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ORIGINAL PAPER

Fine mapping and targeted SNP survey using rice-wheat gene colinearity in the region of the *Bo1* boron toxicity tolerance locus of bread wheat

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Abstract Toxicity due to high levels of soil boron (B) represents a significant limitation to cereal production in some regions, and the *Bo1* gene provides a major source of B toxicity tolerance in bread wheat (*Triticum aestivum* L.). A novel approach was used to develop primers to amplify and sequence gene fragments specifically from the *Bo1* region of the hexaploid wheat genome. Single-nucleotide polymorphisms (SNPs) identified were then used to generate markers close to *Bo1* on the distal end of chromosome 7BL. In the 16 gene fragments totaling 19.6 kb, SNPs were observed between the two cultivars Cranbrook and Halberd at a low frequency (one every 613 bp). Furthermore, SNPs were distributed unevenly, being limited to only two genes. In contrast, RFLP provided a much greater number of genetic markers, with every tested gene identifying polymorphism. *Bo1* previously known only as a QTL was located as a discrete Mendelian locus. In total, 28 new RFLP, PCR and SSR markers were added to the existing map. The 1.8 cM *Bo1* interval of wheat corresponds to a 227 kb section of rice chromosome 6L encoding 21 predicted proteins with no homology to any known B transporters. The co-dominant PCR marker AWW5L7 co-segregated with *Bo1* and was highly predictive of B tolerance status within a set of 94 Australian bread wheat cultivars and breeding lines. The markers and rice colinearity described here represent tools that will assist B tolerance breeding and the positional cloning of *Bo1*.

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Introduction

Phytotoxic levels of boron (B) can be found in soils throughout the world and limit crop yield (Cartwright et al. 1986; Moody et al. 1993; Nable et al. 1997; Rashid and Ryan 2004). Genetic variation and loci for B tolerance have been identified within the cultivated triticeae (Paull et al. 1988; Chantachume et al. 1995; Yau et al. 1997; Jefferies et al. 1999, 2000). In bread wheat (*Triticum aestivum* L.), the *Bo1* gene which likely originated from the early twentieth century varieties Federation and Currawa represents an historically important source of B tolerance in wheats of south-eastern Australia, accounting for much of the B tolerance and success of varieties grown in the B-toxic soils of this region (Paull et al. 1992; Nable et al. 1997; Jefferies et al. 2000; Martin et al. 2004). In field trials of near-isogenic lines, the *Bo1* gene conferred up to 11% yield advantage in B toxic sites (Moody et al. 1993). *Bo1* was mapped as a major QTL on chromosome 7B controlling B toxicity symptoms and shoot B accumulation (Jefferies et al. 2000). Given that *Bo1* is likely to remain an important source of B tolerance in wheat breeding, an easy-to-use diagnostic PCR

marker closely linked to this locus would be of considerable value.

Single nucleotide polymorphisms (SNPs) and small insertion-deletions are commonly regarded as the most abundant form of natural genomic sequence variation in low copy sequences and can be assayed either using generic high-throughput platforms or by facile PCR based procedures requiring common lab equipment such as cleaved amplified polymorphic sequence (CAPS) assays. In efforts to tag and isolate genes of interest from barley and rye, SNP-based PCR markers have provided a highly effective means of mapping genes predicted from the rice genome sequence to be present in particular chromosomal regions (e.g. Collins et al. 2003; Hackauf and Wehling 2005). However, in hexaploid wheat, targeted generation of SNP markers from genes is hampered by the presence in most cases of three homoeologous gene copies. Therefore, strategies to overcome the difficulties in targeted PCR marker generation in wheat would be beneficial.

Our goals in this study were to map *Bo1* accurately and develop PCR markers to assist in selection of the *Bo1* tolerance allele in breeding programs. The information and markers obtained here will also provide a base for the isolation of the *Bo1* tolerance gene from wheat. The first task was to map *Bo1* as a discrete Mendelian locus since it was previously defined as a QTL. Then, to increase the marker density in the region, commonly available markers and genes from the corresponding interval in the rice genome were used, and a novel approach was employed to perform a targeted SNP search in genes from the *Bo1* region.

Materials and methods

Phenotyping and statistical analysis

Genetic mapping was performed using doubled haploid (DH) lines from the Cranbrook/Halberd mapping population (Jefferies et al. 2000). In determining phenotype distributions for DH lines carrying alternate alleles at the *Bo1* locus, the 142 lines which had both complete marker and phenotype data and no recombination between AFLPs *XPAGG/MCAT3* and *XPAGC/MCAA2* were used. To determine the probabilities of individual recombinant lines belonging to either the Halberd or the Cranbrook *Bo1* genotypic class, a finite mixture model with two groups was fitted using the Flexmix package of the R statistical system (R Development Core Team 2006).

Absolute root lengths of recombinant Cranbrook/Halberd DH lines in the presence of toxic B levels (10 mM boric acid) were measured essentially as described by Chantachume et al. (1995), except that seedlings were grown in aerated hydroponics rather than on filter paper. Relative root lengths of the 94 lines in the wheat germplasm collection were determined using the filter paper procedure of Chantachume et al. (1995), and is defined as root length in high B (10 mM boric acid) expressed as a percentage of root length in low B (50 μM boric acid).

SNP survey and sequence tagged site (STS) PCR markers

Seed of Chinese Spring wheat nulli-tetrasomic (CS-NT) chromosome substitution lines (Sears 1966) were kindly supplied by A. Lukaszewski, UC Riverside, USA.

