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ORIGINAL PAPER

Characterization of low-molecular-weight glutenin subunit *Glu-B3* genes and development of STS markers in common wheat (*Triticum aestivum* L.)

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Abstract Low-molecular-weight glutenin subunit (LMW-GS) *Glu-B3* has a significant influence on the processing quality of the end-use products of common wheat. To characterize the LMW-GS genes at the *Glu-B3* locus, gene-specific PCR primers were designed to amplify eight near-isogenic lines and Cheyenne with different *Glu-B3* alleles (*a*, *b*, *c*, *d*, *e*, *f*, *g*, *h* and *i*) defined by protein electrophoretic mobility. The complete coding regions of four *Glu-B3* genes with complete coding sequence were obtained and designated as *GluB3-1*, *GluB3-2*, *GluB3-3* and *GluB3-4*. Ten allele-specific PCR markers designed from the SNPs present in the sequenced variants discriminated the *Glu-B3* proteins of electrophoretic mobility alleles *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h* and *i*. These markers were validated on 161 wheat varieties and advanced lines with different *Glu-B3* alleles, thus confirming that the markers can be used in marker-assisted breeding for wheat grain processing quality.

L. H. Wang and X. L. Zhao contributed equally to this study.

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Introduction

Gluten, the most important storage protein in the endosperm of common wheat (*Triticum aestivum* L.), comprises glutenins and gliadins (Lindsay and Skerritt 1999; Shewry and Halford 2002). Glutenins are separated into high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) according to their mobilities in sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Bietz et al. 1975; Payne and Corfield 1979). These proteins are held together

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by disulphide bonds to form gluten macropolymers (Gras et al. 2001), contribute to fundamental aspects of dough quality such as viscoelasticity and extensibility, and consequently influence the end-use products of wheat (Payne 1987; Luo et al. 2001). The identification of specific HMW-GS and LMW-GS alleles is, therefore, an important target in improving wheat quality (Gupta et al. 1999; Eagles et al. 2001; Gale 2005).

Allelic variation of the HMW-GS and its relationship with processing quality have been studied extensively, and PCR-based DNA markers are available to discriminate the important *Glu-1* alleles *Ax2**, *Bx7*, *Bx7**, *Bx17*, *By8*, *By9* and Dx5 (Ma et al. 2003; Butow et al. 2004; Gale 2005; Lei et al. 2006). Compared with HMW-GS, the extensive allelic variations of LMW-GS and their overlapping mobilities with the more abundant gliadin proteins (Singh and Shepherd 1988) make it difficult to discriminate the roles of individual LMW-GS in wheat quality. Gupta and Shepherd (1990) carried out an extensive survey of LMW glutenin proteins in common wheat cultivars by SDS-PAGE and detected 20 banding patterns. Subsequently, six protein alleles were found for the *Glu-A3* locus (*a*, *b*, *c*, *d*, *e*, *f*), nine for the *Glu-B3* (*a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*) and five for the *Glu-D3* (*a*, *b*, *c*, *d*, *e*). With respect to effects on dough quality, various *Glu-3* alleles were ranked for R_{max} (maximum dough resistance, an indicator of dough strength), and the rankings of alleles were $b > d > e > c$ at the *Glu-A3* locus, $i > b = a > e = f = g = h > c$ at the *Glu-B3* and $e > b > a > c > d$ at the *Glu-D3* (Gupta et al. 1989, 1991; Metakovsky et al. 1990). For dry white Chinese noodle (DWCN) quality, *Glu-A3d* and *Glu-B3d* were considered slightly better than others (He et al. 2005). Cornish et al. (1993) found that the composition bbb for *Glu-A3*, *Glu-B3* and *Glu-D3*, respectively, gave the best extensibility, and the composition bbc was almost as extensible.

Traditionally, SDS-PAGE (Jackson et al. 1996) or RP-HPLC (Margiotta et al. 1993) is used to determine allelic compositions of LMW-GS in wheat. However, difficulties in resolving the multigene families and the overlapping fractions of LMW-GS hinder their routine use, particularly for testing large populations in the early generations of wheat breeding programs. Therefore, it is important to develop functional markers to identify different LMW-GS genes (Andersen and Lübberstedt 2003). Long et al. (2005) classified 69 LMW-GS genes registered in GenBank into nine groups and established nine group-specific primer sets to discriminate the nine groups. Ikeda et al. (2006) constructed 12 group-specific markers according to the 12 groups of LMW-GS genes detected in cultivar Norin 61. Based on the allelic variation of LMW-GS gene at the *Glu-A3* locus, Zhang et al. (2004) developed several PCR markers to distinguish alleles *a*, *b*, *c*, *d*, *e*, *f* and *g*. Zhao et al. (2007a, b) designed several gene-specific markers for discriminating haplotypes of *Glu-D3* genes. Three markers were developed for different *Glu-B3* haplotypes at the DNA level (Zhao et al. 2007b). However, these markers do not discriminate the *Glu-B3* alleles *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h* and *i*, and the association between *Glu-B3* genes and *Glu-B3* protein alleles remained unclear. Here, we report the isolation of LMW-GS genes at the *Glu-B3* locus from common wheat, characterization of the relationship between the genetic haplotypes and *Glu-B3* alleles defined by protein mobility and development of allele-specific STS markers for different *Glu-B3* alleles. This will benefit marker-assisted breeding for wheat quality.

Materials and methods

Plant materials

Aroona (*Glu-B3b*) and its seven near-isogenic lines (NIL), Aroona-B3a (*Glu-B3a*), Aroona-B3c (*Glu-B3c*), Aroona-B3d (*Glu-B3d*), Aroona-B3f (*Glu-B3f*), Aroona-B3g (*Glu-B3g*), Aroona-B3h (*Glu-B3h*) and Aroona-B3i (*Glu-B3i*), and Cheyenne (*Glu-B3e*), with different *Glu-B3* alleles defined by protein mobility, were used to isolate *Glu-B3* genes and develop molecular markers (Table 1). Certain homologous Group 1 Chinese Spring nulli-tetrasomic lines and five Chinese varieties with the 1BL.1RS translocation were used to confirm the chromosomal locations of identified genes. Twenty varieties from our laboratory collection and 141 wheat varieties and advanced lines from the International Maize and Wheat Improvement Centre (CIMMYT) with different *Glu-B3* protein alleles detected by SDS-PAGE were employed to validate the allele-specific markers.

