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ORIGINAL PAPER

A second 'overexpression' allele at the *Glu-B1* high-molecular-weight glutenin locus of wheat: sequence characterisation and functional effects

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Abstract Bread is one of the major constituents of the human diet and wheat (*Triticum aestivum* L.) is the most important cereal for bread making. The gluten proteins (glutenins and gliadins) are recognised as important components affecting the processing quality of wheat flour. In this research, we investigated a particular glutenin subunit allele in an Australian cultivar, H45. Based on protein and DNA assays, the *Glu-B1* allele of H45 seems to be *Glu-B1al,* an allele that includes a functional duplication of a gene encoding an x-type high-molecular-weight glutenin subunit, and is thought to increase dough strength through overexpression of that subunit. Yet H45 does not have the dough properties that would be expected if it carries the *Glu-B1al* allele. After confirming that H45 overexpresses Bx subunits and that it has relatively low un-extractable polymeric protein (an indicator of weak dough), we cloned and sequenced two Bx genes from H45. The sequences of the two genes differ from each other, and they each differ by four single-nucleotide polymorphisms (SNPs) from the sequence that has been reported for the *Glu-B1al* x-type glutenin genes of the Canadian wheat cultivar Glenlea. One of the SNPs leads to an extra cysteine residue in one of the subunits. The presence of this additional cysteine may explain the dough properties of H45 through effects on cross-linkage within or between glutenin subunits. We propose that the *Glu-B1* allele of H45 be designated *Glu-B1br*, and we present evidence that *Glu-B1br* is co-inherited with low un-extractable polymeric protein.

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Introduction

Gluten proteins are important components governing the processing quality of wheat flour for the production of bread and other food products. Based on their solubility in aqueous alcohol, gluten proteins are classified as gliadins or glutenins. Gliadins are monomeric, while glutenins are polymeric, with both high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS). These glutenin proteins interact to make dough elastic, allowing it to trap the gas bubbles produced by yeast and enabling the bread to rise. Variation in the amount and properties of HMW-GS accounts for much of the variation in the processing quality of wheat flour (Branlard and Dardevet 1985; Payne et al. 1988; He et al. 2005) even though HMW-GS represent only 8–10% of the protein in wheat grain.

Many different HMW-GS have been detected in wheat (Mclntosh et al. 2008). They have highly conserved structures, with each subunit containing a long repetitive

region flanked by two highly conserved non-repetitive terminal domains. Within the terminal domains, cysteine residues provide sites for disulphide bonds that connect HMW-GS with each other and with LMW-GS, stabilising glutenin macropolymers and contributing to gluten viscoelasticity (Shewry and Halford 2002). Cross-linkage can also occur between tyrosine residues, but according to Hanft and Koehler (2005), dityrosine residues play only a minor role in the structure of wheat gluten, with less than 0.1% of tyrosine residues being cross-linked. The HMW-GS are known to be encoded at three loci (*Glu-A1*, *Glu-B1* and *Glu-D1* on chromosomes 1A, 1B and 1D, respectively). The alleles at these loci contain tightly linked genes that encode 'x-type' and 'y-type' subunits (Payne et al. 1981). X-type and y-type HMW-GS differ from each other with respect to their cysteine content and the motifs represented within their repetitive domains (Shewry 2003).

Allelic variation has been studied in great detail for HMW-GS, particularly for the *Glu-B1* locus, at which many alleles have been discovered (Payne and Lawrence 1983; Mclntosh et al. 2008, 2009). Among the subunits that are encoded at *Glu-B1*, two x-type subunits (designated Bx7 and Bx7*) differ only very slightly in their electrophoretic mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Marchylo et al. 1992). Similarly, two of the y-type subunits (By8 and By8*) are very similar to each other. Accordingly, the *Glu-B1* alleles that encode a Bx7 or Bx7* subunit and a By8 or By8* subunit are not readily distinguished from each other using SDS-PAGE. These alleles are designated *Glu-B1b* (7 + 8), *Glu-B1u* (7* + 8), *Glu-B1ak* (7* + 8*) and *Glu-B1al* (7 + 8*).

When *Glu-B1al* is present, the Bx7 subunit is overexpressed (7^{OE} + 8*) (Marchylo et al. 1992) as a percentage of the total amount of HMW glutenin present, and this overexpression is associated with greater dough strength (Butow et al. 2003; Vawser and Cornish 2004; Eagles et al. 2004). D'Ovidio et al. (1997) presented evidence that the *Glu-B1al* allele includes two copies of its x-type glutenin gene. Sequencing of the BAC clone TaBAC1215C06 (EU157184) later demonstrated that the *Glu-B1al* allele of Glenlea includes two copies of a 10.3 kb region that includes an x-type glutenin gene (Ragupathy et al. 2008). Molecular markers have been developed that detect insertions in the coding region (Butow et al. 2003) and matrix attachment region (MAR) (Radovanovic and Cloutier 2003) of the *Glu-B1al* x-type gene that encodes the overexpressed $Bx7^{OE}$ subunit. Ragupathy et al. (2008) designed markers that detect the right and left junctions of a long terminal repeat (LTR) retro-element that lies between the two x-type glutenin gene copies in the Glenlea allele and reported that these markers were diagnostic of the $Bx7^{OE}$ phenotype across a panel of diploid, tetraploid and hexaploid accessions of *Triticum* spp.

The Australian cultivar H45 (sometimes known as Galaxy, or Galaxy H45) has been classified as carrying the *Glu-B1al* allele. Nevertheless, it has been observed that H45 does not have the rheological properties that would be expected based on its apparent glutenin alleles. We hypothesised that H45 carries a functionally different allele at the *Glu-B1* locus. We investigated this hypothesis by cloning and sequencing Bx genes from H45, from Glenlea and from a control line (VQ0437) that carries *Glu-B1al* in combination with the same glutenin, puroindoline, serpin and storage protein activator alleles as H45.

Materials and methods

Plant materials

The main plant materials used were the Canadian wheat cultivar Glenlea (pedigree Pembina*2/Bage//CB100), the Australian cultivar H45 (pedigree B1814//WW15/QT7605), the Australian breeding line VQ0437 (pedigree BD200/CD87//Silverstar) and a random sample of 209 F_2 plants derived from a cross between H45 and VQ0437. For some parts of the work, the cultivars Chinese Spring, Gabo, Aroona, Wilgoyne, Janz, Frame, Currawong, Stiletto and/or Glover were used as controls, as they carry known alleles at genes that encode specific grain proteins. Seeds of the cultivars were obtained from the Australian Winter Cereals Collection (Tamworth, NSW, Australia). Seeds of VQ0437 were kindly provided by Dr. Russell Eastwood of Australian Grain Technologies Pty. Ltd. (Horsham, VIC, Australia).