PCRs were performed using ∼100 ng genomic DNA template and hot start thermal-stable polymerase (Immolase, Bioline, Australia), requiring an initial step at 95°C for 7 min prior to thermal cycling to activate the enzyme, followed by 35 cycles of 94°C for 10 s, 58–68°C for 30 s, 68°C for 45 s to 1 min 30 s, and a final extension at 72°C for 10 min. Primers, including those containing locked nucleic acid (LNA™) modifications, were synthesized by Proligo (Sigma-Aldrich, Australia). Primers with a 3′ LNA™ modification can allow higher PCR discrimination between templates containing a match/mismatch with the 3′ most primer base (Latorra et al. 2003). PCR products were direct-purified using the QIAquick PCR Purification Kit (Qiagen, Germany), and sequenced using BigDye™ V 3.1 (ABI) or cloned into the pGemT plasmid vector (Promega, USA). Sequences were aligned using the Contig Express software (Vector NTI Suite 8; InforMax, Bethesda, MD, USA).

Eighteen cloned AWW5L7 fragments amplified using the 7B-nonspecific primer pair AWW11-right plus AWW11-left (Suppl. Table 2) were sequenced from each parent. Sequence differences that were unique to individual clones were attributed to PCR errors and were excluded from the consensus sequences. The AWW5L7 PCR reaction was performed using the three primers AWW5L7-Left2, AWW5L7-Left3 and AWW5L7-Right mixed in equal proportions, with a touchdown cycling regime: after the initial 7 min at 95°C, 10 cycles of 94°C for 10 s, 65°C for 30 s, decreasing by 0.5°C each cycle, 68°C for 1 min 30 s, followed by 25 cycles of 94°C for 10 s, 60°C for 30 s, 68°C for 1 min 30 s, followed by a final extension at 72°C for 10 min. Products were separated at 100 V for approximately 75 min in 2% agarose gels containing 0.5× TBE.

Probes and RFLP analyses

Genomic DNA extraction was performed as described by Pallotta et al. (2000). Gene fragments for probe generation were PCR-amplified from a cDNA library made from developing grain of wheat *cv.* CS kindly provided by Andrew Milligan, University of Adelaide, or from CS genomic DNA, and cloned into pGemT plasmid vector.

PCR-amplified inserts were sequenced to confirm identity and used as template for making RFLP probes. Other probes were obtained from A. Graner, IPK Gatersleben (MWG2062), M. Gale, JIC, Norwich (PSR121) and M. Sorrells Cornell University, Ithaca (CDO347).

Probe copy number estimation and polymorphism screening was performed on filters containing genomic DNA of Cranbrook, Halberd, CS and line CS-N7B/T7D digested separately with the restriction enzymes *Afl*II, *Ase*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nco*I, *Nde*I, *Sca*I, *Sph*I, *Ssp*I, *Stu*I and *Xba*I. All molecular markers generated in this study contain the prefix AWW (Adelaide, Waite Campus, Wheat), as the lab identifier according to the catalogue of gene symbols for wheat (McIntosh et al. 1998).

Microsatellite markers

Sixteen microsatellite markers were tested for polymorphism: GWM577, WMC276, WMC581, BARC10, BARC32, BARC182, GWM344, GWM146, WMC10, WMC70, WMC166, WMC232, WMC273, WMC500, BARC123 and CFA2040. Primer sequences and annealing temperatures for microsatellite markers are described by Röder et al. (1998) or at http://wheat.pw.usda.gov/GG2/index.shtml. After the initial 7 min at 95°C, SSR marker amplifications comprised 10–15 touchdown cycles of 94°C for 30 s, annealing for 30 s, decreasing the temperature by 0.5°C per cycle until the specified annealing temperature was reached, and 72°C for 30 s. This was then followed by 25–35 cycles of amplification with the specified annealing temperature, and a final extension at 72°C for 10 min. PCR products were separated by electrophoresis for three to four hours at 200 V in 8.5% polyacrylamide gels and then visualized with UV light after staining with ethidium bromide.

Results

Mapping *Bo1* as a discrete Mendelian locus

In the Cranbrook/Halberd DH mapping population, Jefferies et al. (2000) found a strong bimodal frequency distribution for shoot B content in plants grown in 100 ppm B, reflecting segregation at the major *Bo1* B tolerance QTL on chromosome 7B. Using the original marker data, lines were selected that were non-recombinant for the QTL interval defined by the AFLP marker loci *XPAGG/MCAT3* and *XPAGC/MCAA2* and these were used with the original [B] (boron concentration) data to establish phenotype frequency distributions for lines carrying alternate alleles at the *Bo1* locus. Average shoot [B] was calculated at 163.7 ± 93.4 or 545.3 ± 142.6 mg/kg dry weight for lines carrying the Halberd or Cranbrook *Bo1* allele, respectively. Almost all (134) of the 142 non-recombinant lines exhibited values with a low probability ($P < 0.06$) of belonging to the opposite genotypic class to that which they were assigned using molecular markers. The remaining eight lines had values which were highly consistent ($P > 0.99$) with the opposite class, possibly representing labeling errors. Therefore it appeared that the *Bo1* genotype of individual recombinant Cranbrook/Halberd DH lines could be determined with a reasonable degree of accuracy.

The [B] distributions of the non-recombinant DH lines were used to predict *Bo1* genotypes of lines that were recombinant for the *Bo1* interval. Each of the 12 recombinant lines for which shoot [B] data were available had a [B] value, which was strongly suggestive of one or the other *Bo1* genotypic class, with the probabilities of mis-classification being very low ($P < 0.00003$). New root length data in high B (10 mM boric acid) for all 13 recombinant DH lines was consistent with the initial classifications (Fig. 1).

