed of PpBOR1 and PpBOR2. SmBOR1 and SmBOR2 were classified into clade I, while SmBOR3 and SmBOR4 belonged to clade II, although the BOR sequences from *S. moellendorffii* were far from those of angiosperms. It is also notable that the average number of amino acid substitutions per site in clade I was significantly lower than that in clade II (unpaired *t*-test

with Welch's correction, $P < 0.05$) (Fig. 2B). Unfortunately, the order of these three clades was unclear because of low bootstrap support. We used several rooted methods and several outgroup sequences, but were unable to obtain sufficient statistical supports.

Conservation of amino acid residues required for polarity and B-dependent vacuolar sorting

Previously, we demonstrated that the tyrosine-based motifs in the large loop region are required for the polarity and B-dependent vacuolar sorting of AtBOR1 (Takano et al. 2010). The tyrosine-based motif YxxΦ, where Y is tyrosine, x is any amino acid, and Φ is any bulky hydrophobic residue, is recognized by the μ subunit of adaptor protein (AP) complexes and is required for selective sorting into clathrin-coated vesicles (Bonifacino and Traub 2003). AtBOR1 variants with single substitutions of tyrosine to alanine in Y398DNM401 and Y405HHM408 showed weak polarity and were localized on the plasma membrane even under high-B conditions in root tip cells (Takano et al. 2010). Furthermore, an AtBOR1 variant with double substitutions of tyrosine to alanine in these motifs showed non-polar localization and was not degraded in response to high B supply (Takano et al. 2010), suggesting that the two tyrosine-based motifs are important for binding to AP complexes. Recently, AtBOR2 was also shown to have polarity toward the stele side in the plasma membrane under low-B conditions and was degraded in response to high B supply (Miwa et al. 2013). Consistent with these observations, the amino acid residues corresponding to the tyrosine-based signals were conserved in AtBOR1 and AtBOR2. The tyrosine-based motifs were conserved among transporters in clade I, although the bulky hydrophobic residue was not methionine but leucine in SmBOR1 and SmBOR2 (Fig. 3). In clade II, the tyrosine-based signals corresponding to Y398xxM401 in AtBOR1 were highly conserved, while most proteins had FxxM at the position corresponding to Y405HHM408 in AtBOR1 (Fig. 3). It was reported

that the FQQI motif, instead of the tyrosine-based motif, of the glucose transporter GLUT4 binds to the μ subunits of AP1 and AP2 complexes in mammal adipocytes (Al-Hasani et al. 2002, Schmidt et al. 2006). Taken together, the observations indicated that most BORs in clade II had the tyrosine- or phenylalanine-based AP binding motifs. In clade III, PpBOR1 and PpBOR2 had QxxL and YxxT, which do not fit the rule of tyrosine-based signals, at the corresponding positions (Fig. 3). Therefore, the tyrosine-based signals are common in the BORs of tracheophytes but not of the putative BORs in the moss *P. patens*.

Ubiquitination at the K590 residue is essential for degradation and vacuolar sorting of AtBOR1 in response to high B supply (Kasai et al. 2011). The amino acid residue corresponding to K590 was conserved in clade I, while various amino acid residues, such as lysine/aspartic acid/glutamic acid/asparagine/serine, were located in clade II (Fig. 3). In clade III, PpBOR1 has a lysine residue at position 590, while PpBOR2 has an asparagine residue.

Conservative acidic di-leucine motif in clade I is essential for the polarity and B-dependent vacuolar sorting of AtBOR1

The acidic di-leucine motif [D/E]xxxL[L/I], where D is aspartic acid, E is glutamic acid, x is any amino acid, L is leucine, and I is isoleucine, is characterized as a signal recognized by AP2 complex in mammals (Schmidt et al. 2006). We noticed the presence of an acidic di-leucine motif in the same loop region as the tyrosine-based motifs in AtBOR1 (Fig. 4A). The acidic di-leucine motif containing L455/L456 in AtBOR1 was highly conserved in clade I, but not in clade II (Fig. 3). The acidic di-leucine motif is expected to be another factor to distinguish between the functions of BOR in clades I and II. To examine the roles of L455/L456 in the polarity and B-dependent vacuolar sorting of AtBOR1, transgenic plants expressing AtBOR1(L455A/L456A)-GFP under the control of the AtBOR1 promoter were generated. In contrast to the polar localization of AtBOR1-GFP,

AtBOR1(L455A/L456A)-GFP showed apparently non-polar localization in the plasma membranes of various cells in the root tip (Fig. 4, B and C). The polarity was carefully examined by comparison with the dye FM4-64, which stains the plasma membrane, and quantified in transverse (apical and basal) plasma membrane domains of epidermal cells in the meristem zone (Fig. 4B – D). The polarity index for AtBOR1-GFP was calculated to be about 2.0, while those for BOR1(L455A/L456A)-GFP and BOR1(Y373A/Y398A/Y405A)-GFP were 1.2 (Figure 4D). The polarity indexes for BOR1(L455A/L456A)-GFP and BOR1(Y373A/Y398A/Y405A)-GFP were significantly lower than that of the wild-type ($P < 0.01$, Student's *t*-test). We then examined whether the acidic di-leucine motif is involved in endocytosis or later endocytic pathways using brefeldin A (BFA), which is a specific inhibitor of a subclass of ARF-GEF and inhibits the trafficking of membrane proteins from the TGN/EE to the plasma membrane and to the MVB/LE but not endocytosis from the plasma membrane (Robinson et al. 2008). In the presence of cycloheximide (CHX), which inhibits new synthesis of proteins, AtBOR1-GFP, AtBOR1(Y373A/Y398A/Y405A)-GFP, and AtBOR1(L455A/L456A)-GFP accumulated in the BFA-induced endosomal aggregations within 60 min (Fig. 4, E – G). This result suggested that at least the rate of constitutive endocytosis from the plasma membrane is unaffected in these AtBOR1 variants. We also analyzed the response of AtBOR1(L455A/L456A)-GFP to high B concentrations. Application of 100 μM boric acid diminished the fluorescence of AtBOR1-GFP but had little effect on that of AtBOR1(L455A/L456A)-GFP in the root tip within 3 h (Fig. 5, A and B). Time course analysis of epidermal cells showed that AtBOR1(L455A/L456A)-GFP was stably localized in the plasma membrane for 60 min, while AtBOR1-GFP was transferred to endosomes and subsequently degraded (Fig. 5, C and D). Western blotting confirmed the stable accumulation of AtBOR1(L455A/L456A)-GFP after high B supply (Fig. 5E). After 60 min, the accumulation of AtBOR1-GFP was decreased

and a signal corresponding to putative ubiquitinated BOR1-GFP (Kasai et al. 2011) appeared. The accumulation of AtBOR1-GFP was further decreased after 180 min. However, the accumulation of AtBOR1(L455A/L456A)-GFP was stable for 180 min. These results indicated that the acidic di-leucine motif of AtBOR1 is required for maintenance of polarity and rapid degradation, and the presence/absence of this motif may determine the physiological functions of BORs in clades I and II.