Size-exclusion high-performance liquid chromatography (SE-HPLC)

Samples of approximately 30 grains harvested from each of 229 individual glasshouse-grown plants (10 H45 plants, 10 VQ0437 plants and 209 H45/VQ0437 F_2 plants) were milled using a FQC-200 (Metefem, Budapest, Hungary) micromill and sifted for 30 s using a 280 μm sieve. After 7 days, the protein was extracted from the sieved flour extracts according to the methods of Singh et al. (1990a, b) and the two-step method of Gupta et al. (1993) with minor modifications. For the first extraction, 1 ml of 0.5% SDS in 0.05 M phosphate extraction buffer (PEB, pH 6.9) was added to 25 mg (±1 mg) of sample in 1.5 ml microfuge tubes. Samples were vortexed for 30 s and subjected to a 10 min centrifugation (10,730×*g*) to pellet SDS-insoluble protein and flour residue. The supernatant (SDS-soluble fraction) was decanted into clean 1.5 ml microfuge tubes and set aside.

The remaining pellet was subsequently resuspended in 1 ml of PEB and subjected to a 30 s sonication using a sonifier (Branson model B-12 cell disrupter, Danbury, CT) fitted with a 3 mm diameter stepped microtip probe which generated ultrasonic vibrations with a frequency of 22 kHz. The samples were centrifuged (10,730×*g*) for 10 min to pellet the flour residue. The supernatant (SDS-insoluble fraction) was transferred to clean 1.5 ml microfuge tubes. Prior to SE-HPLC, both extracts were filtered into 1 ml glass HPLC vials (Waters Corp., Milford, MA) using a PVDF 0.45 µm Gelman Acrodisk LC13 Minispike (Gelman, Ann Arbor, MI) and placed in an 80°C waterbath for 2 min to inactivate proteases (Larroque and Bekes 2000).

SE-HPLC analysis was performed on a Waters (Milford, MA) Protein-Pak 300TM column (C18, 300 Å pore size, 3.5 µm particle size, 150 mm × 4.6 mm id) using a Waters 717plus Auto sampler, a Waters 600 system controller and a Waters 486 detector. Millennium32 (version 3.2) software was used for acquisition and reprocessing of data generated from the detector.

Separation of the SDS-soluble and SDS-insoluble fractions of polymeric protein was achieved in 50 and 40 min, respectively, by loading 10 µl of sample into an eluent of 50% (v/v) acetonitrile and distilled water containing 0.1% trifluoroacetic acid (TFA) (Batey et al. 1991) at a flow rate of 0.5 ml/min. Protein was detected by UV absorbance at 214 nm (Stone and Nicolas 1996). The percentage of SDS-unextractable polymeric protein (UPP, Gupta et al. 1993) was calculated.

SDS sedimentation test

Samples of grain were harvested from glasshouse-grown plants of Glenlea, H45 and VQ0437. A 50 g sample of grain from each line was milled and the flour was sifted as described above for the SE-HLPC separation. SDS sedimentation tests were conducted according to the procedure outlined by Carter et al. (1999) with minor modifications. Samples were placed in 15 ml centrifuge tubes, which were placed in racks with each rack having a measurement scale background. Two stock solutions were prepared as described by Carter et al. (1999). Distilled water (3 ml) was added to each sample. The samples were mixed for 20 s on a high-speed vortex mixer, allowed to hydrate for 5 min, mixed again on the high-speed vortex for 10 s, and then allowed to hydrate for another 5 min. Lactic acid/SDS solution (9 ml) was added to each sample and the tubes were agitated on a Zeleny type rocker (40 cycles/min) for 40 s, rested for 2 min, and agitated again for 40 s. The racks were left in an upright position for 10 min and the height (mm) of the sediment was recorded. Each SDS sedimentation test was performed twice to account for error in procedure.

SDS-PAGE analysis of glutenin and gliadin

SDS-PAGE analysis was conducted to identify individual glutenin and gliadin subunits. Individual grains of each of H45, VQ0437, Glenlea, Gabo, Chinese Spring, Janz, Aroona and Wilgoyne wheat were crushed with a hammer to produce powder samples. Each of these samples was transferred to a 1.5 ml microfuge tube. Extraction of the gliadin fraction was conducted by adding 0.4 ml of 15% (v/v) ethanol to the samples. The samples were incubated at 65°C in an Eppendorf Thermomixer Comfort 1.5 ml (Eppendorf AG, Germany) for 15 min and vortexed for 15 s. The incubation and vortexing steps were then repeated, and the sample residue was pelleted by centrifugation for 10 min (1,968×*g*, Eppendorf Centrifuge 5424, Eppendorf AG, Germany). The supernatant was decanted immediately and any excess liquid was removed from the pellet by keeping the tube inverted for 1 min. The pellet was reserved for subsequent extraction of glutenins. Extraction of gliadins was done by adding 0.4 ml of distilled water to the supernatant and incubating the sample at 4°C for 2 h. This was followed by a 10 min centrifugation (1,968×g) to pellet the gliadin. This pellet was dissolved in 0.1 ml of 1% SDS at 65°C for 60 min with regular vortexing at approximately 10 min intervals. Once the pellet was completely dissolved, 0.1 ml of sample buffer (0.02% bromophenol blue, 80 mM Tris–HCl (pH 8.0), 69 mM SDS) was added and the sample was vortexed for 10 s and incubated for 15 min in the Thermomixer at 65°C. These gliadin samples were frozen to be used later in electrophoresis.

The remaining gliadins present in the flour residue were removed by two subsequent wash steps using 1 ml of 50% (v/v) propan-1-ol. The glutenin fraction was extracted from pelleted flour residue using 0.1 ml of 50% (v/v) propan-1-ol: 80 mM Tris–HCl (pH 8.0) containing 1% (w/v) dithiothreitol (DTT) followed by 30 min incubation in the Thermomixer at 65°C. To prevent the reformation of disulfide bonds and improve band resolution, the protein subunits were alkylated for 15 min at 65°C with 0.1 ml of 50% propan-1-ol: 80 mM Tris–HCl (pH 8.0) containing 1.4% (v/v) 4-vinylpyridine (4-VP). This was followed by a 10 min centrifugation (1,968×*g*) to pellet the residue. The pellet was discarded and the supernatant was decanted into a clean microfuge tube, then 0.2 ml of sample buffer was added, the sample was vortexed for 10 s and incubated for 15 min in the Thermomixer at 65°C. The resulting glutenin fraction was stored at −20°C until electrophoresis was conducted.