DNA extraction and PCR amplification

Genomic DNA was extracted from seedlings or seeds using a modified CTAB procedure (Gale et al. 2001). PCR was performed using *TakaRa Taq* DNA polymerase (1.0 unit) in 20 μl reaction volumes containing approximately 50 ng of genomic DNA, 1× PCR buffer (1.5 mM $MgCl_2$), 100 μM of each dNTPs and 10 pmoles of each PCR primer. PCR cycling conditions for gene-specific primers were 94°C for 5 min followed by 38 cycles at 94°C for 45 s, 56–61°C for 45 s, 72°C for 90 s and a final extension at 72°C for 8 min. PCR conditions for allele-specific markers are shown in Table 2.

Development of locus-specific primers for isolation of *Glu-B3* genes

Locus-specific primers for cloning *Glu-B3* genes were developed from the descriptions of Zhang et al. (2003,

Table 1 Primers used for amplifying *Glu-B3* genes

Target gene	Primer	Sequence (5′ → 3′)	Reference gene	Primer location[a]	Annealing temperature (°C)
GluB3-1	LB1F	GCACAAGCATCAAAACCAAGA	AB262661	−13[b]	58
	LB1R	GACACTTTATTTGTCACCGCTG	AB262661	1,129	
GluB3-2	LB2F	AACCTAACGCATTGTACCAAAAATC	AY542898	208	61
	LB2R	GGCGGGTCACACATGACA	AY542898	1,499	
GluB3-3	LB3F	CATCACAAGCACAAGCATCAA	Y14104	472	58
	LB3R	CATATCCATCGACTAAACAAA	AB119006	709	
GluB3-4	LB4F	CACCCTATACAAGGTTCCAAAAT	Y14104	77	60
	LB4R	TATTTCCATAATTTAAACTAGTTTGT	AB062852	+1,346[b]	

[a] The location was counted from the first nucleotide of the reference gene fragments

[b] Location in the new sequence after in silico cloning

Table 2 Allele-specific PCR markers for the discrimination of *Glu-B3* alleles defined by protein mobility in common wheat

Marker name	Primer set	Sequence (5′ → 3′)[a]	Target allele	Fragment size	PCR conditions
gluB3a	SB1F	CACAAGCATCAAAACCAAGA	a	1,095	94°C/35 s–55°C/35 s–72°C/90 s
	SB1R	TGGCACACTAGTGGTGGTC			
gluB3b	SB2F	ATCAGGTGTAAAAGTGATAG	b	1,570	94°C/35 s–56°C/35 s–72°C/90 s
	SB2R	TGCTACATCGACATATCCA			
gluB3c	SB3F	CAAATGTTGCAGCAGAGA	c	472	94°C/35 s–56°C/35 s–72°C/90 s
	SB3R	CATATCCATCGACTAAACAAA			
gluB3d	SB4F	CACCATGAAGACCTTCCTCA	d	662	94°C/35 s–58°C/35 s–72°C/90 s
	SB4R	GTTGTTGCAGTAGAACTGGA			
gluB3e	SB5F	GACCTTCCTCATCTTCGCA	e	669	94°C/35 s–58°C/50 s–72°C/90 s
	SB5R	GCAAGACTTTGTGGCATT			
gluB3fg	SB6F	TATAGCTAGTGCAACCTACCAT	fg[b]	812	94°C/35 s–62°C/35 s–72°C/90 s
	SB6R	CAACTACTCTGCCACAACG			
gluB3g	SB7F	CCAAGAAATACTAGTTAACACTAGTC	g	853	94°C/35 s–60°C/35 s–72°C/90 s
	SB7R	GTTGGGGTTGGGAAACA			
gluB3h	SB8F	CCACCACAACAAACATTAA	h	1,022	94°C/35 s–60°C/35 s–72°C/90 s
	SB8R	GTGGTGGTTCTATACAACGA			
gluB3i	SB9F	As SB6F	i	621	94°C/35 s–58°C/35 s–72°C/90 s
	SB9R	TGGTTGTTGCGGTATAATTT			
gluB3bef	SB10F	GCATCAACAACAAATAGTACTAGAA	bef[c]	750	94°C/35 s–60°C/35 s–72°C/90 s
	SB10R	GGCGGGTCACACATGACA			

[a] Mismatched nucleotides are underlined

[b] Specific for *Glu-B3f* and *g* alleles

[c] Specific for *Glu-B3b*, *e* and *f* alleles

2004) and Zhao et al. (2006). Eight reference genes with complete coding regions, including seven LMW-GS genes (GenBank accessions AB119006, AB164415, AB164416, AB262661, Y14104, AB062852 and AJ007746) located on the short arm of chromosome 1B, and one (AY542898) with high similarity to *Glu-B3* available in GenBank, were used for primer development (http://www.ncbi.nlm.nih.gov). The genes AB062852, AB164416 and AB262661 were selected as probes for the in silico cloning of *Glu-B3* genes according to He et al. (2007).

Based on the alignment of the reference genes, 63 primers were designed and 378 primer combinations were tested with the NILs and Cheyenne with different *Glu-B3* alleles. Primer screening was conducted as described by Zhao et al. (2006). Finally, four *Glu-B3* genes were isolated by four pairs of primers with annealing temperatures ranging from 56 to 61°C (depending on primer set) (Fig. 1). Primer sequences (5′–3′) and their relative positions in reference genes are shown in Table 1.

Sequencing of PCR products

PCR fragments with expected sizes were recovered from agarose gels and cloned into the pGEM-T Easy Vector. Recombinant clones with expected sizes were sequenced after a PCR test. To eliminate cloning pitfalls (Masci et al. 1998), the recovered fragments were also sequenced directly using the corresponding PCR primers. Each PCR and sequence procedure was repeated three to six times to avoid any technical errors. All the sequencings were performed by the Sangon Biotechnology (Shanghai, China). Sequence analysis and characterization were performed using software DNAMAN (http://www.lynnon.com).

Allele-specific PCR marker design and validation

Allele-specific PCR markers were designed based on the allelic variants of *Glu-B3* following the method of Zhang et al. (2003). These markers were firstly validated with the eight Aroona NILs and Cheyenne, and then with 161 wheat varieties and advanced lines from CIMMYT, Australia and France with *Glu-B3* protein mobility alleles previously identified in SDS-PAGE by other workers.

Results

Allelic variants at the *Glu-B3* locus

Four *Glu-B3* genes, designated *GluB3-1*, *GluB3-2*, *GluB3-3* and *GluB3-4*, including 17 allelic variants at the DNA level were identified at the *Glu-B3* locus in the eight NILs and Cheyenne.