	DH lines Cranbrook/Halberd														
Loci	DH_#26	DH_#76	DH_#79	DH_#82	DH_#172	DH_#97	DH_#164	DH_#20	DH_#170	DH_#143	DH_#34	DH_#87	DH_#156	Cranbr.	Halberd
XPAGG/MCAT3, etc.	A	A	A	A	A	B	B	B	B	B	B	B	A	A	B
Xbarc32	A	A	A	A	A	A	B	B	B	B	B	B	A	A	B
Xaww254	A	A	A	A	A	A	B	B	B	B	B	B	B	A	B
Xaww256	A	A	A	A	A	A	A	B	B	B	B	B	B	A	B
Xaww228, etc.	A	A	A	A	A	A	A	A	A	B	B	B	B	A	B
Bo1	A	A	A	A	A	A	A	A	A	B	B	B	B	A	B
Xaww246	A	A	A	A	A	A	A	A	A	A	B	B	B	A	B
Xpsr680, etc.	A	A	A	A	A	A	A	A	A	A	A	B	B	A	B
Xaww151	B	B	B	A	A	A	A	A	A	A	A	B	B	A	B
XPAGC/MCAA2, etc.	B	B	B	B	B	A	A	A	A	A	A	A	B	A	B
Shoot [B] (mg/kg)[a]	560	593	815	446	556	n.a.	550	513	616	120	131	140	128	580	125
Rel. Root length (RRL)	22.7	8.1	11.9	20.1	19.8	27.0	15.5	22.7	13.0	73.7	56.5	52.6	73.9	17.0	72.7
Root length (mm)[b]	55.6±9.9	59.1±8.7	67.4±9.5	65.2±9.7	64.7±8.9	67.5±8.3	57.6±6.8	74.5±5.6	56.5±5.8	136.0±16.8	121.0±15.1	115.2±9.0	121.6±25.5	61.2±5.5	130.2±12.7

a=data from Jefferies et al. (2000)
b=mean of two replications with 16 plants each ± SD
n.a.=not available

Fig. 1 Mapping of *Bo1* as a discrete locus. Recombinant DH lines are from the Cranbrook (B-intolerant) × Halberd (B-tolerant) population. Marker alleles: *A* Cranbrook and *B* Halberd. *Bo1* genotypes were assigned on the basis of the phenotype scores

Recombinant lines predicted from shoot [B] to carry the Cranbrook *Bo1* allele exhibited root length values of 55.6 ± 9.9 to 74.5 ± 5.6 mm, while those predicted to carry the Halberd allele had root lengths of 115.2 ± 9.0 to 136.0 ± 16.8 mm (Fig. 1). Upon scoring the recombinants for new molecular markers in the interval (see below), *Bo1* was narrowed to a smaller marker interval without a requirement for any double recombinants (Figs. 1, 2), consistent with correct *Bo1* genotype classifications being made for the recombinants.

A targeted survey of SNPs in the *Bo1* region, and generation of PCR markers

Xpsr680-7B was the RFLP marker Jefferies et al. (2000) found to be most closely associated with the *Bo1* QTL, and had been mapped to the distal end of the long arm of chromosome 7B (Erayman et al. 2004). The ends of the long arms of wheat group 7 chromosomes are related to the distal portion of rice 6L (Hossain et al. 2004). Accordingly, genes from 100.0 to 124.4 cM on rice 6L (http://rgp.dna.affrc.go.jp/cgibin/statusdb/irgspMini.pl? chr = 6) were used to identify genes, which were likely to be close to *Bo1* for use in the SNP search. Segments of the rice genome sequence were used in BLASTn searches to identify wheat ESTs corresponding to the rice genes. Those wheat ESTs identifying single-copy genes in the rice genomic sequence (chromosome 6) in reciprocal BLAST searches were selected for primer design. The rice–wheat alignments provided by BLASTn at NCBI also allowed prediction of intron–exon structure. PCR primers were designed from exons to amplify predicted wheat genomic