Electrophoresis was conducted using a discontinuous polyacrylamide gel system (Singh et al. 1991) that had been modified (Cornish et al. 2001) to employ a 3% stacking gel and a 8–12% acrylamide gradient separating gel with 1.5% cross-linker concentration (bisacrylamide:acrylamide).

The electrophoresis apparatus included a Hoeffer SE600 vertical electrophoresis unit (San Francisco, CA), with 16 × 18 cm glass plates separated by a 1 mm spacer. Gels were loaded with 12 µl of sample and electrophoresis was carried out at approximately 10°C and 40 mA per gel for 3.5 h.

Quantification of HMW-GS expression

Quantification of glutenin subunits was performed using the Experion™ automated electrophoresis system using a Pro260 Analysis kit (Bio-Rad Laboratories, USA) following the method described by Wehr et al. (2008). 4 µl of each sample (or molecular-weight ladder) was mixed with 2 µl of Experion™ Pro260 sample buffer containing 0.03% β-mercaptoethanol, heated at 95°C for 5 min, and then diluted with 84 µl of deionized water and loaded onto a primed chip. In this system, proteins are detected and quantified based on laser-induced fluorescence of a dye that interacts with lithium dodecyl sulphate micelles that coat the proteins.

Molecular marker assays for puroindoline, serpin, storage protein activator and *Glu-B1*

Genomic DNA was isolated from leaf tissues of 3-week-old seedlings using a DNA midi-prep method outlined in Rogowsky et al. (1991) with modifications as described by Pallotta et al. (2000). Genomic DNA was quantified with a spectrophotometer (NanoDrop ND-1000, Thermo) and diluted to 100 ng/µl.

Sequence polymorphisms in the *Pina-D1* and *Pinb-D1* puroindoline genes were assayed according to the methods described by Cane et al. (2004) with minor modifications. Reactions were carried out in 12.5 µl volumes containing 12.5 pmol of each primer, 100 µM of each dNTP, 1× buffer, and 1.5 mM MgCl2 supplied by the manufacturer (Qiagen), 0.1 unit of Taq DNA polymerase (Qiagen), and 50 ng of genomic DNA. The amplification profile involved one cycle at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 58°C for *Pina-D1* or 60°C for *Pinb-D1* for 1.5 min and 72°C for 2 min, and a final extension step at 72°C for 10 min. The assay methods and allele classification for the *Srp5B* serpin locus were the same as those used by Cane et al. (2008). A single-nucleotide polymorphism that generates a premature stop codon in the *Spa-B* storage protein activator gene was assayed using the Pmut marker (Guillaumie et al. 2004). PCR reactions were conducted in 10 µl volumes containing 1 pmol of each primer, 100 µM of each dNTP, 1× buffer, and 1.5 mM MgCl2 supplied by the manufacturer (Qiagen), 0.2 units of Taq DNA polymerase (Qiagen), and 50 ng of genomic DNA. The amplification profile involved one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 64°C for 40 s and 72°C for 45 s, and a final extension step at 72°C for 5 min. Sequence polymorphisms at the *Glu-B1* locus were assayed using BxFp and MAR markers, both according to the methods described by Butow et al. (2004), and using the LTR left-junction and right-junction markers designed by Ragupathy et al. (2008).

All the primers were ordered from GeneWorks (Australia) and all PCR products were separated on 1% agarose/TAE gels.

Cloning and sequencing Bx genes from H45, VQ0437 and Glenlea

As the HMW-GS genes have no introns, genomic DNA isolated as described above was used as template for PCR amplification of the entire coding region. In order to amplify both Bx genes from each of three varieties, degenerate primers were designed based on the DNA sequence of TaBAC1215C06 (EU157184). The primers were

Bx7^{OE}copy1-F (5′-CGCGCTCAACTCTTCTAGTC TAA-3′) for the first gene,
Bx7^{OE}copy2-F (5′-CCACTCCAACTCTCCTTCC A-3′) for the second gene, and
Bx7^{OE}-R (5′-CACTCTCGTGCCGATCATTA-3′) for both genes.

The PCR amplification was carried out using QIAGEN LongRange PCR Kit (QIAGEN). The amplification profile was one cycle at 93°C for 3 min, followed by 35 cycles of 93°C for 15 s, 55°C for 30 s and 68°C for 5 min, and a final extension step at 72°C for 7 min. The amplified products were recovered from 1.0% agarose gels, cloned into the pGEM-T vector, and transformed into *E. coli* JM109 competent cells. Both amplification and cloning were repeated at least three times to minimise the possibility of errors present in amplification and sequencing. For amplification of fragments for sequencing, the following nested primers were designed based on the EU157184 sequence using Vector NTI software (Invitrogen):

P1 (5′-TGAACTCATTTGGGAAGTAAAC-3′),
P2 (5′-CATCCACACTTCTGCAAACAA-3′),
P3 (5′-ATGGCTAAGCGCCTGGTCCTCTTTG-3′),
P4 (5′-CGCAGCAACTCCAACAAATG-3′),
P5 (5′-CGCAGCAGTCGGAACAAG-3′) and
P6 (5′-CTATCACTGCCTGGTCGACAATGCG-3′).

Sequencing was performed commercially (Australian Genome Research Facility). Sequence analyses were performed using MEGA (Kumar et al. 2004), BLAST and ClustalW2 from the NCBI (http://www.ncbi.nlm.nih.gov/Tools/) and EBI (http://www.ebi.ac.uk/Tools/sequence.html).

Restriction assay to detect a DNA polymorphism

Based on the results of the SE-HPLC analysis, the 20 flour samples with the highest UPP and the 20 flour samples with the lowest UPP were selected from the 209 H45/VQ0437 flour samples that had been evaluated for UPP as described above. Each of these samples consisted of flour milled from F_3 grains harvested from an individual F_2 plant. The method described by Pallotta et al. (2003) was used to isolate genomic DNA from subsamples of these flour samples. For each of the resulting 40 DNA samples, primers $Bx7^{OE}$-copy1-F, $Bx7^{OE}$copy2-F and $Bx7^{OE}$-R were used to amplify a fragment containing the first Bx gene. Primers P3 and P6 were then used to amplify the entire coding region of the gene. The resulting PCR product was incubated with the restriction enzyme *RsaI* at 37°C for 2 h, and the digested products were separated on a 2.5% agarose gel.