The *GluB3-1* gene had five haplotypes or allelic variants, designated as *GluB3-11*, *GluB3-12*, *GluB3-13*, *GluB3-14* and *GluB3-15* (GenBank accessions EU369699, EU369700, EU369701, EU369702 and EU369703), amplified with the primer set *LB1F/LB1R* from Aroona-B3a (*Glu-B3a*), Aroona (*Glu-B3b*), Cheyenne (*Glu-B3e*), Aroona-B3f (*Glu-B3f*) and Aroona-B3g (*Glu-B3g*), respectively. Compared with *GluB3-12*, four, one and two triplet-nucleotide (CAA) deletions at positions 319–330, 328–330 and 325–330 in the coding region were found in *GluB3-11*, *GluB3-13* and *GluB3-14* (Appendix Fig. A1 in the Electronic Supplementary Material), leading to four, one and two glutamine deletions in the glutamine-rich repetitive domains of the deduced peptides BP1-1, BP1-3 and BP1-4, respectively (Fig. A2). At position 292–354, *GluB3-15* had a 63-bp deletion, resulting in a 21-amino acid deletion in the deduced peptide *BP1-5*. *GluB3-11* and *GluB3-15* showed an additional 3-bp (CAA) deletion at position 556–558, leading to a glutamine deletion. In addition, *GluB3-11* had two SNPs, one with A–G transition at position 1,092 and the other at position 1,113 with a C–T transition; *GluB3-13* had a SNP at the position 69 with a C–A substitution; *GluB3-15* showed four SNPs in the coding region at positions 360, 363, 918 and 1,056, respectively; the 360th SNP of *GluB3-15* resulted in a change of phenylalanine to leucine at position 105 in the N-terminal domain of the deduced peptides, whereas all the others represented synonymous changes.

The *GluB3-2* gene, amplified with primer set *LB2F/LB2R*, had three allelic variants at the DNA level, designated as *GluB3-21*, *GluB3-22* and *GluB3-23* (GenBank accessions EU369704, EU369721 and EU369705), respectively. *GluB3-21* was detected in Aroona-B3a, *GluB3-22* in genotypes with protein mobility alleles *b*, *e* and *f* and *GluB3-23* was present in Aroona-B3g. Compared with *GluB3-21*, both *GluB3-22* and *GluB3-23* had a triplet nucleotide deletion (CAA) at position 378–380 in the coding region (Fig. A3), leading to a glutamine deletion in the repetitive domain of the deduced peptides BP2-2 and BP2-3, respectively (Fig. A4). In addition, *GluB3-22* contained two SNPs at positions 671 and 1,246, and the latter resulted in an amino acid change from serine to asparagine in the C-terminal conserved region.

GluB3-3 had four allelic variants, designated as *GluB3-31*, *GluB3-32*, *GluB3-33* and *GluB3-34* (GenBank accessions EU369715, EU369716, EU369717 and EU369718, respectively), amplified with the primer set *LB3F/LB3R* from Aroona-B3c, Aroona-B3d, Aroona-B3h and Aroona-B3i, respectively. Compared with *GluB3-31*, four SNPs were detected at positions 197, 505, 836 and 1,030 in the coding region of both *GluB3-32* and *GluB3-34* (Fig. A5). *GluB3-34* and *GluB3-32* contained an additional SNP at positions 662 and 692, respectively. *GluB3-33* had SNPs at positions 186, 505, 836 and 1,180 and a double-base substitution at positions 1,030 and 1,031. In addition, *GluB3-34* showed a 3-bp (CAA) deletion at position 603–605 and a triplet code (CAA) insertion between positions 651 and 652, leading to a glutamine deletion and insertion in the deduced peptide BP3-4, respectively (Fig. A6).

GluB3-4 gene was amplified with the primer set *LB4F/LB4R* in all the eight NILs and Cheyenne, and had five allelic variants at the DNA level, designated as *GluB3-41*, *GluB3-42*, *GluB3-43*, *GluB3-44* and *GluB3-45* (GenBank accessions EU369724, EU369719, EU369727, EU369729 and EU369720, respectively). *GluB3-41* was detected in NILs with protein mobility alleles *a*, *c* and *d*, *GluB3-42* in Aroona-B3b, *GluB3-43* in Cheyenne and Aroona-B3h, *GluB3-44* in Aroona-B3f and Aroona-B3g and *GluB3-45* in Aroona-B3i. Compared with *GluB3-41*, five, four and two SNPs were present in the upstream noncoding region of *GluB3-42*, *GluB3-43* and *GluB3-45*, respectively (Fig. A7). In the coding region, a SNP with a C–T base

transition was found at position 669 in *GluB3-45* and at position 714 in *GluB3-42* and *GluB3-43*, respectively, leading to an amino acid substitution from serine to leucine in the deduced proteins of BP4-5, BP4-2 and BP4-3 (Fig. A8). *GluB3-42*, *GluB3-43*, *GluB3-44* and *GluB3-45* had a common SNP (A–G) at position 731, leading to a change from isoleucine to valine in the deduced glutamine-rich repetitive domain. At the position 770–790, a 21-bp deletion was present in *GluB3-45*, resulting in a 7-amino acid (QFPQQQQ) deletion at the protein level. In addition, *GluB3-45* had SNPs at positions 863, 885, 890 and 894, and *GluB3-42* and *GluB3-43* had a SNP at position 894, respectively. At positions 1,056 and 1,135, two SNPs were detected in *GluB3-44*, and the former led to an amino acid mutation from methionine to threonine, whereas the latter was a synonymous mutation in the deduced C-terminal cysteine-rich domain.

Characterization of the *GluB3-1*, *GluB3-2*, *GluB3-3* and *GluB3-4* genes and their deduced amino acid sequences

The 17 identified allelic variants of the four *Glu-B3* genes contain a complete coding sequence, including start codon, termination sequence with double-stop codons TAATAA in *GluB3-2* and *GluB3-3* or TGATAA in *GluB3-1* and *GluB3-4*. *GluB3-1*, *GluB3-2* and *GluB3-4* had the AATAAA polyadenylation signals in the 3′ flanking region. *GluB3-4* was longer than the other three genes at the 5′ flanking region, comprising also the endosperm boxes, CAAT box and double TATA box. Sequence alignments indicated that the homologies of the DNA sequences were 87.4–94.4% among the four gene sequences, and 99.3–99.9% among different allelic variants within each of the four gene sequences (data not shown). At the protein level, the homologies of deduced amino acid sequences of the four *Glu-B3* genes (designated BP1, BP2, BP3 and BP4) were 82.4–93.0% among the four genes and 98.3–100.0% (BP4-2 was the same as BP4-3) among different allelic variants within each of the four genes (data not shown).