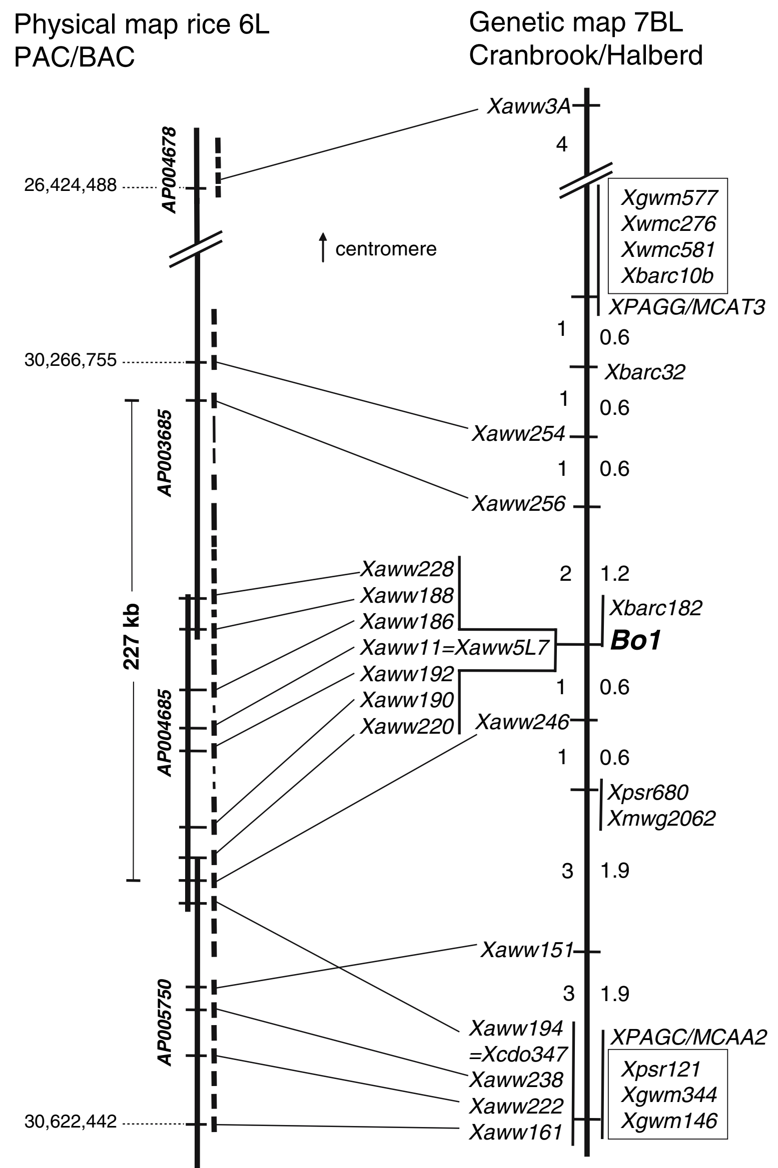

Fig. 2 Map of the *Bo1* region on the distal end of wheat 7BL and its relationship to the distal end of rice 6L. Numbers of recombinants, and genetic distances in cM for marker intervals are indicated on the left and right of the wheat map, respectively. The rice region comprises four BAC clones with corresponding pseudomolecule positions indicated to the left. Predicted rice genes with or without putative orthologous wheat ESTs are represented by *bold* or *non-bold dashes*, respectively. Boxed markers co-segregated with AFLP loci *XPAGG/MCAT3* and *XPAGC/MCAA2* originally used to select those recombinant DH lines for mapping and hence may be located further from the *Bo1* locus than suggested by the map. *Xaww3A* was ordered proximal of *XPAGG/MCAT3* by additional recombination sites present in four of the DH lines. *Xaww3A* and *Xaww5L7* are STS-PCR marker loci, *Xgwm*, *Xwmc* and *Xbarc* are microsatellite marker loci. The remaining markers were scored by RFLP

fragments of 0.8 to 1.6 kb comprising mainly intron sequence (assuming conservation of intron location and size between rice and wheat) to maximize the opportunities for polymorphism detection.

It was anticipated that most primer pairs would amplify from all three homoeologous gene copies present on wheat chromosomes 7A, 7B, and 7D chromosomes. Therefore Chinese Spring wheat nulli-tetrasomic (CS-NT) chromosome substitution lines were used to identify polymorphisms between the homoeologues for the design of primers for 7B-copy-specific amplification. Forty-one primer pairs derived from 26 genes were used to amplify and direct-sequence gene fragment mixtures from genomic DNA of wheat CS-N7D/T7B and CS-N7B/T7D lines. Fifteen primer pairs resulted in mixed sequence chromatograms from each end, possibly due to terminal insertion-deletion differences between amplified orthologues. The remaining primer pairs gave readable sequence chromatograms of between 150 and 750 bp of mostly single peaks, with scattered double peaks representing polymorphisms between the amplified orthologues. For each of the primer pairs, differences in base composition at polymorphic positions were observed in fragment mixtures amplified from the two group-7 NT lines, confirming that the strategy with rice had successfully yielded gene fragments from wheat group seven chromosomes.

Bases present in the CS-N7D/T7B amplicon mixture but absent at the same nucleotide position in the CS-N7B/T7D amplicons were identified as being unique to the 7B orthologue and were used to define the 3′-most base of 24 new primers. When used in combination with the original opposing non-specific primers, eight of the primers amplified products from both the CS-N7B/T7D line and N7D/T7B line, indicating that these primers were not specific under these conditions. 7B-specificity was obtained for the remaining 16 primers, as indicated by amplification from CS-N7D/T7B and non-amplification from CS-N7B/T7D. However, in every case, specificity was only achieved upon either increasing the PCR annealing temperature and/or through inclusion of an LNA™ modification of the 3′-most base (Suppl. Table 1). It was noted that LNA™ modifications were not always sufficient to provide specificity as two of the nine primers containing such a modification remained 7B-nonspecific.

Fifteen of the 7B-specific primer pairs gave amplification products from the mapping parents Cranbrook and Halberd, which were then sequenced directly to identify polymorphisms. In 18.5 kb of total sequence compared, only two inter-varietal SNPs were identified, and these were both from the same gene fragment AWW3A. One of the SNPs altered a *Hin4*I restriction site, resulting in cleavage products of 600 and 700 bp from Cranbrook and an uncleaved product of 1.3 kb from Halberd after digestion with this enzyme. This provided the basis of a AWW3A CAPS marker which was scored in the 13 Cranbrook/Halberd DH recombinant lines for the *Bo1* interval, locating it at least 5.4 cM proximal to *Bo1* (Fig. 2).

The remaining primer pair (AWW5L7-Left2 plus AWW5L7-right) shown to be 7B-specific using the CS-NT substitution lines was found to amplify from Cranbrook and CS but not Halberd, indicating that these primers also covered one or more inter-varietal SNPs. To obtain a co-dominant PCR marker, fragment mixtures were amplified from Cranbrook and Halberd using the original non-discriminatory primer pair and the ~1.1 kb products cloned and sequenced to obtain sequence of all three homoeologues from each parent. Comparison with the sequence traces obtained from the group seven CS-NT substitution lines allowed identification of the 7B copy from each parent. Alignment of the two 7B sequences revealed a high degree of polymorphism between the parents—a total of 30 inter-varietal SNPs and indels. The failure to amplify initially from Halberd using the AWW5L7-Left2 plus AWW5L7-right primer combination was retrospectively found to be due to mismatches between the AWW5L7-Left2 7B-specific primer and Halberd comprising of a G/C difference at the 3′-end (+G, LNA™ modified) and three additional SNPs. An additional primer AWW5L7-Left3 located 79 bp from AWW5L7-Left2 was designed to be Halberd specific using a 4 bp (TGGG) Halberd-specific insertion at its 3′-end, as well as seven additional Halberd specific bases within the primer sequence. Specificity of the respective primers was confirmed by amplification from the CS-NT lines. Use of AWW5L7-Left3 and AWW5L7-Left2 and the non-specific opposing primer AWW5L7-right all in the same PCR reaction provided a co-dominant PCR marker, comprising of a 956 bp fragment representing the Halberd allele, and a 877 bp fragment representing the Cranbrook allele (Fig. 3). The AWW5L7 marker co-segregated with *Bo1* in the 13 recombinant DH lines (Figs. 1, 2).