Discussion

B is an essential micronutrient and the existence and function of RG-II-B has been established in vascular plants. However, plant transporters for B have been characterized only in angiosperms. In this study, we performed phylogenetic analysis and showed that BORs in vascular plants could be classified into two groups, presumably corresponding to different physiological functions (Fig. 1A). We demonstrated that *S. moellendorffii* has BORs belonging to both clades I and II and they export B in yeast cells (Fig. 2), suggesting that *S. moellendorffii* has systems of B translocation similar to those found in angiosperms. This is consistent with the presence of significant amounts of RG-II-B in the cell walls of lycophytes (Matsunaga et al. 2004). By contrast, the presence of a B exporter in *P. patens* has not been established. A database search identified two BOR candidates from *P. patens* and comprised clade III in the phylogenetic tree (Fig. 1A). We performed RT-PCR using mRNA from moss protonema cultured on BCDATG agar and detected *PpBOR1* transcripts. However, in our yeast expression system, neither PpBOR1 expression nor PpBOR1 B transport function were detectable (data not shown). Currently, it is unclear whether B is essential in bryophytes (Hoffman 1966). The cell walls of bryophytes contained similar amounts of B to those of lycophytes and pteridophytes and small amounts of RG-II-like B complex (Matsunaga et al. 2004). Further analysis is needed to reveal potential functions of B exporters for B utilization and/or B exclusion in bryophytes.

As mentioned above, clades I and II may reflect their physiological differences. As both clades I and II contain the BOR of *S. moellendorffi*, it is reasonable to suggest that the physiological differences may have arisen before the divergence of *S. moellendorffi*. In addition, the average number of amino acid substitutions per site in clade I is significantly lower than that in clade II ($P < 0.05$) (Fig. 1B). This clearly indicates that the sequences in clade I are more conserved than those in clade II, suggesting that BORs of clade I may be of

the ancestral type. Moreover, this difference indicates that BORs in clades I and II evolved under different functional and/or environmental constraints.

As a key difference between sequences in clade I and II BORs, we identified the acidic di-leucine motif, which is conserved in clade I but not in clade II (Fig. 3). Importantly, AtBOR1(L455A/L456A)-GFP was not degraded in response to high-B conditions (Fig. 5, B, D, and E), indicating that the acidic di-leucine motif is required for B-dependent vacuolar sorting of AtBOR1. Furthermore, AtBOR1(L455A/L456A)-GFP showed non-polar localization (Fig. 4, C and D). It should be noted that AtBOR1(L455A/L456A)-GFP accumulated in the BFA-induced endosomal aggregations mainly composed of TGN/EE similar to the case of wild-type AtBOR1-GFP (Fig. 4, Takano et al. 2010), suggesting that endocytosis functions properly for AtBOR1(L455A/L456A) under low-B conditions. The acidic di-leucine motif may be required for polar trafficking from TGN/EE to the plasma membrane mediated by the AP complex.

The importance of the acidic di-leucine motif for the polarity and B-dependent vacuolar sorting of AtBOR1 suggest that these features are conserved in BORs in clade I as adaptations to low-B conditions. The cellular function of clade I BORs could be directional export of B for translocation under low-B conditions, which is dependent on polar localization. The BORs in clade I could also share the characteristic of B-dependent vacuolar sorting dependent on the acidic di-leucine motif, the tyrosine-based signals, and the ubiquitin acceptor lysine residue. This characteristic should be important for adjusting the level of B translocation as excessive transport of B toward the shoot causes B toxicity.

By contrast, the physiological functions of AtBOR4 and barley Bot1 in clade II were shown to be export of B out of the tissues to avoid B toxicity (Miwa et al. 2007, Sutton et al. 2007). This is likely mediated by stable localization under high-B conditions, which is dependent on the lack of the acidic di-leucine motif. Among clade II BORs, AtBOR4 shows

slight polar localization toward the soil side in root epidermal cells (Miwa et al. 2007, Łangowski et al. 2010), suggesting that B flux is directed toward the soil. However, the determinants of the polarity of AtBOR4 have not been identified, and it is therefore unclear whether BORs in clade II share polarity. Importantly, clade II contains OsBOR4, which is expressed in pollen and was suggested to be important for normal pollen germination and/or elongation (Tanaka et al. 2013). *A. thaliana* BOR6 and BOR7 are also specifically expressed in pollen (Becker et al. 2003, Bock et al. 2006). As reproductive growth of crop plants generally requires higher concentrations of B (Dell and Huang 1997), pollen-specific BORs may be stably accumulated over wider concentrations of B and functions in cross-linking RG-II to support rapid elongation.

In conclusion, the results of this study indicated that *S. moellendorffii* is equipped with BORs belonging to two clades possibly specified by the presence/absence of the acidic di-leucine motif. Lycophytes are one of the most primitive extant tracheophytes, which are thought to have branched from euphyllophytes ~400 million years ago. The characteristics of BORs are thought to have evolved along with the development of the vasculature to fulfill increasing demand for B in the whole plant body, and at the same time, to avoid excessive accumulation of B in tissues.

Materials and Methods

Collection of amino acid sequences and selection of BOR candidates

A search of plant BOR proteins was performed using PSI-BLAST at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/BLAST/) against the non-redundant protein sequences with seven *A. thaliana* BOR proteins as queries: AtBOR1 (RefSeq ID: NP_850469), AtBOR2 (NP_191786), AtBOR3 (NP_187296), AtBOR4 (NP_172999), AtBOR5 (NP_177619), AtBOR6 (NP_197925), and AtBOR7 (NP_194977). Amino acid sequences derived from *P. patens*, *S. moellendorffii*, *O. sativa*, and *G. max* genomes were collected from the results. The number of transmembrane regions was predicted using TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Amino acid sequences containing eight or more transmembrane regions were regarded as candidates for BOR protein. The locus of each protein in *A. thaliana*, *O. sativa*, and *G. max* was identified. Duplicate sequences and sequences without locus data were excluded. Preliminary phylogenetic analysis was conducted for these primary candidates to exclude proteins outside of the outgroup, *S. cerevisiae* BOR1 (P53838). The remaining proteins were subjected to further analysis.