All the deduced amino acid sequences of the four genes contained a single open reading frame (ORF) encoding a highly conserved signal peptide of 20 amino acids and a short N-terminal conserved region with 13 amino acids, followed by a repetitive domain rich in glutamine and a C-terminal conserved domain. The C domains had three subregions typical of LMW glutenin subunit proteins (Cassidy et al. 1998). The deduced peptides BP1, BP2 and BP3, encoded by *GluB3-1*, *GluB3-2* and *GluB3-3*, respectively, were characterized with the amino acids MENSHIP in the N-terminal domain, and their deduced molecular weight ranged from ~39.0 kDa (BP1-5) to ~44.6 kDa (BP3-1). BP4, corresponding to *GluB3-4*, started with METSHIP in the N-terminal domain with molecular weights of ~39.0 kDa (BP4-5) or ~39.8 kDa (BP4-1, BP4-2, BP4-3 and BP4-4). The deduced amino acid sequences also showed typical eight-cysteine (Cys) residues, and all can be classified into type II based on the distribution of the cysteine residues (D'Ovidio and Masci 2004).

Relationship between *Glu-B3* gene haplotypes and *Glu-B3* protein mobility alleles

Each of the *Glu-B3* protein mobility alleles contained different haplotypes at the DNA level (Table 3). Aroona-B3a possesses the haplotypes *GluB3-11*, *GluB3-21* and *GluB3-41*; Aroona (*Glu-B3b*) has allelic variants *GluB3-12*, *GluB3-22* and *GluB3-42*; Aroona-B3c contains *GluB3-31* and *GluB3-41*; Aroona-B3d has *GluB3-32* and *GluB3-41*; Cheyenne (*Glu-B3e*) contains *GluB3-13*, *GluB3-22* and *GluB3-43*; Aroona-B3f possesses *GluB3-14*, *GluB3-22* and *GluB3-44*; Aroona-B3g contains *GluB3-15*, *GluB3-23* and *GluB3-44*; Aroona-B3h has *GluB3-33* and *GluB3-43*; Aroona-B3i contains *GluB3-34* and *GluB3-45*.

Gene-specific PCR markers for *Glu-B3* alleles defined by protein mobility

Based on the sequence alignment of allelic variants among each of the *Glu-B3* genes, ten primer sets were developed to amplify different *Glu-B3* alleles based on the detected SNPs (Table 2). In the eight NILs and Cheyenne, primer set *SB1F/SB1R* amplified only a 1,095-bp PCR fragment in Aroona-B3a with the *Glu-B3a* allele (Fig. 2a). Primer set *SB2F/SB2R* was designed for the *Glu-B3b* allele in Aroona with a 1,549-bp PCR fragment (Fig. 2b). For the *Glu-B3c* allele in Aroona-B3c, *SB3F/SB3R* generated a unique 472-bp PCR product (Fig. 2c). Primer set *SB4F/SB4R* was used to identify the *Glu-B3d* allele in Aroona-B3d, producing a 662-bp band (Fig. 2d). In Cheyenne with the *Glu-B3e* allele, a specific 669-bp PCR product was generated with the primer set *SB5F/SB5R* (Fig. 2e). Primer set *SB7F/SB7R* was used to detect the *Glu-B3g* allele in Aroona-B3g with an 853-bp PCR fragment (Fig. 2f). To discriminate the *Glu-B3h* allele from others, primer set *SB8F/SB8R* was developed; it generated a 1,022-bp band in Aroona-B3h (Fig. 2g). Primer set *SB9F/SB9R* uniquely amplified a 621-bp PCR fragment in Aroona-B3i with the *Glu-B3i* allele (Fig. 2h). Since it was difficult to design a specific primer set for *Glu-B3f*, primer set *SB6F/SB6R* was developed to amplify *Glu-B3f* and *Glu-B3g* in Aroona-B3f and Aroona-B3g, respectively (Fig. 2i). In combination with *SB7F/SB7R*, this primer set can be used to identify *Glu-B3f*. In addition, primer set *SB10F/SB10R* was designed to amplify *Glu-B3b*, *e* and *f* in Aroona, Cheyenne and Aroona-B3f,

Table 3 Relationship between *Glu-B3* protein mobility alleles and *Glu-B3* haplotypes

NIL/variety	*Glu-B3* allele[a]	Haplotype[b]														
		GluB3-11	*GluB3-12*	*GluB3-13*	*GluB3-14*	*GluB3-15*	*GluB3-21*	*GluB3-22*	*GluB3-23*	*GluB3-31*	*GluB3-32*	*GluB3-33*	*GluB3-34*	*GluB3-41*	*GluB3-42*	*GluB3-43*	*GluB3-44*	*GluB3-45*
Aroona-B3a	a	+					+							+				
Aroona	b		+					+							+			
Aroona-B3c	c									+				+				
Aroona-B3d	d										+			+				
Cheyenne	e			+				+								+		
Aroona-B3f	f				+			+									+	
Aroona-B3g	g					+			+								+	
Aroona-B3h	h											+				+		
Aroona-B3i	i												+					+

Sequences have been submitted to GenBank, viz. *GluB3-11* (EU369699), *GluB3-12* (EU369700), *GluB3-13* (EU369701), *GluB3-14* (EU369702), *GluB3-15* (EU369703), *GluB3-21* (EU369704), *GluB3-22* (EU369721), *GluB3-23* (EU369705), *GluB3-31* (EU369715), *GluB3-32* (EU369716), *GluB3-33* (EU369717), *GluB3-34* (EU369718), *GluB3-41* (EU369724), *GluB3-42* (EU369719), *GluB3-43* (EU369727), *GluB3-44* (EU369729) and *GluB3-45* (EU369720)

[a] *Glu-B3* alleles defined by protein electrophoretic mobility in SDS-PAGE

[b] "+" means the gene haplotype is present in the corresponding NIL or variety

respectively (Fig. 2j), and this set can be used to verify the former primer sets.

Validation of *Glu-B3* allele-specific markers

Eight NILs and Cheyenne used in cloning the *Glu-B3* genes, and additional 161 wheat varieties and advanced lines were used to validate the ten *Glu-B3* allele-specific markers (Table 4). The results were in accordance with those detected by SDS-PAGE except for five genotypes (entries 28, 68, 121, 152 and 165). In particular, the entry 121, which was detected to possess *Glu-B3j* or *Glu-B3g* allele in SDS-PAGE (Fig. 3), was determined to have *Glu-B3g* allele by the markers *gluB3fg* and *gluB3g*. Thirty-two genotypes with the 1BL.1RS translocation showed no target PCR product in the test with molecular markers, indicating the presence of the protein allele *Glu-B3j*.