Results

Comparison of the flour properties, protein composition and genotypes of H45 and VQ0437

With SE-HPLC analysis (Online Resource 1), the percentage UPP was significantly lower for H45 (30.12 ± 1.78) than for VQ0437 (44.29 ± 1.92) or for a control sample of bakers' flour (45.48 ± 1.39). In the SDS sedimentation test, the SDS sedimentation volume for H45 (19.3 ± 0.14 ml g^{-1}) was significantly lower than for VQ0437 (23.6 ± 0.28 ml g^{-1}) and Glenlea (24.5 ± 0.14 ml g^{-1}), even though all three flour samples had similar protein concentrations.

SDS-PAGE results for H45 and VQ0437 appeared identical to each other in all respects. Both of these lines carry the Ax1, Bx7 (or similar), By8*, Dx2, and Dy12 HMW-GS and the A3c, B3h and D3b LMW-GS (Fig. 1). No differences in gliadin profiles were detected (Fig. 2). The proportion of Bx subunits relative to total HMW-GS, calculated from the Experion™ automatic electropherogram trace (Fig. 3) was 0.543 for H45, 0.563 for VQ0437 and 0.567 for Glenlea, compared to only 0.412 for Chinese Spring, confirming overexpression of the Bx subunits in H45, VQ0437 and Glenlea.

None of the molecular marker assays conducted for polymorphisms in puroindoline, serpin and storage protein activator genes detected any differences between H45 and VQ0437. The genes *Pina-D1* and *Pinb-D1* (Giroux and Morris 1998), which are both located at the hardness (*Ha*) locus on chromosome 5D, encode puroindoline a and puroindoline b proteins. Their wild-type alleles have been designated *Pina-D1a* and *Pinb-D1a*. Two alleles with highly conserved mutations have been identified and named *Pina-D1b* and *Pinb-D1b* (Giroux and Morris 1998).

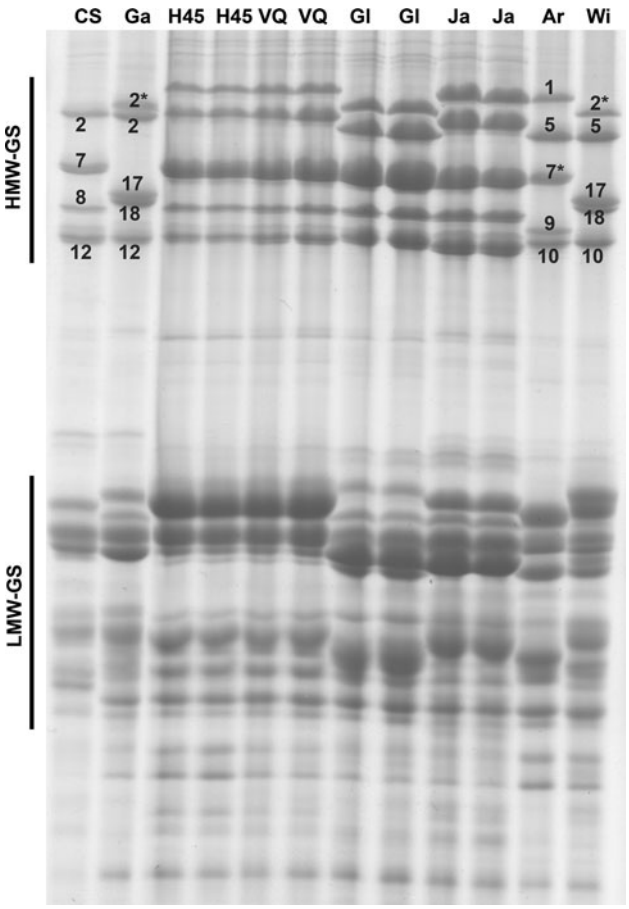

Fig. 1 One-dimensional SDS-PAGE of glutenins extracted from flour of Chinese Spring (CS), Gabo (Ga), H45, VQ0437 (VQ), Glenlea (Gl), Janz (Ja), Aroona (Ar) and Wilgoyne (Wi) wheat. HMW-GS and LMW-GS high- and low-molecular-weight glutenin subunits, respectively. *1* and *2** HMW-GS encoded by *Glu-A1a* and *Glu-A1b*, respectively. *7* and *8* HMW-GS encoded by *Glu-B1b*. *17* and *18* HMW-GS encoded by *Glu-B1i*. *7** and *9* HMW-GS encoded by *Glu-B1c*. *2* and *12* HMW-GS encoded by *Glu-D1a*. *5* and *10* HMW-GS encoded by *Glu-D1d*

With Currawong (*Pina-D1b/Pinb-D1a*) and Stiletto (*Pina-D1a/Pinb-D1b*) as standards, it was shown that the puroindoline genotype of both H45 and VQ0437 is *Pina-D1a/Pinb-D1b* (Fig. 4). At the *Srp5B* serpin locus, the genotype of H45 and VQ0437 is the same as the standard Glover: the type named *a* by Cane et al. (2008) (Fig. 5). For the *Spa-B* storage protein activator gene, a product of 311 bp (which is indicative of an allele with a premature stop codon) was amplified from Glenlea but no product was amplified from H45, VQ0437 or Chinese Spring (Fig. 5).