In summary, rice colinearity was effective at enabling identification of genes linked to *Bo1*, and the approach using the CS-NT lines allowed design of primers specific to 7B orthologues. A total of 19.6 kb from 16 fragments (from 13 genes) was scanned and 32 polymorphisms identified—a frequency of one polymorphism every 613 bp. However, these polymorphisms were limited to only two of the genes, with the vast majority (30) occurring in a single gene. The uneven distribution of polymorphism meant that only two of these genes could be converted to PCR markers.

RFLP mapping of genes in the *Bo1* region identified using rice

The low rate of success in generating markers from SNPs motivated exploration of marker generation by using Southern blot analysis and RFLPs. By this stage, the PCR

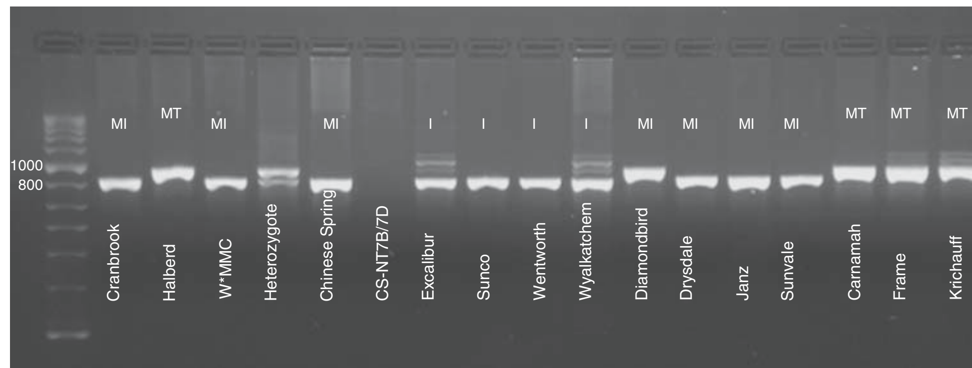

Fig. 3 AWW5L7 PCR marker in a selection of bread wheat lines. *I* B intolerant, *MI* moderately intolerant and *MT* moderately tolerant. First lane, molecular weight marker (Bioline HyperLadder II)

markers had refined the region of interest on rice chromosome 6 to be distal of nucleotide position 26,424,488 (AWW3A) but closely linked to position 30,430,721 (AWW5L7). Fragments of wheat orthologues of 15 rice genes located between 30,266,755 (AWW254) and 30,622,442 (AWW161) were obtained by PCR amplification from wheat genomic or cDNA and cloned for use as probes. To avoid potential repetitive sequence elements, only exon sequences were used. Southern blot hybridization to wheat CS-NT lines revealed at least one hybridizing fragment on chromosome 7B for each of the probes (not shown). Probe AWW228, representing a gene family in rice, hybridized to more than 10 restriction fragments. The remaining probes represented low copy genes in rice (Suppl. Table 2) and also appeared to be low copy in wheat, identifying around 3–6 fragments (not shown). RFLPs between Cranbrook and Halberd were identified for all probes, and were mapped in the 13 Cranbrook/Halberd DH recombinant lines. The 15 probes were all genetically mapped close to *Bo1* (Fig. 2). Probe AWW11 co-segregated with *Bo1*, as did the AWW5L7 PCR marker developed from the same gene. Six other probes (AWW186, 188, 190, 192, 220, 228) also co-segregated with *Bo1* (Figs. 1, 2).

Mapping of commonly available RFLP and SSR markers

Three RFLP probes known to map to 7BL (MWG2062, PSR121, CDO347) were also mapped using the recombinants (Fig. 2). The oat cDNA probe CDO347 was homologous to wheat probe AWW194 and did not recombine with it (Fig. 2). The recombinants were also re-scored for *Xpsr680-7BL*, the closest RFLP locus to *Bo1* previously identified by Jefferies et al. (2000).

SSR markers previously reported to be located on 7BL were also assessed. Of the 16 SSR primer pairs tested, eight identified polymorphisms between Cranbrook and Halberd and were mapped close to *Bo1* (Fig. 2). Three of the markers (BARC182, GWM146 and GWM577) were dominant markers, requiring being scored on the basis of ± amplification in the recombinant DH lines (not shown).

Comparison between wheat chromosome 7BL and rice 6L

A high degree of conservation in gene order was observed between the *Bo1* region on 7BL and rice 6L. However, there were some breaks in the colinearity (Fig. 2). Gene-derived probes PSR121 (1,3;1,4-beta-glucanase), MWG2062 (MatE domain containing protein) and PSR680 (leucine-rich repeat containing protein) were mapped in the *Bo1* region but BLASTn searches showed that these genes lacked a homologue in the expected location in rice. The order of the genes *Xaww151* and *Xaww194* were different in wheat and rice (Fig. 2), indicating that one of these genes had moved, or that an interstitial inversion of a chromosome segment containing these genes had occurred since the wheat-rice evolutionary divergence.

Bo1 was located to an interval in wheat of around 1.8 cM, defined by the RFLP loci *Xaww246* (one recombination distal), and *Xaww256* (two recombination events proximal), corresponding to an interval in rice of around 227 kb (Fig. 2) or 0.5 cM (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/irgspMini.pl? chr = 6). BLAST analysis against protein and EST sequence databases predicted 21 genes in the rice interval. Seven of these were used to generate markers in wheat, and all co-segregated with *Bo1* (Fig. 2). Wheat ESTs corresponding to the majority of the remaining genes were identified (Fig. 2) but these were not mapped. None of the 21 predicted proteins encoded by the rice *Bo1* interval showed similarity to any proteins previously shown to be required for B uptake in Arabidopsis (Takano et al. 2002, 2006).