Discussion

The LMW-GS proteins are critical components of the gluten complex of wheat and are encoded by a highly variable gene family. Because of their importance in wheat flour quality and difficulties in discriminating them by traditional SDS-PAGE techniques, it was necessary to develop allele-specific markers to identify different LMW-GS alleles (Gupta et al. 1999; Gale 2005; Bagge et al. 2007). Zhang et al. (2004) reported seven *Glu-A3* markers to discriminate different *Glu-A3* alleles. Several STS markers were also developed for different *Glu-D3* gene haplotypes (Zhao et al. 2007a, b). In this study, ten allele-specific primer sets were successfully designed for the nine *Glu-B3* alleles defined by protein mobility. The ten markers were validated with eight Aroona NILs and Cheyenne, and 161 wheat varieties and advanced lines from CIMMYT, Australia and France.

The *GluB3-1* and *GluB3-2* genes were present in varieties with protein based alleles *a*, *b*, *e*, *f* and *g*, and the *GluB3-3* allele was present in those with *c*, *d*, *h* and *i* only. As the three genes were isolated by the gene-specific primer sets *LB1F/LB1R*, *LB2F/LB2R* and *LB3F/LB3R*, respectively, we also designed new primer sets from within the coding regions of these genes to perform PCR, and confirmed the distinctions between the genes. In a previous study, Zhao et al. (2007b) found that the primer set *T13F4/T13R3* was null in varieties with *Glu-B3c*, *d*, *h* and *i*. The *GluB3-4* genes were present in varieties with the protein alleles *a*, *b*, *c*, *d*, *f*, *g*, *h*, and *i*, and thus the haplotypes of gene variants present in different varieties share common genes as well as carrying unique variants.

Currently, more than 200 LMW-GS genes have been registered in GenBank. About 30 sequences were located

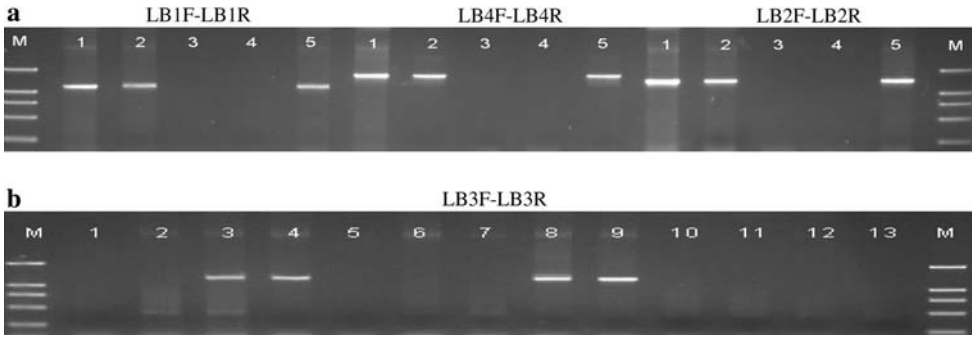

Fig. 1 Electrophoresis of PCR products of four gene-specific primer sets on agarose gels. **a** *GluB3-1*, *GluB3-4* and *GluB3-2*; *1* Chinese Spring, *2* N1AT1B, *3* N1BT1A, *4* N1BT1D, *5* N1DT1B. **b** *GluB3-3*; *1*, *2*, *5–7* 1BL.1RS lines, *3* Aroona-B3c, *4* Aroona-B3d, *8* Aroona-B3h, *9* Aroona-B3i, *10* N1AT1B, *11* N1BT1A, *12* N1BT1D, *13* N1DT1B. *M* DNA Ladder 2000 (100, 250, 500, 750, 1,000 and 2,000 bp)

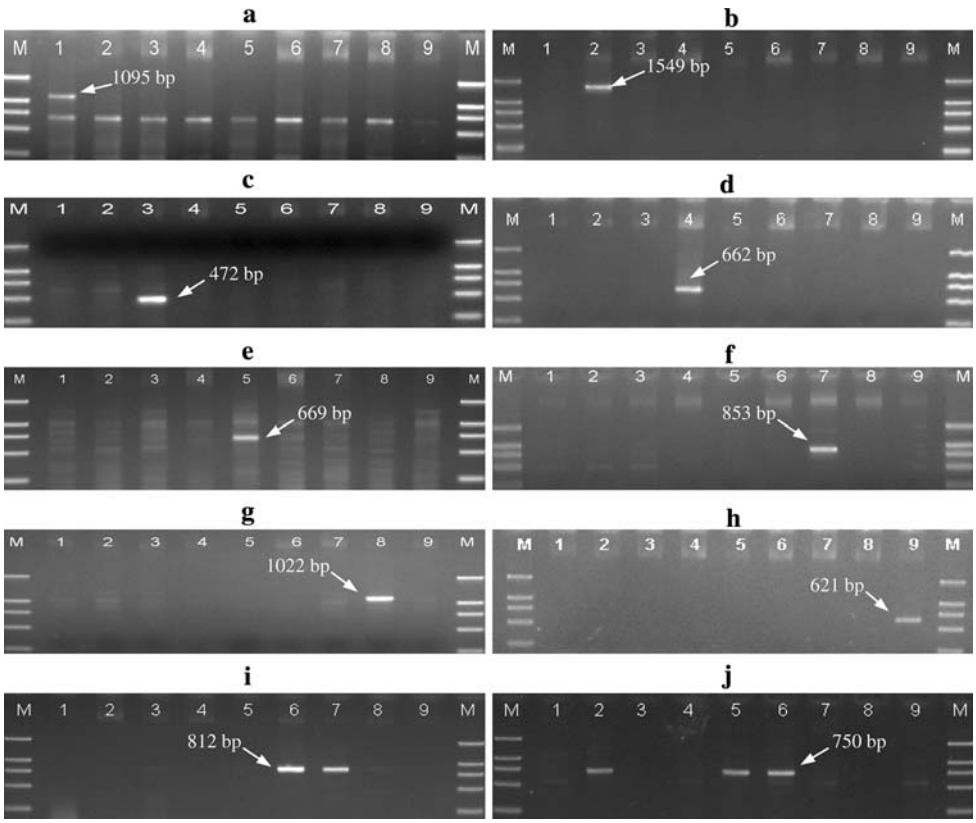

Fig. 2 Electrophoresis of PCR products amplified from the eight NILs and Cheyenne on agarose gels using ten allele-specific markers: **a** *gluB3a*, **b** *gluB3b*, **c** *gluB3c*, **d** *gluB3d*, **e** *gluB3e*, **f** *gluB3g*, **g** *gluB3h*, **h** *gluB3i*, **i** *gluB3fg*, **j** *gluB3bef*. Materials used as PCR templates were as follows: *1* Aroona-B3a (**a**), *2* Aroona-B3b (**b**), *3* Aroona-B3c (**c**), *4* Aroona-B3d (**d**), *5* Cheyenne (**e**), *6* Aroona-B3f (**f**), *7* Aroona-B3g (**g**), *8* Aroona-B3h (**h**), *9* Aroona-B3i (**i**). PCR product sizes and conditions are as listed in Table 2. *M* DNA Ladder 2000 (100, 250, 500, 750, 1,000 and 2,000 bp)