None of the molecular marker assays conducted for sequence polymorphisms at the *Glu-B1* locus detected any differences among H45, VQ0437 and Glenlea. Using a marker (BxFp) in the Bx coding region, PCR amplicons from both H45 and VQ0437 were the same size as those from Glenlea ($Bx7^{OE}$) and Chinese Spring (Bx7), both of which are

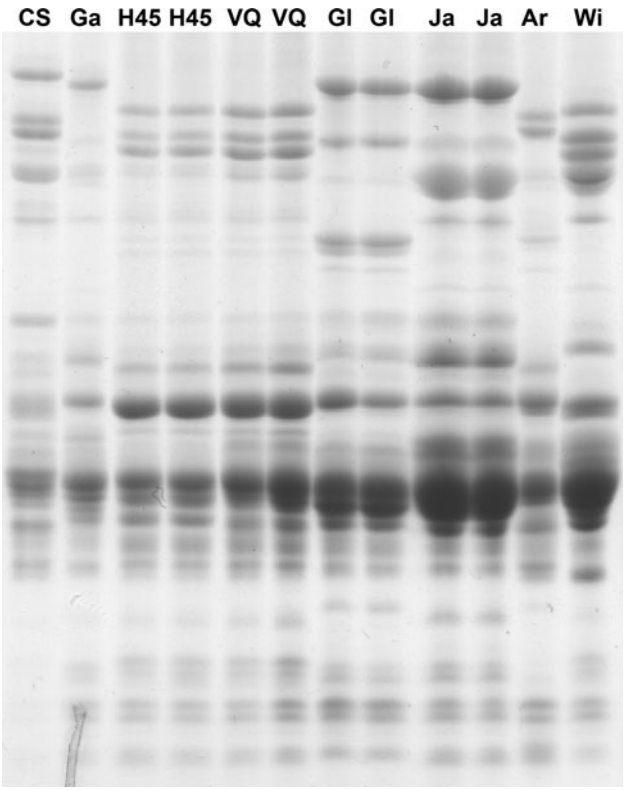

Fig. 2 One-dimensional SDS-PAGE of gliadins extracted from flour of Chinese Spring (CS), Gabo (Ga), H45, VQ0437 (VQ), Glenlea (Gl), Janz (Ja), Aroona (Ar) and Wilgoyne (Wi) wheat

known to contain an 18 bp insertion that is not present in Janz (Fig. 6). Similarly, PCR amplicons derived using a MAR primer pair showed that H45, VQ0437 and Glenlea all have a 43 bp insertion, which is not present in Chinese Spring or Janz (Fig. 6). With primers that Ragupathy et al. (2008) designed to amplify regions flanking a retrotransposon that lies between two copies of the $Bx7^{OE}$ gene in Glenlea, H45, VQ0437 and Glenlea ($Bx7^{OE}$) all exhibited 447 and 884 bp amplicons. These products were not amplified from Chinese Spring (Bx7) or Janz (Bx7*) (Fig. 7).

Sequences of Bx7 alleles

Two regions (4.3 and 3.9 kb in length), each including a Bx gene, were cloned from each of H45, VQ0437 and Glenlea and were fully sequenced. For Glenlea, the sequences obtained for the two gene copies were the same as each other and the same as those in GenBank (EU157184). Alignment of the H45 sequences (JF938070, JF938071) with those of Glenlea revealed four single nucleotide polymorphisms (SNPs) in the first gene and four SNPs in the second gene (Online Resource 2). All four SNPs in the first gene are non-synonymous (Fig. 8), with one altering a lysine into an arginine in the C-terminal region of the protein and the others altering amino acids in the repetitive domain: a tyrosine into a cysteine, a glutamine into an arginine, and a serine into a proline. Only one of the four SNPs in the second gene is non-synonymous; it alters a proline into a serine in the repetitive domain. Alignment of the VQ0437 sequences (JF938072 JF938073) with those of Glenlea revealed two SNPs in the first gene and one SNP in the second gene (Online Resource 2). Of these, one of the SNPs in the first gene is non-synonymous, altering a lysine into a threonine in the N-terminal region (Fig. 8).

According to the sequences obtained for the first gene, the SNP that alters a tyrosine (in Glenlea and VQ0437) into a cysteine (in H45) eliminates an *RsaI* restriction site. This restriction site polymorphism provided the basis for the design of a molecular marker assay to distinguish the H45 allele from each of the other two alleles. After amplification of a 2,391 bp product representing the coding region of the first gene, digestion with *RsaI* should result in 22 products for Glenlea and VQ0437, but only 21 for H45 (Table 1). The H45 digestion products should include a 216 bp fragment that is not present in the Glenlea or VQ0437, and should lack a 135 bp fragment and one of three 81 bp fragments that are present in Glenlea and VQ0437.

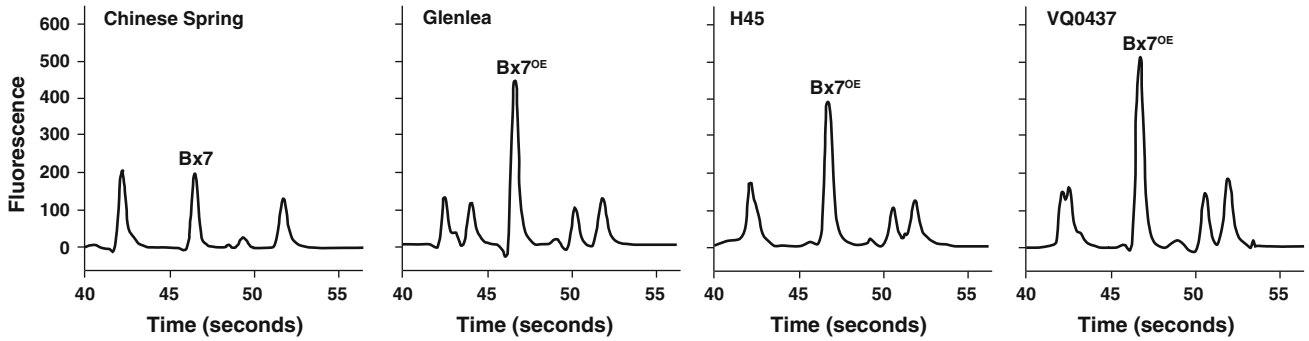

Fig. 3 Experion™ automatic electropherograms of high-molecular-weight glutenin subunits extracted from flour of Chinese Spring, Glenlea, H45 and VQ0437 wheat

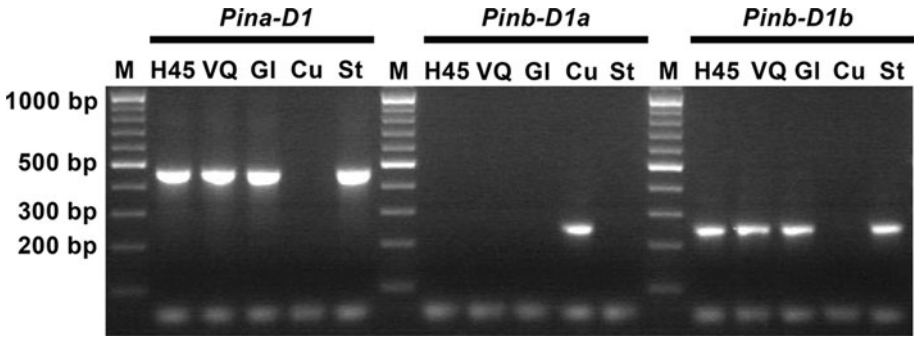

Fig. 4 PCR products amplified with primers for the puroindoline genes *Pina-D1* and *Pinb-D1* from genomic DNA of H45, VQ0437, Glenlea (Gl), Currawong (Cu, *Pina-D1b*/*Pinb-D1a*) and Stiletto (St, *Pina-D1*/*Pinb-D1b*) wheat. *M* 100 bp ladder