Multiple sequence alignment and phylogenetic analysis

The amino acid sequences of known BORs and predicted BOR candidates were aligned by CLUSTAL X (Larkin et al., 2007) and refined by Gblocks (Talavera and Castresana, 2007) using the default parameters. A total of 512 positions in the final dataset were used. For this alignment, Poisson-corrected amino acid distances (Zuckerkandl and Pauling, 1965) were used as an amino acid substitution model. The phylogenetic tree of the BORs was reconstructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987). Reliability of the topology was examined by the bootstrap method (Felsenstein, 1985), which generated the

bootstrap probability by 1000 pseudo-replications at each interior branch of the tree. Evolutionary analyses were conducted using MEGA6 (Tamura et al., 2013).

cDNA cloning and construction of yeast expression vectors

Total RNA was extracted from *S. moellendorffii* grown at 22°C under continuous light. Based on the amino acid sequences of SmBOR1, SmBOR2, SmBOR3, and SmBOR4 corresponding to XP_002962848.1, XP_002989430.1, XP_002968573.1, XP_002975908.1, XP_001759676.1, and XP_001766018.1, respectively, primers were designed as follows. For *SmBOR1*, 5'-atggaagagacattcgttcctttccg-3' and 5'-ttaagagccgggagaggagctgtc-3'; for *SmBOR2*, 5'-atggaagagacattcgttcctttccg-3' and 5'-tcacgaagtcaagcttactctcattgc-3'; for *SmBOR3*, 5'-atggcggcgtctcatccctttc-3' and 5'-aacctgaagctcaaaatctttgtcattg-3'; for *SmBOR4*, 5'-atggcggcgtttcatccctttc-3' and 5'-ttaagcttcatatgttgcaacatccaattc-3'. The DNA fragments were inserted into pGEM-T easy vector (Promega, Madison, WI), and sequenced.

Expression plasmids were constructed to produce SmBOR1, SmBOR3, and SmBOR4 under the control of the *GAL1* promoter. The ORFs were amplified with restriction linker sequences from the cDNA clones using the following primers. For *SmBOR1*, 5'-aaagagctcaccatggaagagacattcgttcc-3' and 5'-ttttctagattaagagccgggagaggag-3'; for *SmBOR3*, 5'-aaaggtaccatggcggcgtctcatcc-3' and 5'-tttgaattcctaagcttcatatgttgcaacatc-3'; for *SmBOR4*, 5'-aaaggtaccatggcggcgtttcatccc-3' and 5'-tttgaattcttaagcttcatatgttgcaacatcc-3' (restriction sites underlined). These PCR products were inserted into the corresponding restriction sites of pYES2 (Invitrogen, Carlsbad, CA), resulting in pSW68 (SmBOR1), pSW69 (SmBOR3), and pSW70 (SmBOR4), respectively.

B transport activity in yeast cells

The *S. cerevisiae* strains, Y01169, were transformed by the lithium acetate method with

pYES2, pSW68, pSW69, and pSW70. Transformants were selected on solid synthetic minimal medium (Sherman 1991) supplemented with 2% D-glucose. The synthetic minimal medium contained 8 μM boric acid, and was supplemented with 20 mg/L histidine, 30 mg/L leucine, and 20 mg/L methionine to grow transformed cells. Growth of transformants was examined in solid medium (Nozawa et al. 2006, Cañon et al. 2013). *S. cerevisiae bor1* mutants carrying each expression vector were cultured at 30°C for 48 h in synthetic minimal medium supplemented with 2% (w/v) D-raffinose instead of D-glucose. The optical density at 590 nm (OD_{590}) of the yeast cultures was diluted to 1.0 and a dilution series (1/10, 1/100, 1/1000, and 1/10000) was spotted on SG medium supplemented with 0 or 20 mM boric acid. The plates were incubated at 30°C for 6 days. B concentrations in yeast cells in liquid medium were determined as described (Takano et al. 2002). Briefly, transformants grown in SG medium at 30°C until the OD_{590} reached 1.0 – 1.5 were centrifuged at 3000 × *g* for 5 min. The cell pellets were resuspended in 20 mL of SG media containing 0.5 mM boric acid to OD_{590} 4.0. After 60 min of incubation, the cells were centrifuged at 3000 × *g* for 5 min and washed twice with ice-cold water. The yeast cells were boiled for 30 min and centrifuged at 3000 × *g* for 20 min, and the concentrations of B in the supernatants were measured by inductively coupled plasma mass spectrophotometry (ELAN DRC-e; Perkin Elmer, Waltham, MA). Experiments were performed with three independent transformants.

Construction of expression vectors and transgenic *A. thaliana*

DNA fragments encoding AtBOR1(L455A/L456A)-GFP were generated by fusion PCR using the AtBOR1-GFP (Takano et al. 2005) construct as a template and the following primers: 5'-caccatggaagagacttttgtgccgtttg-3', 5'-ccgttgttctttgacttcaactggtgctgcatcatctatttctttctctatgtcgaaca-3', 5'-tgttcgacatagagaaagaaatagatgatgcagcaccagttgaagtcaaagaacaacgg-3', and

5'-ttacttgtacagctcgtccatgcc-3' (introduced mutations are underlined). The PCR fragments were subcloned into the pENTR-D-TOPO vector (Life Technologies, Carlsbad, CA) and then subcloned into pAT100 containing the AtBOR1 promoter (Takano et al. 2010) by Gateway LR reaction (Life Technologies), resulting in pAT97. The plasmid was used for transformation of the *bor1-1* mutant using the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998).

Plant materials and growth conditions

A. thaliana plants were grown on vertically placed solid MGRL medium containing 1% (w/v) sucrose, 1.5% (w/v) gellan gum, and 1 μM boric acid in growth chambers at 22°C under fluorescent lamps with a 16-h light/8-h dark cycle. The *bor1-1* mutants expressing AtBOR1-GFP and AtBOR1(Y373A/Y398A/Y405A)-GFP under the control of AtBOR1 promoter were described previously (Takano et al. 2010). Transgenic plants in the T3 generation were used for analysis.

Imaging analysis

Laser scanning confocal microscopy was performed using a Leica TCS SP8 instrument (Leica Microsystems, Wetzlar, Germany) equipped with an HCPL APO CS2 ×40 water immersion lens with the following excitation and detection wavelengths: 488 and 500 – 530 nm for GFP and 488 and 600 – 700 nm for FM4-64 (Life Technologies). FM4-64 was prepared as a 10 mM stock solution in water. BFA (Sigma, St. Louis, MO) was prepared as 50 mM stock solution in dimethyl sulfoxide (DMSO). Plants were transferred from solid to liquid medium containing the dye or inhibitors and incubated at room temperature.