on chromosome 1B and 13 of them had complete coding sequences (Van Campenhout et al. 1995; D'Ovidio et al. 1997, 1999; Masci et al. 1998; Ikeda et al. 2002; Maruyama-Funatsuki et al. 2005; Huang and Cloutier 2008). In the present study, four *Glu-B3* genes were identified, including the complete coding sequences of 17 allelic variants. Sequence alignments indicated that *GluB3-1* was 99.1–99.7% identical in sequence to AB262661 and EU189088. In particular, *GluB3-11* had only three SNP differences from AB262661 and EU189088 in the aligned domain. The three allelic variants of *GluB3-2* showed lower identities to the genes in GenBank, with the highest similarity of 93.4% between *GluB3-23* and AB164415, indicating that *GluB3-2* might be a new LMW-GS gene identified at the *Glu-B3* locus. *GluB3-3* shared 99.5–100.0% identity with *AB164415*, and *GluB3-33* had the same sequence as *AB164415*. *GluB3-4* shared 99.1–99.9% of identities with *AB062852* and *X84960*, and *GluB3-41* had only 1 bp difference from *X84960* at position 1,303 in the coding region.

Based on the first N-terminal amino acid of the mature protein, LMW-GS were divided into three types: LMW-m,

Table 4 Validation of the *Glu-B3* allele-specific markers with eight NILs and Cheyenne, and 161 wheat varieties and advanced lines

No.	NIL, variety or line	Seed source	Allele[a]	*gluB3a*	*gluB3b*	*gluB3c*	*gluB3d*	*gluB3e*	*gluB3g*	*gluB3h*	*gluB3i*	*gluB3fg*	*gluB3bef*
1	Aroona-B3a	Australia	a	+									
2	Aroona	Australia	b		+								+
3	Aroona-B3c	Australia	c			+							
4	Aroona-B3d	Australia	d				+						
5	Cheyenne	USA	e					+					+
6	Aroona-B3f	Australia	f									+	+
7	Aroona-B3g	Australia	g						+			+	
8	Aroona-B3h	Australia	h							+			
9	Aroona-B3i	Australia	i								+		
10	Halberd	Australia	c			+							
11	Silverstar	Australia	h							+			
12	Declic	Australia	b		+								+
13	Leichhardt	Australia	h							+			
14	Dagger	Australia	h							+			
15	Hartog	Australia	h							+			
16	Chinese Spring	France	a	+									
17	Gabo	France	b		+								+
18	Orca	France	d				+						
19	Magali Blondeau	France	f									+	+
20	Brimstone	France	g						+			+	
21	Ruso	France	i								+		
22	Apollo	France	j										
23	Cappelle-Desprez	France	g						+			+	
24	Festin	France	b		+								+
25	Manital	France	b		+								+
26	Pepital	France	d				+						
27	Petrel	France	h							+			
28	Salmone	France	c						+			+	
29	Etoile De Choisy	France	i								+		
30[b]	SERI/RAYON	CIMMYT	b		+								+
31	KRONSTAD F2004	CIMMYT	h							+			
32	KAMBARA1	CIMMYT	h							+			
33	WHEATEAR	CIMMYT	b		+								+
34	WEEBILL1	CIMMYT	h							+			
35	WEEBILL1	CIMMYT	h							+			
36	SERI.1B*2/3/KAUZ*2/BOW//KAUZ	CIMMYT	h							+			
37	ATTILA*2/PBW65	CIMMYT	h							+			
38	WAXWING	CIMMYT	b		+								+

Table 4 continued

No.	NIL, variety or line	Seed source	Allele[a]	*gluB3a*	*gluB3b*	*gluB3c*	*gluB3d*	*gluB3e*	*gluB3g*	*gluB3h*	*gluB3i*	*gluB3fg*	*gluB3bef*
39	PRL/2*PASTOR	CIMMYT	g						+			+	
40	ATTILA/3*BCN//BAV92/3/PASTOR	CIMMYT	j										
41	BABAX//IRENA/KAUZ/3/HUITES	CIMMYT	b		+								+
42	BABAX/LR42//BABAX*2/3/KURUKU	CIMMYT	h							+			
43	BABAX/LR42//BABAX*2/3/VIVITSI	CIMMYT	h							+			
44	BABAX/LR42//BABAX*2/3/VIVITSI	CIMMYT	h							+			
45	BABAX/LR42//BABAX*2/3/VIVITSI	CIMMYT	h							+			
46	BL2064//SW89-5124*2/FASAN/3/TILHI	CIMMYT	j										
47	CAL/NH//H567.71/3/SERI/4/CAL/NH//H567.71 /5/2*KAUZ/6/PASTOR	CIMMYT	j										
48	CNO79//PF70354/MUS/3/PASTOR/4/BABAX	CIMMYT	h							+			
49	FRET2*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ	CIMMYT	h							+			
50	FRET2*2/BRAMBLING	CIMMYT	h							+			
51	FRET2*2/BRAMBLING	CIMMYT	h							+			
52	FRET2/TUKURU//FRET2	CIMMYT	h							+			
53	FRET2/WBLL1//KAMB1	CIMMYT	g						+			+	
54	IRENA/2*PASTOR	CIMMYT	g						+			+	
55	KAMB1*2/BRAMBLING	CIMMYT	h							+			
56	KAMB1*2/KIRITATI	CIMMYT	h							+			
57	KAUZ//ALTAR 84/AOS/3/MILAN /KAUZ/4/HUITES	CIMMYT	b		+								+
58	KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ/4/HUITES	CIMMYT	b		+								+
59	KAUZ//ALTAR 84/AOS/3/MILAN /KAUZ/4/HUITES	CIMMYT	b		+								+
60	KAUZ/PASTOR//PBW343	CIMMYT	j										
61	KIRITATI//ATTILA*2/PASTOR	CIMMYT	i								+		
62	KIRITATI//PRL/2*PASTOR	CIMMYT	i								+		
63	KIRITATI/WBLL1	CIMMYT	i								+		
64	MILAN/S87230//BABAX	CIMMYT	h							+			
65	OASIS/SKAUZ//4*BCN*2/3/PASTOR	CIMMYT	g						+			+	
66	OASIS/SKAUZ//4*BCN/3/2*PASTOR	CIMMYT	g						+			+	
67	OASIS/SKAUZ//4*BCN/3/PASTOR/4/KAUZ*2 /YACO//KAUZ	CIMMYT	g						+			+	
68	PFAU/SERI.1B//AMAD/3/WAXWING	CIMMYT	b						+			+	
69	PFAU/WEAVER*2//BRAMBLING	CIMMYT	g						+			+	
70	PFAU/WEAVER*2//KIRITATI	CIMMYT	g						+			+	
71	PFAU/WEAVER*2//KIRITATI	CIMMYT	i								+		
72	PRINIA/PASTOR	CIMMYT	g						+			+	
73	SITE/MO//PASTOR/3/TILHI	CIMMYT	g						+			+	
74	TAM200/PASTOR//TOBA97	CIMMYT	g						+			+	
75	THELIN/2*WBLL1	CIMMYT	h							+			