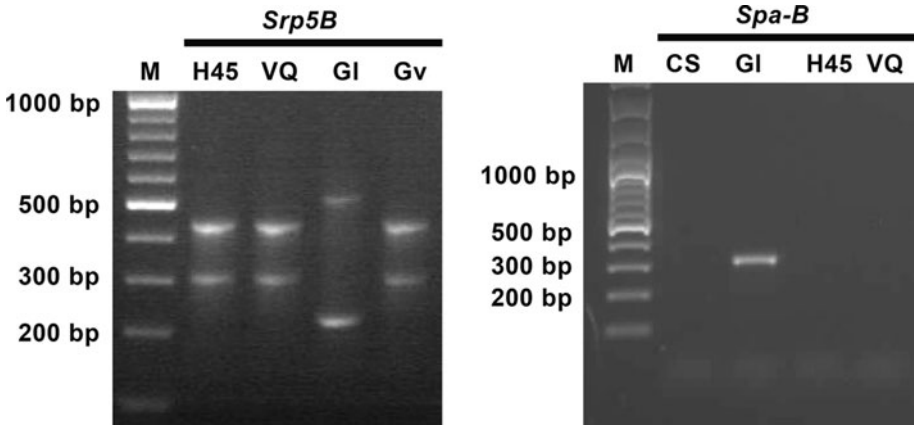

Fig. 5 PCR products amplified with primers for the serpin gene *Srp5B* from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl, *Srp5Bb*) and Glover (St, *Srp5Ba*) wheat and products amplified with primers for the storage protein activator gene *Spa-B* from Chinese Spring (CS), Glenlea (Gl), H45 and VQ0437 (VQ) wheat. *M* 100 bp ladder

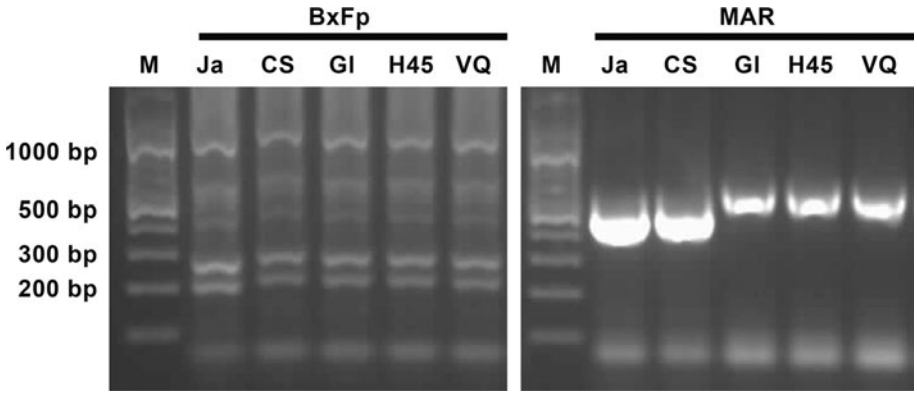

Fig. 6 PCR products amplified with BxFp and MAR primers for Bx glutenin genes from genomic DNA of Janz (Ja, Bx7*), Chinese Spring (Cs, Bx7), Glenlea (Gl, $Bx7^{OE}$), H45 and VQ0437 (VQ) wheat. *M* 100 bp ladder

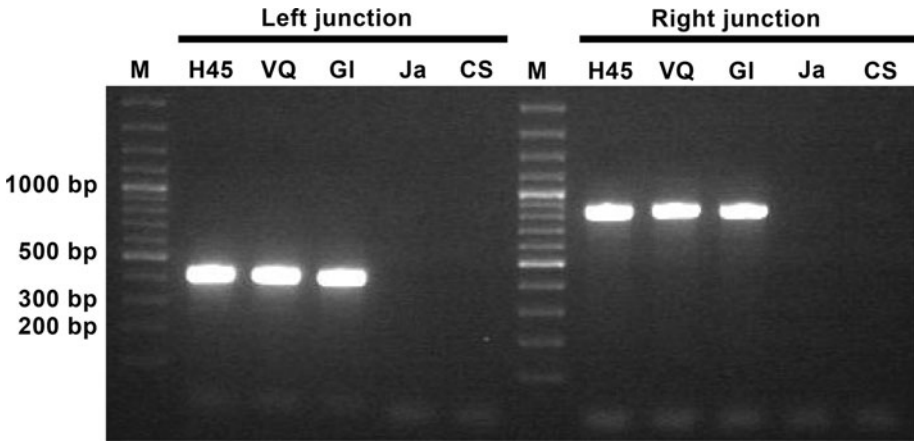

Fig. 7 PCR products amplified from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl, $Bx7^{OE}$), Janz (Ja, Bx7*) and Chinese Spring (CS, Bx7) wheat using primers designed at the left and right junctions of a retroelement between duplicate copies of the Bx gene in the *Glu-B1al* allele of Glenlea. *M* 100 bp ladder