Preparation and immunoblotting analysis of microsomal proteins

The transgenic plants were grown on vertically placed solid medium containing 1 μM boric acid for 14 days and then transferred to solid medium containing 100 μM boric acid. All steps in the preparation of proteins were conducted at 4°C or on ice. Samples of approximately 300 mg of root tissues were homogenized in 1 mL of buffer (250 mM Tris, pH 8.5, 290 mM sucrose, 25 mM EDTA) supplemented with 50 mM dithiothreitol, 0.5 mg/mL Pefabloc SC (Roche, Indianapolis, IN), and protease inhibitors (CompleteMini; Roche) using a Multi Beads Shocker (Yasui Kikai, Osaka, Japan). The lysates were centrifuged at 10000 × *g* for 15 min at 4°C. The resultant supernatants were transferred to 1.5-mL tubes (Beckman Coulter, Brea, CA) and centrifuged at 100,000 × *g* for 30 min at 4°C. The pellets, representing the microsomal fraction, were resuspended in storage buffer containing 50 mM potassium phosphate buffer (pH 6.3), 1 mM magnesium sulfate, and 20% glycerol supplemented with 0.5 mg/mL Pefabloc SC and protease inhibitors. The protein concentration was measured by protein assay (Bio-Rad, Hercules, CA). NuPAGE LDS sample buffer (Invitrogen) and 50 mM dithiothreitol were added to the samples, followed by incubation at 90°C for 10 min. Microsomal proteins (5 μg) were separated on NuPAGE 4% – 12% Bis-Tris gels (Invitrogen) and transferred onto polyvinylidene difluoride membranes by electroblotting. The membranes were blocked by incubation in Blocking One (Nacalai Tesque, Kyoto, Japan). Mouse anti-GFP monoclonal antibody (Nacalai Tesque) was used at 1:10000 dilution in Can Get Signal Solution 1 (Toyobo, Osaka, Japan) and horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare, Little Chalfont, UK) was used at 1:20000 dilution in Can Get Signal Solution 2 (Toyobo). Detection was performed using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA). The membranes were stained with 0.25% Coomassie Brilliant Blue R-250 after detection.

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Disclosures

No conflicts of interest declared.

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Legends to figures

Fig. 1

Boron export activities of BORs in *S. moellendorffii.*

(A) Tolerance of yeast *bor1* deletion mutant cells expressing SmBOR1, SmBOR3, and SmBOR4 under high-B conditions. SG medium supplemented without boric acid and with 10 and 20 mM boric acid were used. Tenfold dilutions were dropped on the plate from left to right in each panel and the plates were incubated at 30°C for 6 days. (B) B concentrations in yeast *bor1* deletion mutant cells expressing SmBOR1, SmBOR3, and SmBOR4. The concentrations of soluble B in yeast cells (mmol B kg^{-1} dry weight) are shown. Data are means ± standard deviation for three independent transformants. Asterisks indicate significant differences between BORs and empty vector control by Student's *t* test (**P* < 0.01; ***P* < 0.05).

Fig. 2

Phylogenetic analysis of BOR family in land plants.

(A) Evolutionary relationships of BOR family. The evolutionary history was inferred using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 32 amino acid sequences. Inferred clades are indicated by the bars and numbers. (B) Comparison of evolutionary distances between clades I and II. The means ± standard deviation of the evolutionary distance within each group are shown. Asterisks indicate significant differences according to the unpaired *t* test with Welch's correction (*P* < 0.05).

Fig. 3

Multiple alignments of the amino acid sequences of the motifs required for the polarity and vacuolar sorting.

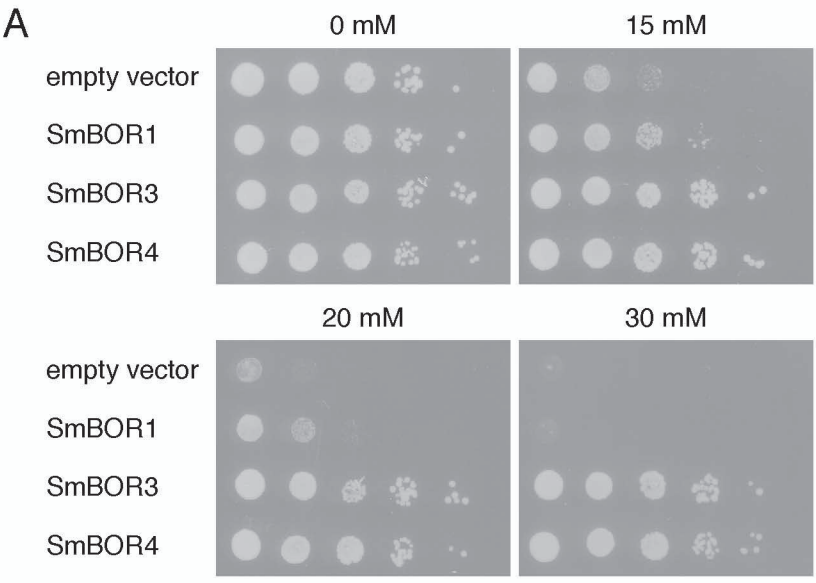
The tyrosine-based motif, the acidic di-leucine motif, and the lysine residue in AtBOR1 and corresponding sequences in homologs are shown. The essential residues in the motifs are highlighted in black.

Fig. 4

Effects of the L455A/L456A mutation on polar localization of AtBOR1. (A) Topological model of AtBOR1. (B – D) Involvement of L455/L456 in polar localization of AtBOR1. (B) AtBOR1-GFP and (C) AtBOR1(L455A/L456A)-GFP in root tips (*Left*) and epidermal cells in the meristem zone (*Right*) under low-B conditions (1 μM boric acid). The GFP and FM4-64 signals are shown in the *Top* and *Middle* rows, respectively. In the merged images, the GFP (*green*) and FM4-64 (*red*) overlapping signals appear in yellow. (D) Polarity index. Ratio of fluorescence intensity at the stele side and soil side halves of transverse (apical and basal) plasma membrane in the epidermis of AtBOR1-GFP ($n = 30$ cells from three roots), AtBOR1(Y373A/Y398A/Y405A)-GFP ($n = 30$ cells from three roots), and AtBOR1(L455A/L456A)-GFP ($n = 30$ cells from three roots). Fluorescence intensity at the stele side was divided by that at the soil side. FM4-64 was used as an internal standard. Error bars represent standard deviation. Asterisks indicate significant differences between AtBOR1-GFP and AtBOR1 variants by Student's *t* test ($P < 0.01$). (E) AtBOR1-GFP, (F) AtBOR1(Y373A/Y398A/Y405A)-GFP, and (G) AtBOR1(L455A/L456A)-GFP grown on low-B medium (1 μM boric acid) treated with liquid medium containing 50 μM CHX (and inhibitor of new protein synthesis) for 30 min and then with 50 μM CHX and 50 μM BFA for 1 h (Scale bars, 50 μm).