Table 4 continued

No.	NIL, variety or line	Seed source	Allele[a]	*gluB3a*	*gluB3b*	*gluB3c*	*gluB3d*	*gluB3e*	*gluB3g*	*gluB3h*	*gluB3i*	*gluB3fg*	*gluB3bef*
76	THELIN/3/2*BABAX/LR42//BABAX	CIMMYT	g						+			+	
77	TOBA97/PASTOR	CIMMYT	g						+			+	
78	TOBA97/PASTOR	CIMMYT	g						+			+	
79	TUKURU//BAV92/RAYON	CIMMYT	i							+			
80	VORB/FISCAL	CIMMYT	g						+			+	
81	WAXWING*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP //KAUZ	CIMMYT	b		+								+
82	WAXWING*2/KIRITATI	CIMMYT	b		+								+
83	WAXWING*2/KUKUNA	CIMMYT	b		+								+
84	WAXWING*2/KUKUNA	CIMMYT	b		+								+
85	WAXWING*2/VIVITSI	CIMMYT	b		+								+
86	WAXWING*2/VIVITSI	CIMMYT	b		+								+
87	WAXWING/4/SNI/TRAP#1/3/KAUZ*2/TRAP //KAUZ	CIMMYT	b		+								+
88	WBLL1*2/4/SNI/TRAP#1/3/KAUZ*2/TRAP//KAUZ	CIMMYT	h							+			
89	WBLL1*2/4/YACO/PBW65/3/KAUZ*2/TRAP //KAUZ	CIMMYT	h							+			
90	WBLL1*2/BRAMBLING	CIMMYT	h							+			
91	WBLL1*2/BRAMBLING	CIMMYT	h							+			
92	WBLL1*2/BRAMBLING	CIMMYT	h							+			
93	WBLL1*2/BRAMBLING	CIMMYT	h							+			
94	WBLL1*2/CHAPIO	CIMMYT	h							+			
95	WBLL1*2/KIRITATI	CIMMYT	h							+			
96	WBLL1*2/KUKUNA	CIMMYT	h							+			
97	WBLL1*2/KUKUNA	CIMMYT	h							+			
98	WBLL1*2/KURUKU	CIMMYT	h							+			
99	WBLL1*2/TUKURU	CIMMYT	h							+			
100	WBLL1/3/STAR//KAUZ/STAR/4/BAV92/RAYON	CIMMYT	b		+								+
101	WBLL1/KUKUNA//KAMB1	CIMMYT	h							+			
102	WBLL4/KUKUNA//WBLL1	CIMMYT	h							+			
103	BOW/NKT//CBRD/3/CBRD	CIMMYT	g						+			+	
104	CROC_1/AE.SQUARROSA (224)//OPATA/3/BJY /COC//PRL/BOW/4/ BJY/COC//PRL/BOW	CIMMYT	d				+						
105	HEILO	CIMMYT	g						+			+	
106	RABE/LAJ3302	CIMMYT	b		+								+
107	V763.2312/V879.C8.11.11.11(36)//STAR/3/STAR	CIMMYT	g						+			+	
108	PAVON F 76	CIMMYT	h							+			
109	KIRITATI	CIMMYT	i								+		
110	PFAU/WEAVER*2//KIRITATI	CIMMYT	g						+			+	
111	TAM200/TUI	CIMMYT	g						+			+	
112	HAR3116	CIMMYT	g						+			+	

Table 4 continued

No.	NIL, variety or line	Seed source	Allele[a]	*gluB3a*	*gluB3b*	*gluB3c*	*gluB3d*	*gluB3e*	*gluB3g*	*gluB3h*	*gluB3i*	*gluB3fg*	*gluB3bef*
113	CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/PASTOR	CIMMYT	g							+			
114	K6295.4A	CIMMYT	i								+		
115	YANAC	CIMMYT	b		+								+
116	PVN//CAR422/ANA/5/BOW/CROW//BUC /PVN/3/YR/4/TRAP#1	CIMMYT	h							+			
117	BAU/TNMU	CIMMYT	j										
118	CATBIRD	CIMMYT	j										
119	GONDO	CIMMYT	j										
120	GUAM92//PSN/BOW	CIMMYT	j										
121	IVAN/6/SABUF/5/BCN/4/RABI//GS/CRA/3/AE.SQUARROSA (190)	CIMMYT	J/g[c]						+			+	
122	KAUZ//TRAP#1/BOW	CIMMYT	j										
123	NG8675/CBRD	CIMMYT	j										
124	NING MAI 9558	CIMMYT	j										
125	SHA3/CBRD	CIMMYT	g						+			+	
126	SHA3/SERI//SHA4/LIRA	CIMMYT	j										
127	SHA5/WEAVER	CIMMYT	j										
128	SHA8/GEN	CIMMYT	j										
129	TINAMOU	CIMMYT	j										
130	WUH1/VEE#5//CBRD	CIMMYT	j										
131	ACHTAR*3//KANZ/KS85-8-4	CIMMYT	g						+			+	
132	ACHTAR*3//KANZ/KS85-8-5	CIMMYT	g						+			+	
133	ALTAR 84/AEGILOPS SQUARROSA (TAUS)//OPATA	CIMMYT	i								+		
134	KANZ*4/KS85-8-4	CIMMYT	h							+			
135	KANZ*4/KS85-8-4	CIMMYT	h							+			
136	PRL/SARA//TSI/VEE#5	CIMMYT	i								+		
137	BH1146*3/ALD//BUC/3/DUCULA/4/DUCULA	CIMMYT	h							+			
138	PF839197/BR35//BR23/3/PASTOR	CIMMYT	j										
139	TNMU/6/PEL74144/4/KVZ//ANE/MY64/3/PF70354/5/BR14/7/BR35	CIMMYT	g						+			+	
140	ALTAR 84/AE.SQ//OPATA/3/2*WH 542	CIMMYT	j										
141	CHUM18/BORL95//CBRD	CIMMYT	j										
142	CROC_1/AE.SQUARROSA (205)//KAUZ/3/SASIA	CIMMYT	j										
143	CROC_1/AE.SQUARROSA (205)//KAUZ/3/SASIA	CIMMYT	j										
144	KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ	CIMMYT	j										
145	SW89.3064//CMH82.17/SERI	CIMMYT	j										
146	W462//VEE/KOEL/3/PEG//MRL/BUC	CIMMYT	j										
147	W485/HD29	CIMMYT	g						+			+	
148	CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER/5/2*PASTOR	CIMMYT	g						+			+	