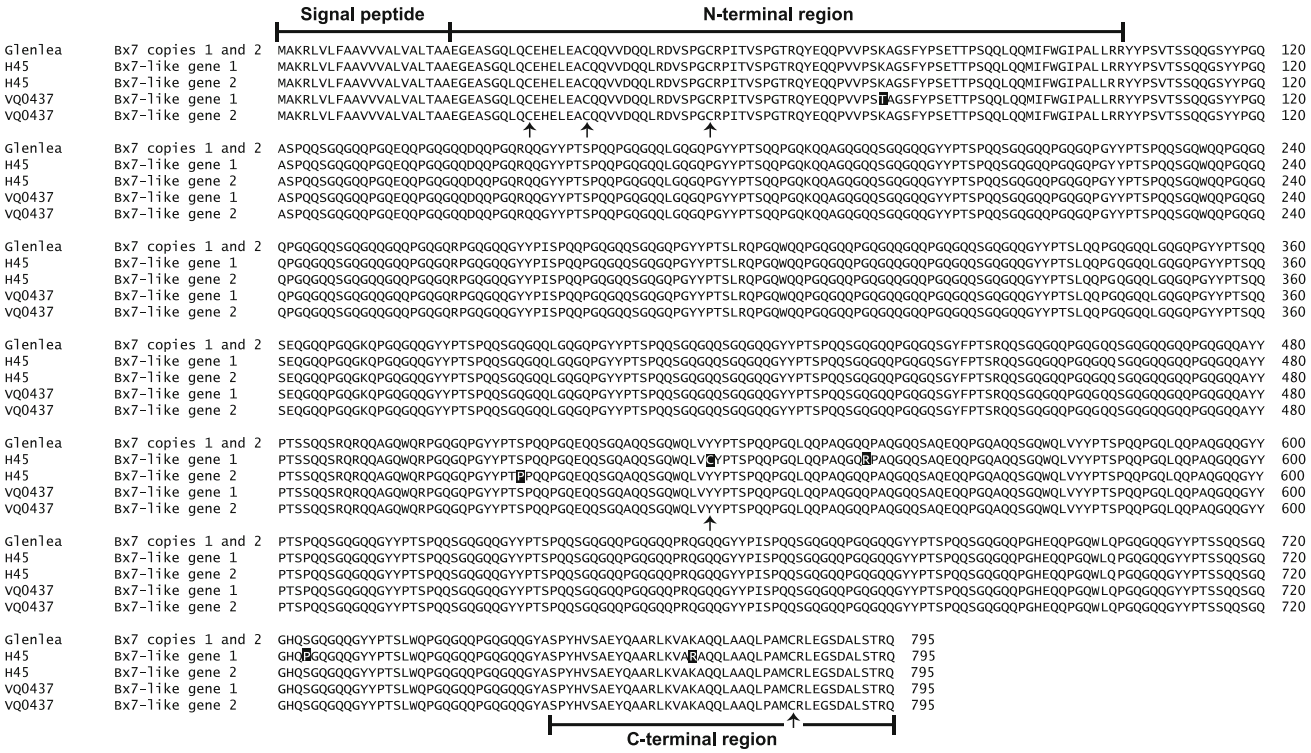

Fig. 8 Amino acid sequences translated from the nucleotide sequence of a duplicated gene at the *Glu-B1* locus that encodes the high-molecular-weight glutenin subunit $Bx7^{OE}$ in Glenlea wheat (EU157184), aligned with the amino acid sequences translated from each copy of the corresponding gene from the wheat variety H45 (JF938070, JF938071) and the wheat breeding line VQ0437 (JF938072 JF938073). The positions of cysteine residues are shown by *arrows*

Variation among H45/VQ0437 F_2 progeny

The UPP values of the flour samples milled from the F_3 grains harvested from individual H45/VQ0437 plants exhibited a continuous range of variation from slightly below the value for H45 to slightly above the value for VQ0437 (Fig. 9). With the *RsaI* digestion assay, the H45 allele could be distinguished from that of VQ0437, principally based on the presence of the additional 216 bp fragment (Fig. 10). All 20 of the $F_{2:3}$ families plants with the lowest UPP gave the same result as H45, while all 20 of the $F_{2:3}$ families plants with the highest UPP gave the same result as VQ0437 (Fig. 10).

Discussion

The results reported here demonstrate that the Australian wheat cultivar H45 has significantly lower SDS-unextractable polymeric protein and SDS sedimentation volume than Glenlea, which carries the Bx-overexpression allele *Glu-B1al*. Further, H45 was found to differ from the breeding line VQ0437 for these traits, even though H45 and VQ0437 seemed to carry exactly the same combination of glutenin alleles. These two lines also carry the same alleles for a storage protein activator gene that is closely linked with *Glu-B1* and has previously been proposed (Guillaumie et al. 2004) as a candidate gene for Bx overexpression, and for puroindoline and serpin genes that affect milling quality and that are routinely assayed in wheat breeding. They also had similar gliadin profiles as assessed by SDS-PAGE. The similarity of these two lines across all of these genes helped ensure that VQ0437 was an appropriate control line to be compared with H45 and to be used as a parent to generate a population in which the effects of *Glu-B1* would not be confounded by the effects of other known grain quality genes. With quantification of Bx subunits as a proportion of HMW-GS, we confirmed that both H45 and VQ0437 overexpress Bx subunits. Further, we found that they both carry the tandem segmental duplication that is thought to be diagnostic for the $Bx7^{OE}$ phenotype (Ragupathy et al. 2008). This led us to investigate the sequence of each of the Bx genes from H45, VQ0437 and Glenlea.

By isolating and sequencing both Bx genes from H45, VQ0437 and Glenlea, we discovered several non-synonymous SNPs. Of these, the one most likely to explain the properties of H45 causes a tyrosine-to-cysteine change in

Table 1 Restriction fragments expected for an assay in which the restriction enzyme *RsaI* is used to digest a 2,391 bp fragment amplified from the first of two x-type high-molecular-weight glutenin genes at the *Glu-B1* locus of Glenlea, H45 and VQ0437 wheat

Fragment length (bp)	Wheat lines and numbers of restriction fragments	
	Glenlea and VQ0437	H45
523	1	1
216		1
189	1	1
162	1	1
144	1	1
135	1	
108	1	1
107	1	1
93	1	1
81	3	2
78	2	2
63	7	7
45	2	2

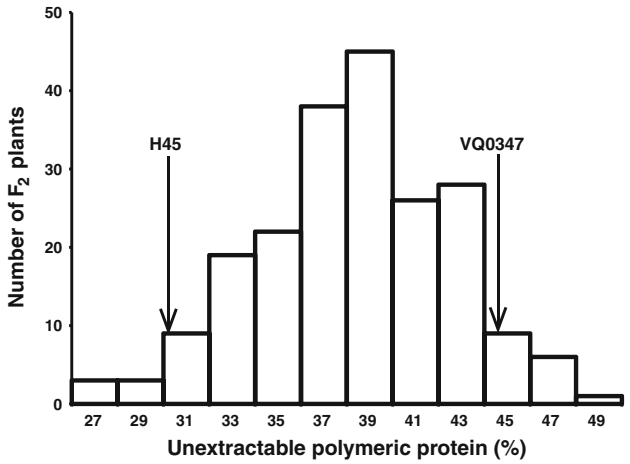

Fig. 9 Frequency distribution of percentage SDS-unextractable polymeric protein in flour samples of F_3 grains harvested from F_2 plants from a cross between H45 and VQ0437

the HMW-GS encoded by the first Bx gene. The results from assaying this SNP on DNA extracted from flour milled from F_3 grains harvested from high-UPP and low-UPP F_2 plants demonstrated that the H45 *Glu-B1* allele is strongly associated with low UPP. Given the continuous range of variation observed for UPP (Fig. 9), it seems that *Glu-B1* is not the sole factor affecting this trait. Although there may be genetic factors involved, much of the variation is probably due to error variation among measurements taken on flour samples from individual F_2 plants.