Fig. 5

Effects of the L455A/L456A mutation on the B-dependent vacuolar sorting of AtBOR1. (A) AtBOR1-GFP in the root tips under low-B conditions (1 μM boric acid, –B) (*Left*) and high-B conditions (100 μM boric acid, +B) (*Right*). (B) AtBOR1(L455A/L456A)-GFP in the root tips under low-B conditions (1 μM boric acid, –B) (*Left*) and high-B conditions (100 μM boric acid, +B) (*Right*). Plants were grown on low-B medium (1 μM boric acid, –B) and then transferred to low-B (1 μM boric acid, –B) or high-B medium (100 μM boric acid, +B) for 3 h. Time course analysis of the B-dependent vacuolar sorting of (C) AtBOR1-GFP and (D) AtBOR1(L455A/L456A)-GFP. Plants were grown on low-B medium (1 μM boric acid) and then transferred to high-B medium (100 μM boric acid). Scale Bars, 50 μm. (E) Immunoblotting analysis of AtBOR1-GFP and AtBOR1(L455A/L456A)-GFP. The plants were grown under low-B conditions (1 μM boric acid) and then treated with high-B medium (100 μM boric acid). The asterisk represents the presumed mono-ubiquitinated AtBOR1-GFP. The sizes of the molecular markers are in kDa. Coomassie Brilliant Blue-stained membranes are shown as a loading control.



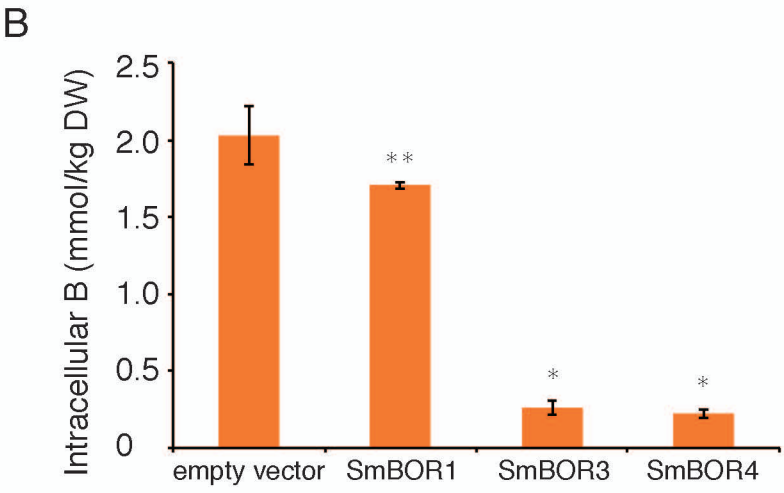


Fig 1 Wakuta et al.