Association of the AWW5L7 PCR marker with B tolerance in Australian bread wheat germplasm

Since the AWW5L7 PCR marker was simple to use and co-segregated perfectly with *Bo1* in the mapping population,

its association with B tolerance within a collection of 94 hexaploid bread wheat lines, representing a large portion of the current Australian bread wheat germplasm, was tested. Each of the lines was assessed for its B tolerance by measuring root length under high (10 mM) boric acid relative to that under low (50 µM) boric acid (relative root length; RRL). Sixty-nine lines were classified as intolerant (I) or moderately intolerant (MI), whereas the remaining 25 lines were classified as moderately tolerant (MT) or tolerant (T) (Table 1). A PCR product similar in size to that observed in either Halberd (956 bp) or Cranbrook (877 bp) was amplified from each line (subset of lines represented in Fig. 3). All of the MT and T lines gave Halberd-like products. Of the I or MI lines, 63 showed Cranbrook-like products, while the remaining six showed Halberd-like products (VS0363, WI24014, Diamondbird, RAC1261, 1263, 1284; Table 1). Therefore, within the germplasm surveyed, the Halberd-like and Cranbrook-like alleles of the AWW5L7 PCR marker were strongly associated with B tolerance and susceptibility, respectively.

Discussion

The *Bo1* map

Twenty-eight new molecular markers were added to the map of the *Bo1* region and *Bo1* was mapped as a discrete Mendelian locus. This allowed delimitation of *Bo1* to a 1.8 cM interval. This represents a significant step towards the positional cloning of this gene. To date, no B toxicity tolerance gene has been cloned and the molecular mechanisms of B toxicity tolerance are unknown. However, genes required for B uptake under low B conditions have been identified in Arabidopsis. These encode *AtBOR1* belonging to a family of B transporters (Takano et al. 2002) and the *AtNIP5;1* aquaporin (Takano et al. 2006). In barley it was shown that B toxicity tolerance was associated with the ability to maintain low [B] in the shoot and is likely to result from a mechanism of active efflux of B from the roots (Hayes and Reid 2004). Hence, it is conceivable that components of B deficiency tolerance and B toxicity tolerance in plants could be related. However, none of the 21 genes from the rice interval, including the seven so far shown to co-segregate with *Bo1* in wheat, encode proteins with any similarity to *AtBOR1*, a characterized aquaporin, or to any protein annotated as a transporter. Therefore, either a homologue of *Bo1* is missing from the expected interval in rice, or *Bo1* belongs to an as yet unknown class of protein involved directly or indirectly in B transport.

The positioning of the *Bo1* QTL near the top of the whole-chromosome 7B Cranbrook × Halberd genetic map

Table 1 Association between AWW5L7 PCR marker allele type and boron tolerance in some Australian bread wheat genotypes

Line	Rel. root length[a]	Boron rating[b]	Marker allele size (kb)[c]
WI21108	0.13	I	0.9
SUN434G	0.15	I	0.9
SUN436F	0.16	I	0.9
TAMMARIN ROCK	0.16	I	0.9
TMB1806F	0.17	I	0.9
SUN498F	0.18	I	0.9
VR0168	0.18	I	0.9
VS0519	0.18	I	0.9
WI24068	0.18	I	0.9
CHARA	0.19	I	0.9
LANG	0.19	I	0.9
SUN434A	0.19	I	0.9
KENNEDY	0.20	I	0.9
SUN431B	0.20	I	0.9
SUNCO	0.20	I	0.9
VS0718	0.20	I	0.9
AGT SCYTHE	0.21	I	0.9
H46	0.21	I	0.9
SUN434D	0.21	I	0.9
SUN498H	0.21	I	0.9
SUNSTATE	0.21	I	0.9
VS0363	**0.22**	**I**	**1**
WYALKATCHEM	0.22	I	0.9
RAC1192	0.23	I	0.9
RAC1309	0.23	I	0.9
VS0457	0.23	I	0.9
WESTONIA	0.23	I	0.9
WI24014	**0.23**	**I**	**1**
SUN431A	0.24	I	0.9
SUN434H	0.24	I	0.9
VS0427	0.24	I	0.9
WI21108	0.13	I	0.9
EXCALIBUR	0.25	I	0.9
SUN485A	0.25	I	0.9
VR1128	0.25	I	0.9
VS0038	0.25	I	0.9
VS0367	0.25	I	0.9
RUBY	0.26	I	0.9
SUN421T	0.26	I	0.9
SUN435D	0.26	I	0.9
SUN502C	0.26	I	0.9
VQ4127	0.26	I	0.9
H45	0.28	I	0.9
RAC1267	0.28	I	0.9
WENTWORTH	0.28	I	0.9
RAC1261	**0.29**	**MI**	**1**

Table 1 continued

Line	Rel. root length[a]	Boron rating[b]	Marker allele size (kb)[c]
RAC1229	0.30	MI	0.9
SUN431D	0.30	MI	0.9
SUNVALE	0.30	MI	0.9
VS0284	0.30	MI	0.9
VS0486	0.30	MI	0.9
SUN498A	0.31	MI	0.9
VS0039	0.31	MI	0.9
SUN498D	0.31	MI	0.9
DIAMONDBIRD	**0.32**	**MI**	**1**
RAC1220	0.32	MI	0.9
RAC1263	**0.32**	**MI**	**1**
SUN434E	0.32	MI	0.9
RAC1250	0.32	MI	0.9
VS1408	0.34	MI	0.9
JANZ	0.36	MI	0.9
STRZELECKI	0.36	MI	0.9
SUN389A	0.36	MI	0.9
SUN503A	0.36	MI	0.9
DRYSDALE	0.37	MI	0.9
ELLISON	0.37	MI	0.9
VENTURA	0.37	MI	0.9
RAC1284	**0.38**	**MI**	**1**
VQ0326	0.40	MI	0.9
WI24050	0.41	MT	1
YITPI	0.43	MT	1
FRAME	0.44	MT	1
WI24003	0.44	MT	1
VO1225	0.45	MT	1
RAC1257	0.46	MT	1
VS2177	0.46	MT	1
WI24065	0.46	MT	1
WI22100	0.47	MT	1
WI23322	0.47	MT	1
VR5190	0.48	MT	1
WI24001	0.48	MT	1
WI24033	0.49	MT	1
RAC1262	0.50	MT	1
VS2284	0.51	MT	1
WI24055	0.51	MT	1
KRICHAUFF	0.52	MT	1
RAC1199	0.54	MT	1
WI24013	0.55	MT	1
WI24053	0.55	MT	1
CARNAMAH	0.56	MT	1
WI24072	0.64	T	1
WI24034	0.67	T	1
VP1081	0.76	T	1
RAC1311	0.88	T	1

