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Rapid paper

Identification of Boron Transporter Genes Likely to be Responsible for Tolerance to Boron Toxicity in Wheat and Barley

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Tolerance to boron (B) toxicity in cereals is known to be associated with reduced tissue accumulation of B. Genes from roots of B-tolerant cultivars of wheat and barley with high similarities to previously reported B efflux transporters from Arabidopsis and rice were cloned. Expression of these genes was strongly correlated with the ability of tolerant genotypes to lower the concentration of B in roots. The gene from barley located to chromosome 4. Backcross lines containing a B tolerance locus on chromosome 4 showed tolerance in proportion to the level of expression of the transporter gene, whereas those lacking the locus were sensitive to B and had very low levels of gene expression. The results are consistent with a widespread mechanism of tolerance to high B based on efflux of B from root cells.

Keywords: *BOR* genes — Boron tolerance — Boron toxicity — *Hordeum vulgare* — Membrane transporter — *Triticum aestivum*.

Abbreviations: EST, expressed sequence tag; ICP-OES, inductively coupled plasma–optical emission spectroscopy.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers EU220225 and EU223365.

Introduction

Boron (B) is an essential element for plant growth, and a number of roles for B in plant development have been proposed (Blevins and Lukaszeski 1998, Goldbach et al. 2007). The main demand for B is for cell wall development where it forms a central component of the structure of the rhamnogalacturonan II complex (Matoh 1997, Matoh 2000). At physiological pH, B is present as uncharged boric acid which, combined with its relatively high lipid solubility (Raven 1980), allows rapid penetration of biological membranes, as demonstrated by Stangoulis et al. (2001) and Hayes and Reid (2004). Under conditions of adequate B nutrition, active uptake of B would therefore seem unnecessary. However, under conditions of B deficiency, its predominantly apoplastic role would require redistribution of B from the symplasm to the apoplasm, either to support local cell wall development or for translocation from the site of uptake in the roots to expanding tissue in the shoot. This latter function was shown to be mediated in Arabidopsis by At*BOR*1, a B efflux transporter expressed in roots under deficiency conditions, which increases B supply to the shoots by loading of B from the xylem parenchyma into the xylem (Takano et al. 2002). It is now known that there are six other genes related to At*BOR1* in Arabidopsis (Miwa et al. 2005) and four in rice (Nakagawa-Yokoi et al. 2005), with different patterns of expression in tissues and in response to B nutrition.

Most research to date has focused on the role of *BOR* genes in maintaining function under conditions of B deficiency or adequacy, but a number of recent studies have pointed to a role for B efflux transporters in tolerance to B toxicity. In yeast, knock-out and overexpression studies have demonstrated that Bor1p is a B efflux transporter that confers tolerance to B toxicity (Nozawa et al. 2006, Takano et al. 2007). It has long been known that in wheat and barley, B tolerance is related to the reduced accumulation of B in shoot tissue (Nable 1988, Nable et al. 1990). In barley, Jefferies et al. (1999) used restriction fragment length polymorphism (RFLP) mapping to identify a locus on chromosome 4 that correlated with low B accumulation in shoots under high B supply and proposed that this was due to exclusion of B from the roots. More recently, Hayes and Reid (2004) showed that a barley cultivar that was tolerant to B toxicity was able to maintain root B concentrations much lower than in the external medium, whereas a sensitive cultivar was not, and that the reduced root concentration translated into lower concentrations in the xylem and much lower concentrations in the shoots. Since the apparent membrane permeability to B was similar in the tolerant and sensitive cultivars, it was concluded from that study that the reduced B concentration in the roots resulted not from an exclusion mechanism, but from active efflux pumping in the tolerant cultivar. This proposal was supported by experiments in which the

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Table 1 B tolerance of eight wheat cultivars

Cultivar	Relative DW (%)	
	Roots	Shoots
India	100 ± 8	100 ± 6
Greek	58 ± 29	111 ± 25
Gk*24/1	92 ± 25	95 ± 22
Gk*7/1	71 ± 25	79 ± 9
BT Schomburgk	88 ± 25	90 ± 30
Schomburgk	58 ± 8	77 ± 20
WIMMC*10	25 ± 8	40 ± 2
Kenya Farmer	25 ± 8	32 ± 4

Plants were grown hydroponically in nutrient solution supplemented with 5 mM H_3BO_3 for 28 d. Each value is the mean ± SE of three determinations.

ability of the tolerant cultivar to maintain low root B concentrations was lost following application of metabolic inhibitors. The transporter responsible for B efflux was not identified or characterized.