Table 4 continued

No.	NIL, variety or line	Seed source	Allele[a]	*gluB3a*	*gluB3b*	*gluB3c*	*gluB3d*	*gluB3e*	*gluB3g*	*gluB3h*	*gluB3i*	*gluB3fg*	*gluB3bef*
149	PASTOR/TEERI	CIMMYT	g						+			+	
150	PASTOR/TEERI	CIMMYT	g						+			+	
151	PASTOR/TEERI	CIMMYT	g						+			+	
152	ENEIDA F94	CIMMYT	b								+		
153	HUW468	CIMMYT	i								+		
154	INQALAB 91	CIMMYT	g						+			+	
155	INQALAB 91*2/KUKUNA	CIMMYT	g						+			+	
156	INQALAB 91*2/TUKURU	CIMMYT	g						+			+	
157	KANCHAN	CIMMYT	i								+		
158	PBW343	CIMMYT	j										
159	PBW343*2/KHVAKI	CIMMYT	j										
160	PBW343*2/KUKUNA	CIMMYT	b		+								+
161	PBW343*2/TUKURU	CIMMYT	i								+		
162	58769	CIMMYT	j										
163	SW 8488 (W)	CIMMYT	j										
164	SW00-91382	CIMMYT	j										
165	SW02-90137	CIMMYT	b						+			+	
166	SW03-81497	CIMMYT	b		+								+
167	SW22725	CIMMYT	g						+			+	
168	YUNMAI 47	CIMMYT	j										
169	80.8	CIMMYT	i								+		
170	SW2148	CIMMYT	j										

[a] *Glu-B3* alleles defined by protein electrophoretic mobility in SDS-PAGE

[b] Numbers 30–170 are varieties and advanced lines from CIMMYT

[c] The *Glu-B3* allele of No. 121 could not be distinguished between j and g in SDS-PAGE

"+" corresponds to PCR products present

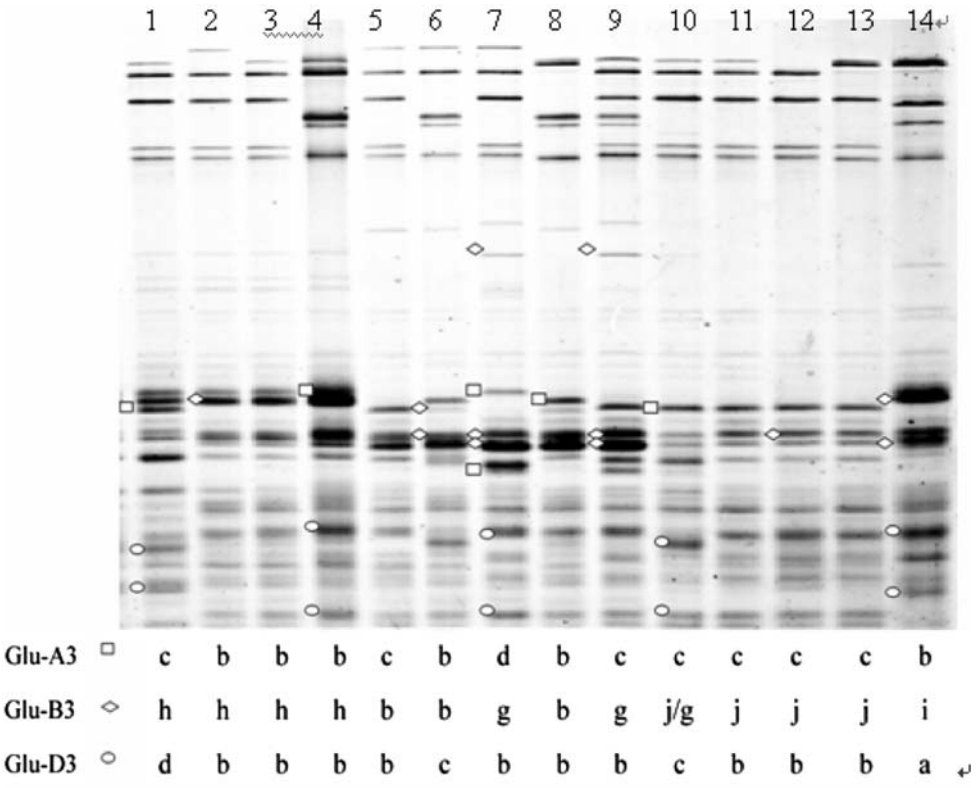

Fig. 3 SDS-PAGE patterns of glutenin subunits of wheat varieties and advanced lines. *1* entry no. 43 in Table 4, *2* no. 48, *3* no. 52, *4* Pavón (Control), *5* no. 30, *6* no. 33, *7* no. 39, *8* no. 41, *9* no. 53, *10* no. 121, *11* no. 40, *12* no. 46, *13* no. 47, *14* Opata (Control)

LMW-s and LMW-i, corresponding to methionine, serine and isoleucine, respectively (Lew et al. 1992). In this study, BP4 started with the amino acids METSHIP in the N-terminal domain and could therefore be classified as a LMW-m type. The LMW-m type was found to be the main type by gene sequencing, but the LMW-s type was the predominant type found by N-terminal sequencing of proteins (Lew et al. 1992; Masci et al. 2002). It was suggested that the LMW-s type might originate from post-translational cleavage by an asparaginyl peptidase (Masci et al. 1998; Dupont et al. 2004). Consequently, BP1, BP2 and BP3 starting with MENSHIP in the N-terminal domain could be classified as LMW-s types based on asparagine at the third position (Ikeda et al. 2002, 2006). Previous studies also indicated that such classification of LMW-m and LMW-s type genes might be highly tenuous, as the LMW-m and LMW-s type genes have high homologies in wheat and its relatives (Jiang et al. 2008; Huang and Cloutier 2008).

The overall results of this study showed that the protein mobility alleles determined by SDS-PAGE were consistent with the screening results obtained using the allele-specific markers in 165 of 170 genotypes. The inconsistent results for five genotypes were attributed to the low discrimination power of SDS-PAGE in distinguishing some LMW-GS and the impurity of some materials. The ten *Glu-B3* allele-specific markers can be used in the marker-assisted breeding aimed at the improvement of wheat quality.

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