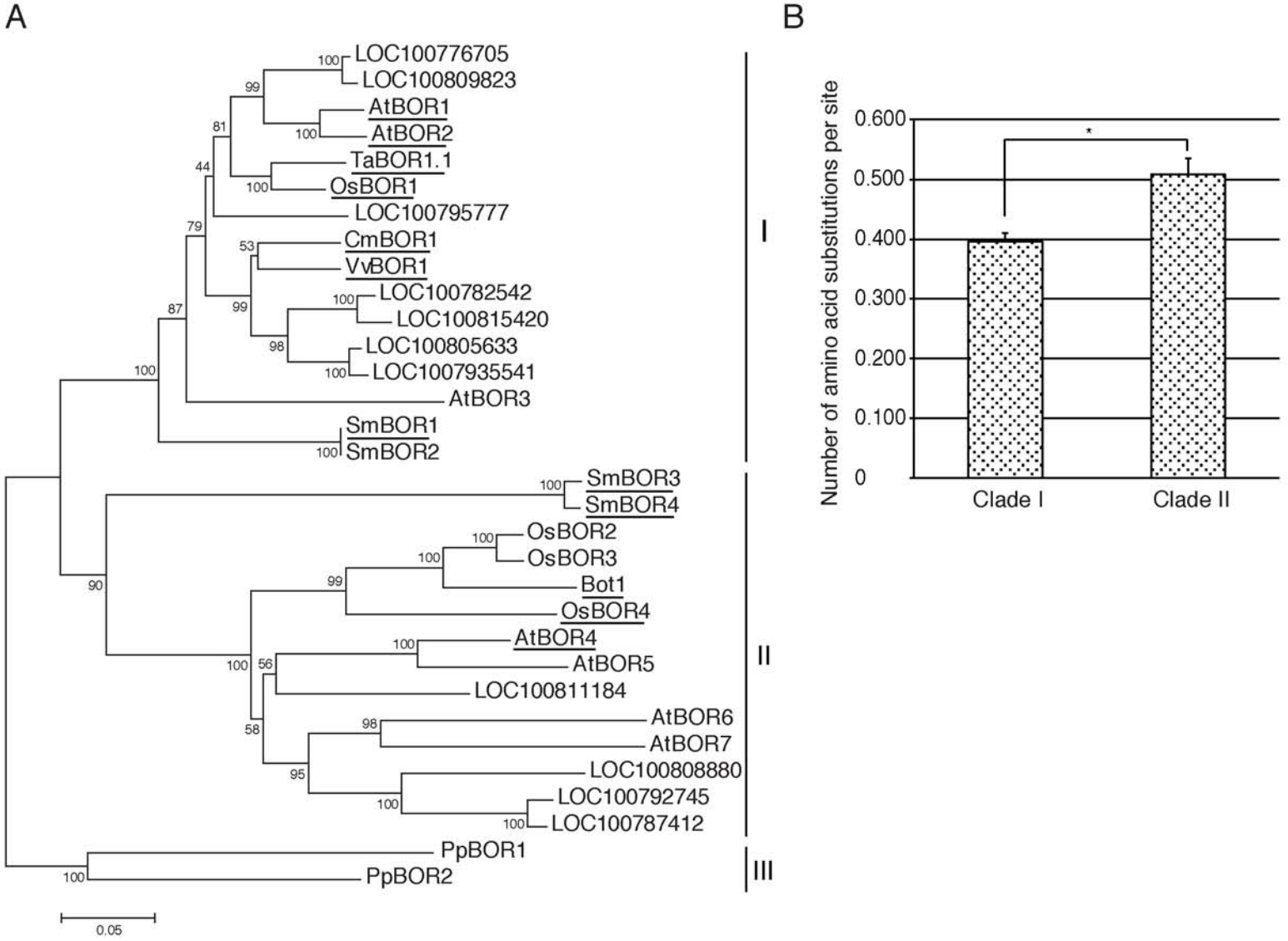


Fig. 2 Wakuta et al

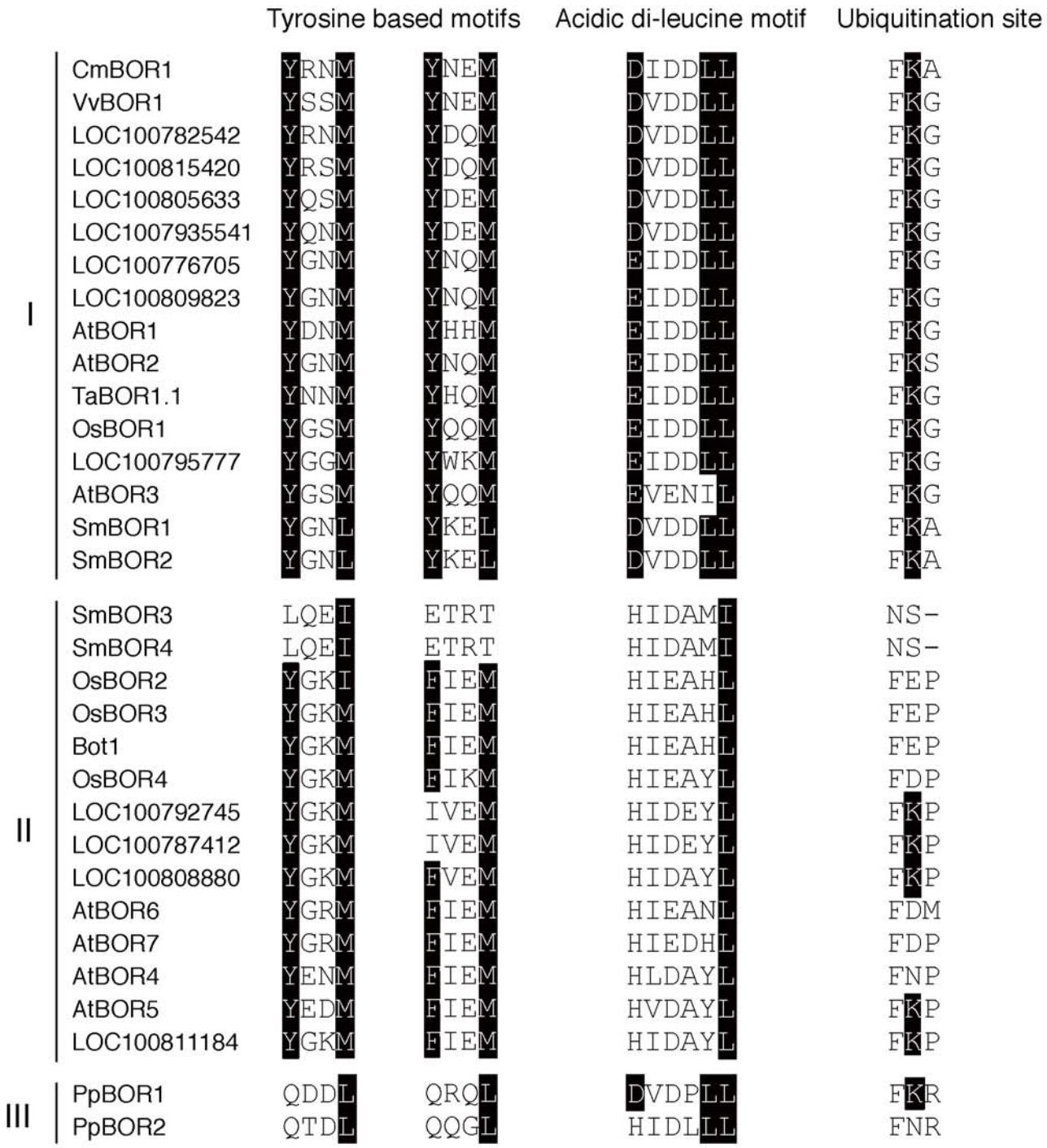


Fig. 3 Wakuta et al