Bold values have a AWW5L7 marker allele type inconsistent with B tolerance status

[a] Relative root length = Root length in high B (10 mM boric acid) expressed as a percentage of root length in low B (50 µM)

[b] Boron rating, *I* Intolerant, RRL < 0.29; *MI* moderately intolerant, 0.30 < RRL < 0.40; *MT* moderately tolerant, 0.41 < RRL < 0.60; *T* tolerant, 0.61 < RRL < 0.90

[c] AWW5L7 marker alleles: 0.9 kb = Cranbrook-like allele; 1 kb = Halberd-like allele

of Jefferies et al. (2000) implied a short arm location for *Bo1*. However, a later version of the map with a revised marker order (Chalmers et al. 2001) put the *Bo1*-associated RFLP marker locus *Xpsr680-7B* on the long arm and near the bottom of the new map. A distal long arm location for *Bo1* is also confirmed by the fact that PSR680, as well as CDO347, PSR121, and MWG2062 probes mapped close to *Bo1* (Fig. 2), had been located to the distal-most bin of a wheat 7L deletion map and near the telomere of a wheat 7L consensus genetic map (Erayman et al. 2004). The corresponding region in rice is also located close to the telomere of 6L, as the most distal locus with a link to rice (*Xaww161*) is located only 110 kb from the end of the rice physical map.

Wheat telomeric regions generally exhibit a low physical to genetic ratio, and the distal-most bin of the wheat 7L deletion map containing the *Bo1* region shows a low overall ratio of 442 kb per cM (Erayman et al. 2004). The *Bo1* genetic interval of 1.8 cM represents 1.5×10^{-3} of the 1,200 cM total genetic length of the B genome (Quarrie et al. 2005), yet corresponds to a 227 kb rice interval representing only 5.3×10^{-4} of the 430 Mb rice genome. This further indicates that the ratio of physical to genetic distance in the immediate vicinity of *Bo1* would be low. Such a low ratio of physical to genetic distance should assist in the positional cloning of *Bo1*.

Overall, gene colinearity with rice in the *Bo1* region was very good (Fig. 2), although there were exceptions. The gene derived probes PSR121, PSR680 and MWG2062 identifying loci in the wheat *Bo1* region had no homologues in the section of rice 6L most related to the *Bo1* interval. These three genes are multi-copy in rice. Multi-copy genes have been shown to break rice–wheat colinearity more often than single-copy genes (Singh et al. 2007). Therefore, our decision to develop markers mainly from genes that were single-copy in rice may have biased our study towards

genes with higher than average level of rice–wheat colinearity. EST mapping using deletion lines has provided information on gross-level colinearity between rice and wheat (e.g. Hossain et al. 2004). However, detailed genetic mapping can reveal colinearity on an entirely different level, often revealing small rearrangements such as inversions between the rice and triticeae species (Li and Gill 2002; Brunner et al. 2003; Caldwell et al. 2004a; Spielmeyer and Richards 2004; Bossolini et al. 2006). Indeed, the reversal of the markers *Xaww151* and *Xaww194* (Fig. 2) indicates a potential wheat–rice inversion in the *Bo1* region. This potential inversion should not complicate higher-resolution mapping of *Bo1* because it is located outside of the smallest *Bo1* marker interval.

Studies in a range of organisms have indicated sub-telomeric regions to be more prone to structural alterations than other chromosome regions (Eichler and Sankoff 2003). For example, a comparison of three yeast species found rearrangements, commonly involving gene families, in addition to translocations, gene deletions and novel genes, to be almost entirely confined to the sub-telomeric regions (Kellis et al. 2003). In a survey of genes on the distal 42% of wheat chromosome arm 4BL, 11% of the genes that showed no DNA sequence homology to rice were found to be confined to the terminal 5% of 4BL, suggesting that cereal genes near telomeres may evolve more rapidly than genes at other locations (See et al. 2006). However, in the same survey, a high (83%) proportion of genes were also found to be syntenic with rice, and only one wheat–rice inversion was identified. We also found good levels of wheat–rice co-linearity in the *Bo1* region, located close to the telomere of 7BL. Therefore, while it is possible that breaks in wheat–rice co-linearity are more common near the telomeres, conservation in these regions still appears to exist at levels sufficient to allow satisfactory exploitation of rice for targeted marker generation in wheat.

Targeted SNP survey

Although there are efforts to investigate SNPs in wheat (http://wheat.pw.usda.gov/ SNP/new/index.shtml), information on SNPs in wheat and their application has been slow to emerge. One reason is the presence of three homoeologous copies for most wheat genes, which necessitates the development of PCR primers specific to individual homoeologues before SNPs can be discovered or assayed. Sequences of homoeologous gene copies can be obtained by various approaches (Bryan et al. 1999; Somers et al. 2003; Ravel et al. 2006), and usually, primers specific to each of the three gene copies need to be made and used for PCR on wheat CS-NT substitution lines before the copies can be assigned to the A, B or D genomes. While this is reasonable if one wishes to discover SNPs throughout the genome, it is wasteful for applications such as positional cloning in which there is a single chromosomal region of interest.