In the current work, genes from wheat and barley related to known B efflux transporters in rice and Arabidopsis have been cloned and sequenced, and their expression in B-tolerant and sensitive cultivars determined.

Results

Tolerance to high B in wheat

The cultivars listed in Table 1 were selected on the basis of known tolerance or sensitivity to B based on growth. Plants were grown in a solution containing a toxic concentration of B (5 mM) and the total root and shoot B concentrations determined. Root B concentrations varied significantly and were strongly correlated with both shoot B concentrations and shoot growth (Fig. 1). The ability of the tolerant cultivars to lower the root B concentration below that of the external medium pointed to the action of a B efflux transporter, for which strong evidence has been reported in barley by Hayes and Reid (2004). A number of B transporter genes have been identified in Arabidopsis and rice, and at least AtBOR1 (Takano et al. 2002) and OsBOR1 (Nakagawa et al. 2007) are known to efflux B across the plasma membrane of root cells. Primers designed from the sequences of four homologous genes in rice (accession Nos. *OsBOR1*, AK070617; Os*BOR2*, DQ421408; Os*BOR3*, AK072421; Os*BOR4*, DQ421409) and related expressed sequence tags (ESTs) were used to amplify cDNA prepared from roots of wheat (cv. India) grown under B toxic conditions. A sequence with 85% identity to Os*BOR2* at the amino acid level was obtained. Phylogenetic analysis (Fig. 2) indicated that the wheat gene was more closely related to Os*BOR2* and Os*BOR3* than to Os*BOR1* or

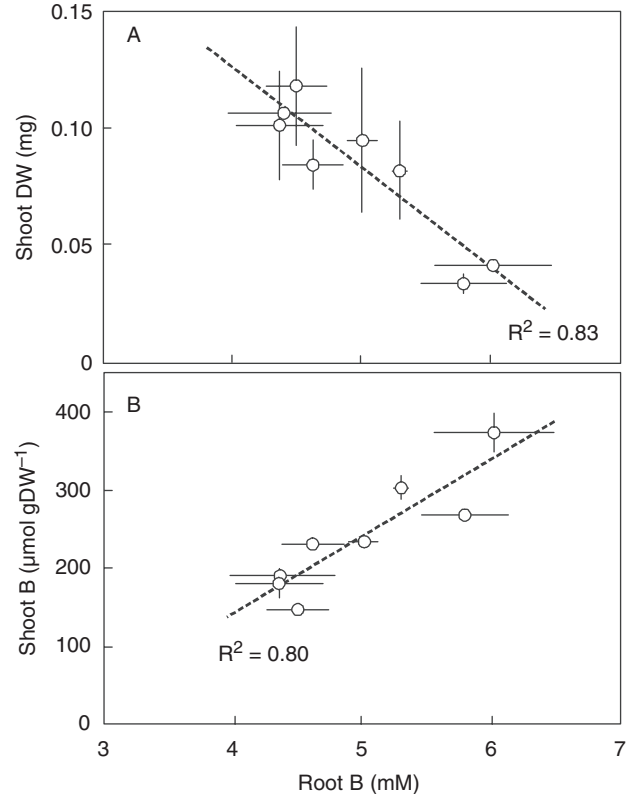

Fig. 1 Correlation between root B concentration and shoot dry weight (A) and shoot B concentration (B) in eight cultivars of wheat grown in solution containing 5 mM B. Each value is the mean ± SE of three determinations.

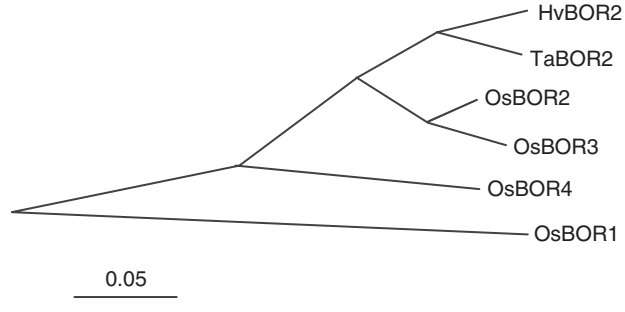

Fig. 2 Phylogenetic relationship between rice, barley and wheat BOR amino acid sequences using the neighbor-joining method.

Os*BOR4*, and will therefore be referred to as Ta*BOR2* (accession No. EU220225). Expression analysis using real-time PCR showed very high expression of this gene in the tolerant cultivar India and very low expression in the sensitive cultivar WIMMC*10 (Table 2), with the level of expression being related to the reduction in root B concentrations. The high expression of Ta*BOR2* in India was observed at both low and high B concentrations, while the low expression in WIMMC*10 increased only slightly under B toxic conditions (Table 2).