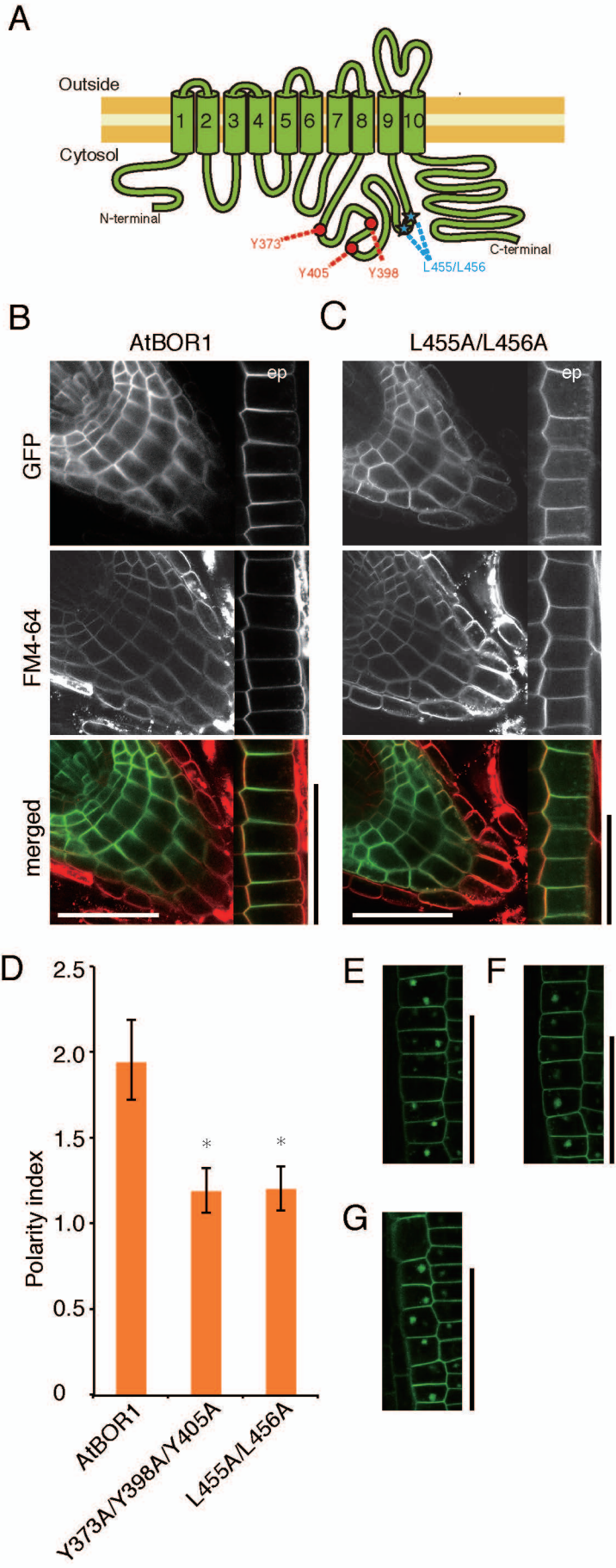


Fig. 4 Wakuta et al

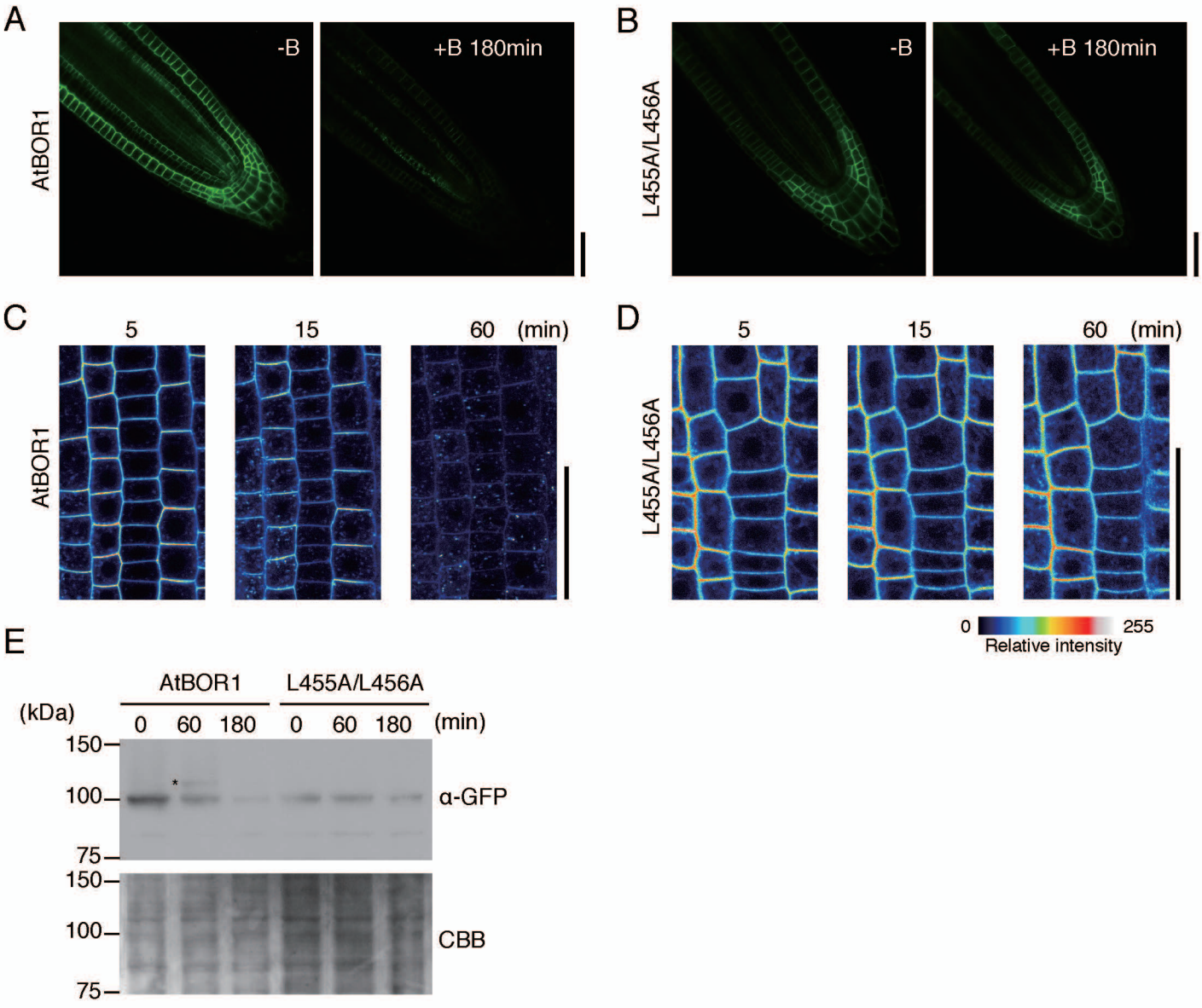
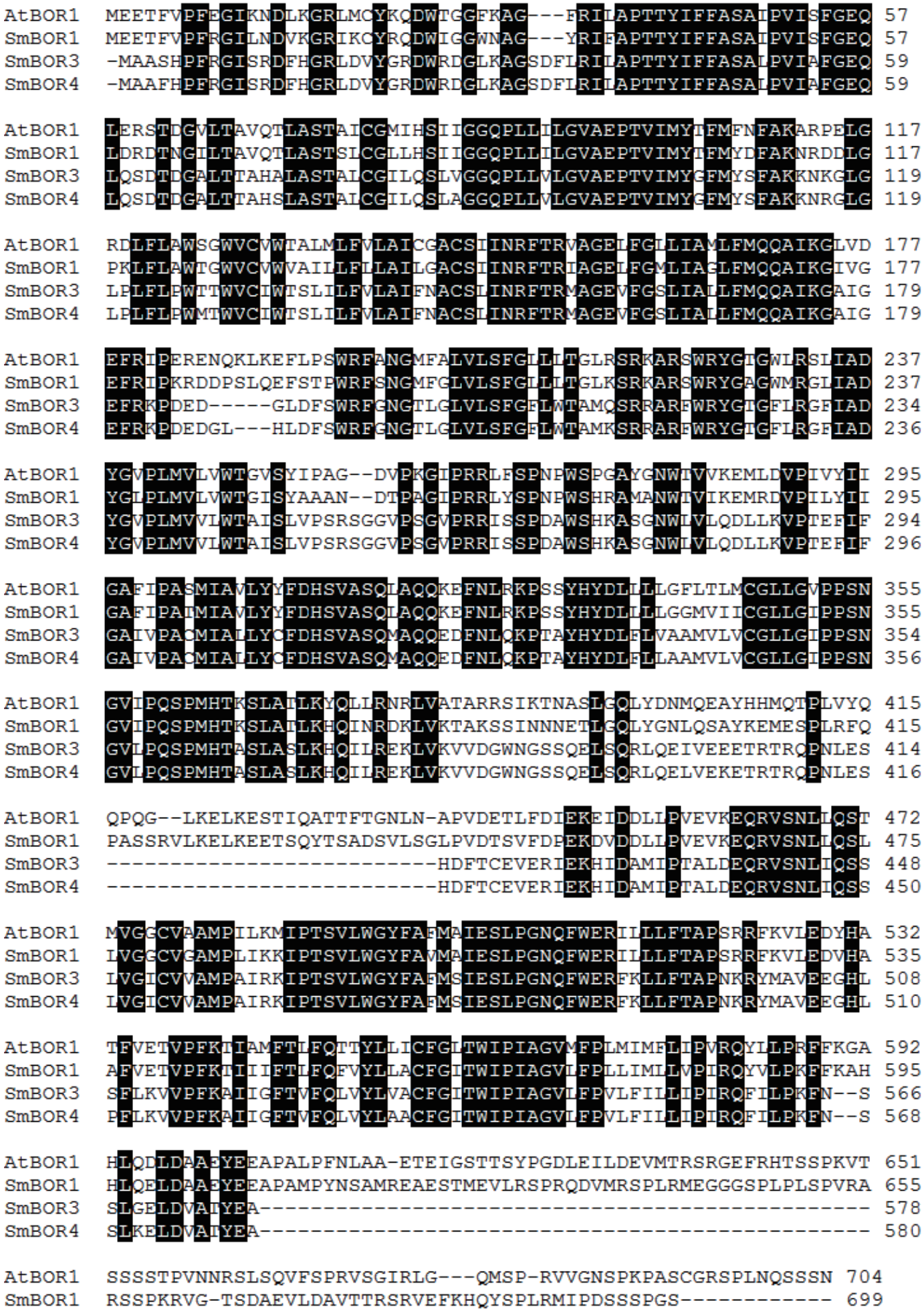