Direct sequencing of gene fragment mixtures amplified from wheat CS-NT substitution lines allowed the identification of bases specific to homoeologous 7B gene copies. This formed the basis for gene specific PCR primer design, thus avoiding the need to specifically amplify and analyze the other two gene copies. The procedure also allows 7B copies to be discriminated from any non-group-7 paralogues that may also be amplified in the initial fragment mixture. Furthermore, it confirms that genes identified via rice are at least located on the desired wheat chromosome (syntenic) before these genes are investigated further. As found by other investigators (Bryan et al. 1999; Caldwell et al. 2004b), the frequency of polymorphism between wheat homoeologues was not a limitation in the development of homoeologoue-specific primers.

While rice co-linearity combined with sequencing of PCR product mixtures from the CS-NT lines was effective in yielding amplicons from the target chromosome segment, there may be scope for making the process more efficient. For example, pyrophosphate-based sequencing could be used to provide readable sequence immediately downstream of the primer in cases where insertion/deletion polymorphisms between homoeologues close to the primers prevent this (Mochida et al. 2004). The ability of *Taq* polymerase to extend 3′ primer-template mismatches was overcome to some extent by the use of LNA™ primer modifications. However, specificity might be further improved by judicious choice of primer-template mismatch base combinations that are least likely to be extended (Huang et al. 1992; Ayyadevara et al. 2000), or by further destabilizing the primer-template duplex by introduction of additional mismatches at the penultimate primer base (Newton et al. 1989). EST sequences from diploid or tetraploid relatives of hexaploid bread wheat could be used to *in silico* assign gene sequences to the A, B or D genome. However, currently this approach would be limited to highly expressed genes, as the databases for these species are not very large (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html).

The survey of 19.6 kb of sequence from genes in the *Bo1* region on 7B revealed an average of one difference every 613 bp between Halberd and Cranbrook. While only one chromosome region and two varieties were examined, this figure is comparable to inter-varietal polymorphism frequencies previously observed in wheat: one every 335–1,000 bp (Bryan et al. 1999; Ravel et al. 2006). SNP frequency in wheat is lower than that reported for other inbreeding crop plants such as barley (one every 78–200 bp; Russell et al. 2004; Rostoks et al. 2005). A principal reason for this is likely to be the genetic bottlenecks created by the

derivation of allohexaploid wheat from a small number of hybridization events involving select diploid and tetraploid progenitor accessions (Dvorak et al. 1998; Caldwell et al. 2004b). The ability to use SNP in wheat for marker generation was further limited by the fact that all the SNPs were confined to only two genes, AWW5L7 (putative catalase) and AWW3A (putative complex 1 family protein-like, Os06g0714100). Notably, 30 of the SNPs were found in AWW5L7. Ravel et al. (2006) also experienced an uneven distribution of polymorphism in wheat, finding that most of their SNPs were limited to only two out of 21 genes. It is not possible to determine if the strategy used here of sequencing mostly intron sequence improved polymorphism detection, as no exon sequences were generated for comparison.

In contrast to the SNP survey, every gene used as a probe detected an RFLP between Cranbrook and Halberd, suggesting that wheat still contains a high level of polymorphism in the intergenic regions. The comparison between the two methods of gene mapping was instructive from a practical perspective, suggesting that RFLP is still a highly efficient method for conducting high-density targeted mapping in particular regions of the wheat genome, although PCR markers may still be worth developing for certain high-throughput applications such as screening for recombinants in large mapping populations, or marker-assisted selection in breeding. Bryan et al. (1999) had reached similar conclusions. Polyploid wheat contrasts with the diploid triticeae species barley and rye, in which PCR markers offer a very efficient way of mapping genes identified using rice (e.g. Collins et al. 2003; Hackauf and Wehling 2005).

The AWW5L7 PCR marker as a tool for B tolerance breeding in wheat

The *Bo1* gene is likely to continue to be an important source of B tolerance in wheat breeding, at least in Australia. The AWW5L7 marker correlated well with B tolerance in a collection of Australian genotypes, as has been the case for the *Bo1*-linked RFLP marker locus *Xpsr680-7BL* (Jefferies et al. 2000). This supports the proposal that much of the variation in B tolerance present in the Australian wheats is dermined by the *Bo1* locus, with the tolerance allele originating from a common source, probably the early twentieth century cultivars Federation and Currawa (Paull et al. 1992). The association was not absolute, but since AWW5L7 co-segregated with *Bo1*, it would be expected to be more diagnostic than the more distantly located microsatellites WMC276 and GWM344, which were PCR markers described previously for *Bo1* (Martin et al. 2004). The advantages to breeders of a molecular marker for *Bo1* have been described previously (Jefferies et al. 2000). AWW5L7 could provide an easier and more accurate alternative for routine selection of *Bo1*. Leaf symptoms of B toxicity can be controlled by other genes that have no effect on yield under B toxic conditions, and boron screening procedures available to breeders give variable results. AWW5L7 could be used to enrich for *Bo1* along with a range of other tagged loci in segregating populations using generic molecular marker procedures, giving breeders more freedom to select for untagged traits that require specialized growing conditions. The co-dominant AWW5L7 marker would allow easier identification of *Bo1* heterozygotes, a task otherwise hampered by the incompletely dominant expression of tolerance at this locus (Paull et al. 1992; Jefferies et al. 2000). For Australian conditions, it would be an advantage to obtain levels of tolerance beyond that provided by *Bo1* alone, and other strong sources of B tolerance unrelated to *Bo1* are known (Paull et al. 1992). The AWW5L7 marker could be useful in helping identify further novel sources of boron tolerance and in the pyramiding of these new genes with *Bo1* to achieve greater levels of tolerance.

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