Table 2 Concentrations of B and corresponding levels of expression of *BOR2* genes in roots and shoots of B-tolerant and B-sensitive cultivars of wheat and barley grown in low or toxic B concentrations

Cultivar	B-tolerance	Root B (mM)	Shoot B (μmol g^{-1} DW)	Relative expression of *BOR2*	
				Low B	High B
Wheat					
India	Tolerant	4.17 ± 0.39	190 ± 9	1.000 ± 0.151	0.281 ± 0.082
WIMMC*10	Sensitive	5.98 ± 0.43	373 ± 25	0.002 ± 0.002	0.030 ± 0.002
Barley					
Sahara	Tolerant	2.36 ± 0.03	90 ± 14	1.000 ± 0.239	0.584 ± 0.077
Schooner	Sensitive	4.90 ± 0.30	291 ± 34	0.002 ± 0.000	0.012 ± 0.003

For total B concentrations in tissues, wheat was grown in 5 mM B for 28 d and barley for 16 d. Values are the mean ± SE ($n = 3$ for wheat; $n = 4$ for barley).
For expression levels, RNA was extracted from roots after 4 d exposure to 5 mM B (barley) or 21 d to 2 mM B (wheat). Low B treatments contained 12 μM B in the nutrient solutions. Expression levels were normalized against tubulin.

Tolerance to high B in barley

The highly B-tolerant barley cultivar Sahara has previously been shown to be capable of maintaining root concentrations less that half of that of a B-sensitive cultivar Schooner (Hayes and Reid 2004). Using an approach similar to that used for cloning of Ta*BOR2*, a barley homolog with 90% similarity to Ta*BOR2* and 84% similarity to Os*BOR2* at the amino acid level was obtained using cDNA extracted from Sahara. Because of the similarities in sequence to Os*BOR2* and Ta*BOR2* (Fig. 2), the barley gene will be referred to as Hv*BOR2* (accession No. EU223365). Expression analysis using real-time PCR showed that this gene was highly expressed in roots of Sahara, while in Schooner its expression was very low. Partial sequencing of Schooner showed that the nucleotide sequence of the region chosen for amplification by the real-time PCR primers was identical to that in Sahara. As with Ta*BOR2*, the strong expression of Hv*BOR2* was observed under both high and low B conditions (Table 2).

Molecular marker studies have identified a locus on barley chromosome 4H which is related to reduced B accumulation in shoots (Jefferies et al. 1999). Barley lines that had been developed by backcrossing Sahara barley with a B-sensitive cultivar Sloop were examined for their ability to reduce root and shoot B concentrations. Two cultivars were known to have the 4H locus and in two it was absent. Measurement of tissue B concentrations showed that those cultivars with the 4H locus had significantly lower root and shoot B concentrations, although not as low as the Sahara parent (Fig. 3). Those cultivars without the 4H locus had root and shoot B concentrations similar to those of the sensitive parent Sloop. Those cultivars with the 4H locus also showed high levels of expression of Hv*BOR2*, while there was negligible expression in the other cultivars (Fig.4). Within the three tolerant cultivars, the level of expression was inversely proportional to the concentration of B in roots and shoots (Figs. 3, 4).

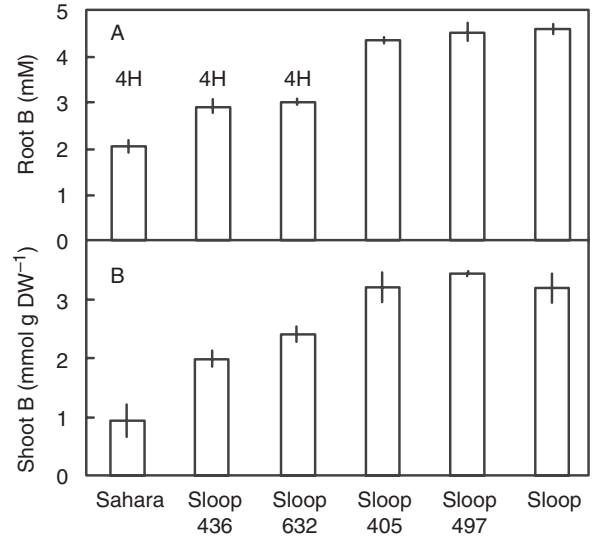

Fig. 3 Concentrations of B in roots (A) and shoots (B) of Sahara–Sloop backcross lines grown in a nutrient solution containing 5 mM B. The three cultivars to the left contain the 4H locus related to B tolerance, while the three cultivars to the right do not contain the locus. Each concentration is the mean ± SE of three determinations.