It seems likely that the tyrosine-to-cysteine mutation is the causal polymorphism underlying the association of *Glu-B1* with UPP. Given the minor role that dityrosine residues play in cross-linking within or between glutenin subunits (Hanft and Koehler 2005), the loss of a tyrosine would be unlikely to have a major effect on glutenin polymerisation. The gain of a cysteine would be more significant. Most x-type HMW-GS contain four cysteines: three in the N-terminal domain and one in the C-terminal domain. Normally, two of the cysteines in the N-terminal domain are connected by an intrachain disulphide bond, while the other two cysteines contribute to glutenin polymerisation via interchain disulphide bonds with cysteines in other HMW- or LMW-GS (Keck et al. 1995; Wieser 2007). In the subunit encoded by the first Bx gene of H45, there is an extra cysteine, located in the repetitive domain of the protein. Differences in the number and location of cysteine residues in HMW-GS are known to affect the pattern of disulphide bond formation, and hence the composition and functionality of glutenin polymers (Tamás et al. 2002; Pirozi et al. 2008). Depending on the number and positions of cysteine residues available to form disulphide bonds, glutenin subunits may act as chain extenders or chain terminators (Tamás et al. 2002), affecting polymer size and thus dough strength and extensibility.

If intrachain links form more rapidly than interchain links (as has been proposed by Kasarda (1999)), the extra cysteine in the Bx subunit encoded by the first Bx gene of H45 might form intrachain disulphide bonds, leading to a conformation that could impede interchain links. In that

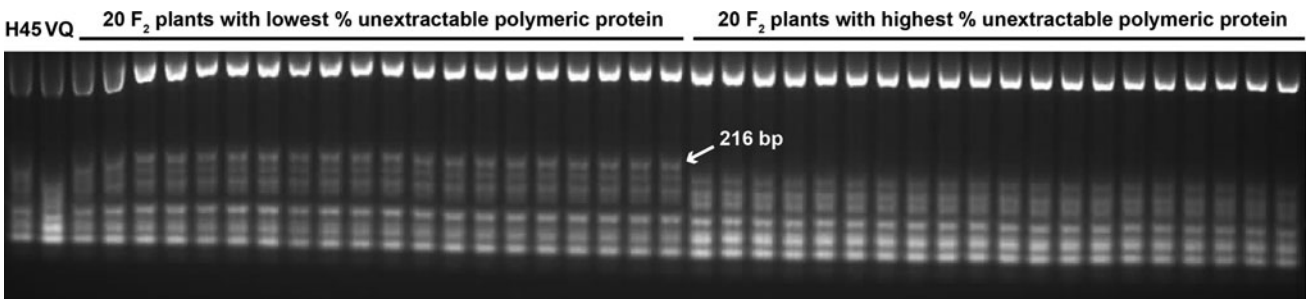

Fig. 10 Restriction fragments from *RsaI* digestion of PCR products of the first of two x-type high-molecular weight glutenin genes at the *Glu-B1* locus of H45, VQ0437 (VQ) and sets of 20 H45/VQ0437 F_2 plants with low and high SDS-unextractable polymeric protein

case, the subunit with the extra cysteine might act as a chain terminator, preventing other HMW-GS from bonding to the gluten matrix and counteracting the favourable effects of Bx overexpression. This would be similar to effects observed by Tamás et al. (2002), who found that chain-terminator proteins could inhibit the effects of incorporating supplemental Bx7 protein into dough. This provides a plausible explanation for the low values of UPP and SDS-sedimentation volume that we observed for H45. Another possible explanation is that the 'extra' cysteine participates in interchain bonds, and that this affects polymerisation in some other way.

We propose to designate the *Glu-B1* alleles of H45 and VQ0437 as *Glu-B1br* and *Glu-B1bs*, respectively. The discovery of these alleles demonstrates that *Glu-B1al* is not the only *Glu-B1* allele with a duplication of an x-type glutenin gene. *Glu-B1al*, *Glu-B1br* and *Glu-B1bs* all confer overexpression of subunits with the same size and electrophoretic mobility as Bx7, but *Glu-B1br* seems to differ from the others in its effect on glutenin polymerisation. Given the sequence similarity between the coding regions of the three alleles, the fact that the same duplication junctions are detected in all three alleles and that those duplication junctions have been detected in diploid, tetraploid and hexaploid species of *Triticum* (Ragupathy et al. 2008), it seems likely that these alleles originated from a common ancestor. The origin of *Glu-B1al* has previously been traced back to the Argentinian wheat cultivar Klein Universal II (Butow et al. 2004; Vawser and Cornish 2004). VQ0437 probably inherited *Glu-B1bs* from the cultivar CD87 (which has previously been classified as carrying *Glu-B1al*). The origin of *Glu-B1br* is not known. We have not been able to detect the gene duplication in any of the known ancestors of H45.

With the *RsaI* digestion assay, *Glu-B1br* can be distinguished from *Glu-B1al* and *Glu-B1br* based on the presence or absence of a restriction site caused by the SNP that generates the tyrosine-cysteine amino acid substitution. As this assay requires long-range PCR and a restriction enzyme, it is not very convenient for large-scale screening of germplasm or for routine use in breeding. Nevertheless, it could be useful in situations where it is important to distinguish *Glu-B1br* from other similar alleles.

The discovery of *Glu-B1br* is of practical significance for wheat breeding, especially in Australia, where H45 has been used as a parent and has been particularly important in hybrid wheat breeding. The presence of a cysteine in the repetitive region of first Bx gene of *Glu-B1br* could explain the weaker dough of H45 and some of its progeny relative to other lines that carry two Bx genes at the *Glu-B1* locus and that overexpress Bx subunits. Knowledge of which parents carry *Glu-B1br* and which carry *Glu-B1al* or *Glu-B1bs* could improve the predictability of cross outcomes in wheat breeding.

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