Fig. 5 Wakuta et al



Supplemental Fig 1

Multiple alignment of the AtBOR1, SmBOR1, SmBOR3, and SmBOR4 amino acid sequences. Conserved residues are highlighted in black.

Supplemental Table 1. The list of proteins collected using PSI-BLAST

Organism	Name	Protein length (aa)	TMD	NCBI protein number
Arabidopsis thaliana	AtBOR1	704	10	NP_850469.1
	-	729	9	NP_001078071.1
	AtBOR2	703	10	NP_191786.1
	AtBOR3	732	8	NP_187296.2
	AtBOR4	683	10	NP_172999.1
	AtBOR5	683	8	NP_177619.2
	AtBOR6	671	11	NP_197925.4
	AtBOR7	673	11	NP_194977.6
	-	344	3	T02172
	-	542	12	AAD26598.1
	-	668	8	AAG51913.1
	-	710	12	CAA22578.1
	-	736	8	AAF08571.1
Oryza sativa	OsBOR1	711	9	AAQ02664.1
	OsBOR3	672	10	ABD78950.1
	OsBOR4	677	10	ABD78951.1
	-	637	10	ABG22050.1
	-	665	10	BAD67809.1
	-	684	10	EEE53967.1
	-	659	10	EEE53968.1
	-	461	5	EEE62518.1
	-	672	10	NP_001042174.2
	-	745	8	NP_001054793.1
	-	711	9	NP_001067049.1
Glycine max	LOC100809823	723	9	XP_003521605.1
	LOC100809823.2	748	8	XP_003521606.1
	LOC100793554	720	9	XP_003523082.1
	LOC100787412	665	10	XP_003523697.1

	LOC100815420	680	10	XP_003525432.1
	LOC100805633	723	9	XP_003527015.1
	LOC100792745	662	8	XP_003527795.1
	LOC100795777	708	9	XP_003533117.1
	LOC100811184	669	9	XP_003546177.1
	LOC100808880	834	10	XP_003549256.1
	LOC100782542	652	10	XP_003550734.1
	LOC100776705	723	9	XP_003554567.1
Selaginella moellendorffii	SmBOR4	501	12	XP_002975908.1
	SmBOR3	578	12	XP_002968573.1
	SmBOR2	640	10	XP_002989430.1
	SmBOR1	685	10	XP_002962848.1
	-	567	0	XP_002989380.1
	-	322	0	XP_002985384.1
	-	318	0	XP_002984513.1
	-	313	0	XP_002983595.1
	-	312	0	XP_002983586.1
	-	318	0	XP_002983506.1
	-	322	0	XP_002979677.1
	-	317	0	XP_002973989.1
	-	312	0	XP_002973811.1
	-	312	0	XP_002973801.1
	-	293	0	XP_002973045.1
	-	322	0	XP_002967234.1
	-	1193	0	XP_002962426.1
	-	485	0	XP_002962425.1
	-	322	0	XP_002960490.1
Physcomitrella patens	-	530	0	XP_001751249.1
	-	195	0	XP_001753790.1
	-	851	0	XP_001755125.1
	-	340	0	XP_001759600.1
	PpBOR1	684	9	XP_001759676.1

	-	144	0	XP_001760050.1
	-	759	0	XP_001760535.1
	-	356	0	XP_001762296.1
	-	640	10	XP_001765760.1
	-	103	0	XP_001765788.1
	PpBOR2	621	9	XP_001766018.1
	-	580	0	XP_001767572.1
	-	483	0	XP_001767574.1
	-	719	0	XP_001771363.1
	-	929	0	XP_001773667.1
	-	206	2	XP_001773686.1
	-	461	0	XP_001775810.1
	-	322	0	XP_001776619.1
	-	540	0	XP_001777150.1
	-	601	0	XP_001778063.1
	-	103	0	XP_001778694.1
	-	766	0	XP_001780195.1
	-	793	0	XP_001780218.1
	-	357	0	XP_001781181.1
	-	540	0	XP_001783014.1
	-	891	0	XP_001784656.1
	-	588	0	XP_001785279.1
	-	131	0	XP_001785597.1
	-	649	0	XP_001785598.1
	-	628	0	XP_001785602.1
	-	645	0	XP_001785735.1
	-	419	0	XP_001785801.1
	-	449	0	XP_001786200.1
	-	183	0	XP_001786227.1
	-	367	1	XP_001787013.1