The sequence of Hv*BOR2* was used to generate a probe for Southern hydridization of genomic DNA from wheat–barley addition lines. These lines were generated by introduction of a single chromosome from barley (cv. Betzes) into wheat (cv. Chinese Spring); individual lines each contained one of the seven barley chromosomes (Islam et al. 1981, Islam and Shepherd 2000). Southern hydridization with the probe prepared from Sahara showed that the gene was located on chromosome 4 of barley (Fig. 5).

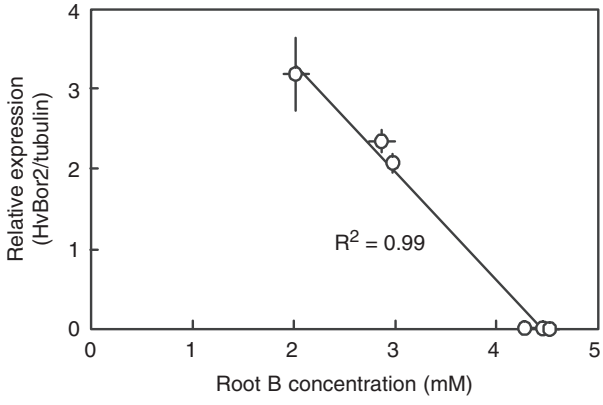

Fig. 4 Relative expression of Hv*BOR2* as a function of root B concentration in Sahara–Sloop backcross lines grown in a nutrient solution containing 5 mM B.

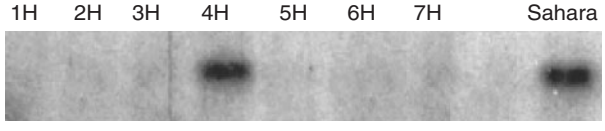

Fig. 5 Southern hybridization of Sahara barley and wheat–barley addition lines with a probe prepared from Hv*BOR2*. Genomic DNA was digested with *Bam*HI.

Discussion

The close correlations between root and shoot B concentrations, and root B concentration and shoot yield (Fig. 1) suggest that the main control over B toxicity is exerted at the root level by regulation of root B concentrations. Because of the high membrane permeability of B, and lack of charge at physiological pH, the tendency in sensitive cultivars is for the intracellular B concentration to equalize with the B concentration in the external medium. Tolerant cultivars of wheat and barley were shown to be able to lower root B concentrations, thereby restricting transfer of B to the shoot and reducing the toxicity in the roots. The close relationship between the degree of reduction in root B and the expression of Ta*BOR2* and Hv*BOR2* suggests that these genes encode B transporters that are expressed in roots and mediate the active efflux of B across the plasma membrane of root cells. The two putative transporters encoded by these genes differ from B transporters so far described, AtBOR1 and OsBOR1, which act to promote B transport to shoots at low B concentrations, but are degraded at adequate or high B concentrations (Takano et al. 2005, Nakagawa et al. 2007). The genes related to B toxicity tolerance were relatively unresponsive to tissue B status, but varied greatly in their expression between tolerant and sensitive genotypes.

It is not possible from the data presented to define the exact mechanism of these B transporters. Nevertheless there are certain attributes that the transporters must possess in order to function. First, the movement of B against a concentration gradient requires the input of metabolic energy; the efflux requires active transport of B. In yeast, active efflux by Bor1p appears to be driven by the proton electrochemical gradient across the plasma membrane (Jennings et al. 2007). Secondly the transporter must operate at high B concentrations, i.e. it must be a low affinity transporter; it is unlikely that the same transporter would be able to mediate B efflux under deficiency conditions. To be most effective, the transporter would need to be preferentially located on the plasma membrane of the epidermal or outer cortical cells. Otherwise, B effluxed across the plasma membrane would build up to high concentrations in the apoplast, resulting in higher B influx and effectively short circuiting the efflux.

The question remains as to whether these genes are really expressed for the purposes of tolerance to B toxicity, or are simply housekeeping genes that have become overexpressed. The main role of B is for synthesis of rhamnogalacturonan II in the cell wall, so outwardly directed movement of B would be expected in most cell types.

Mapping of chromosome regions conferring tolerance to B toxicity identified a region on chromosome 4 of barley (Jefferies et al. 1999) and chromosome 7B of wheat (Jefferies et al. 2000). The use of wheat–barley addition lines was able to confirm that Hv*BOR2* was located on chromosome 4. The location of Ta*BOR2* is unknown. Fine mapping of the Bo1 locus related to B tolerance on wheat chromosome 7B has so far failed to find any genes that could encode a membrane transport protein (Schnurbusch et al. 2007).

The identification and cloning of genes conferring tolerance to B toxicity in cereals is potentially a major advance in the development of varieties capable of withstanding B-toxic soil conditions. It will be interesting to discover whether efflux pumping of B in roots can explain tolerance to B toxicity that is observed in other plants (Stangoulis and Reid 2002).

Materials and Methods

Plant growth

Seeds of barley and wheat were germinated on moist filter paper for 3–4 d then grown hydroponically on a nutrient solution containing (mM) 3.75 NO_3-N, 1.5 K, 1.25 Ca, 0.5 Mg, 0.5 S and 0.25 P; and (in μM): 8.52 Na, 4.6 Cl, 4.5 Fe-EDTA, 2.3 Mn, 0.26 Mo, 0.19 Zn, 0.08 Cu and 11.7 B. In toxicity studies, this solution was supplemented with 5 mM B. Plants were grown in a laboratory at 22°C on a 16 h/8 h day/night cycle and illuminated at a flux density of 240 μmol m^{-2} s^{-1}.

Tissue B analyses

Due to the high permeability of B, roots were not rinsed prior to analysis but simply blotted. Total B was measured by inductively coupled plasma–optical emission spectroscopy (ICP-OES), or soluble B was measured in hot water extracts by a colorimetric assay based on the reaction between B and azomethine-H (Gaines and Mitchell 1979). The B concentration in roots is expressed in mM based on root water content (difference between fresh weight and dry weight) for purposes of comparison with the concentration of B in the external solution. For shoot tissue it was not meaningful to express B concentrations on a fresh weight basis due to the variable extent of necrosis, which depended on the B sensitivity of the genotype. Therefore, for shoots, B concentration is expressed on a dry weight basis.

Sequencing and expression analysis of BOR genes

RNA was extracted from root and shoot tissues using TRIzol (Invitrogen) following the protocol provided by the supplier. RNA was reverse transcribed using Superscript II (Invitrogen) or Omniscript (Qiagen) reverse transcriptases, and poly(dT) for barley and gene-specific primers for wheat. For sequencing of Hv*BOR2*, partial sequences were obtained using primer pairs from the 5′ and 3′ ends based on similarities with OsBor2 and related ESTs, and the full-length cDNA was then cloned using internal primers designed from the partial sequences. The initial primer sets for the 5′ end were 5′GCTTCCTTCCTCCTCCTTG3′ and 5′AGTCCAAATGCAGACCCATC3′, and for the 3′ end were 5′TATGTTTCGGCACAACATGG3′ and 5′CAGTCTGCTGCT CTCACTGC3′. For Ta*BOR2*, primer pairs from the 5′ end were 5′CACCGCTAGCTGATGGATCT3′ and 5′GCTTGTTGCAGG AAGAGGAC3′, and for the 3′ end were 5′TTGGCTGGAGAA TTTGATCC3′ and 5′CCCCTTCCACATCCAATACA3′. Internal primers were then designed using the resultant sequences. PCR products were cloned into pGEM-T Easy vectors and sequenced using Big Dye Terminator v.3.1 (Applied Biosystems). Real-time PCR analysis was conducted on a Corbett Rotor-Gene 6000 using Platinum SYBR Green (Invitrogen). Forward and reverse primers 5′AATCGTGGGCGAGTTCAGTA3′ and 5′CCCAAGTAGGCC ATTGACAT3′ were used for barley, and 5′GATGGGTCTGC ATTTGGACT3′ and 5′CCAAAAAGCTCTCCTGCAAC3′ for wheat. Expression was normalized against tubulin 1β for barley (accession No. AM502849) and tubulin 2β (accession No. U76745) for wheat using primers 5′AGTATGCCACTCCCTTGGTG3′ and 5′GGTGAGGGGAACACTGAGAA3′ for both genes.

Chromosome location of HvBOR2

The chromosomal location of Hv*BOR2* was determined using wheat–barley addition lines generated by Islam et al. (1981) and Islam and Shepherd (2000). Genomic DNA was digested with *Bam*HI, separated by gel electrophoresis, transferred to a nitrocellulose membrane and hybridized with a probe prepared with primers 5′CGATGATGATCGTTGGAGTC3′ and 5′TTGC TGAGCCAACTGTGAAG3′ which amplified a region near the 5′ end of approximately 1 kb starting at 265 bp inside the coding region and included several introns.

Phylogenetic and statistical analysis

Phylogenetic analysis was performed with MEGA 3.1 http://www.megasoftware.net) using the neighbor-joining method on translations of the full open reading frame for each gene.

Statistical analysis was performed by pairwise comparison using the analytical tools of Excel 2000, using a probability of $P < 0.05$ for significant differences between treatments.

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