



Development of STS markers and establishment of multiplex PCR for *Glu-A3* alleles in common wheat (*Triticum aestivum* L.)

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ABSTRACT

Low-molecular-weight glutenin subunits (LMW-GS) play a key role in determining the processing quality of the end-use products of common wheat. The objectives of this study were to identify genes at *Glu-A3* locus, develop the STS markers, and establish multiplex PCR with the STS markers for *Glu-A3* alleles. Gene-specific PCR primers were designed to amplify six near-isogenic lines (NILs) and Glenlea with different *Glu-A3* alleles (*a*, *b*, *c*, *d*, *e*, *f* and *g*) defined by the protein electrophoretic mobility. Three *Glu-A3* genes with complete coding sequence were cloned, designated as *GluA3-1*, *GluA3-2* and *GluA3-3*, respectively. Seven dominant allele-specific STS (sequence tagged sites) markers were designed based on the SNPs (single nucleotide polymorphisms) among different allelic variants for the discrimination of the *Glu-A3* protein alleles *a*, *b*, *c*, *d*, *e*, *f* and *g*. Four multiplex PCRs were established including *Glu-A3b* + *Glu-A3f*, *Glu-A3d* + *Glu-A3f*, *Glu-A3d* + *Glu-A3g*, and *Glu-A3b* + *Glu-A3e*. These markers and multiplex-PCR systems were validated on 141 CIMMYT wheat varieties and advanced lines with different *Glu-A3* alleles, confirming that they can be efficiently used in marker-assisted breeding.

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1. Introduction

Of all the cereal grains, wheat is unique to form dough that exhibits the rheological properties required for the broad diversity of foods (He et al., 2005; Shewry et al., 1995). The unique properties are mainly determined by the composition and amount of storage proteins in seed endosperm, which consist of two major components of the polymeric glutenins and the monomeric gliadins (An et al., 2006). 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). HMW-GS and LMW-GS

are held together by inter-molecular disulfide bonds to form the glutenin polymer. Through non-covalent interactions in the presence of water, the glutenin polymer interacts with gliadins to form gluten, conferring unique visco-elastic properties to wheat flour dough (Lindsay and Skerritt, 1999; Shewry et al., 2001).

The HMW-GS represent approximately 10% of the total seed storage proteins. Their important role has been well established (Shewry et al., 1992, 1995), because of the relatively small number (3–5 subunits per hexaploid variety) and the ease of characterization by SDS-PAGE (Shewry et al., 1995). The typical LMW-GS are encoded by genes at the *Glu-3* loci (*Glu-A3*, *Glu-B3* and *Glu-D3*) on the short arm of the group 1 chromosomes (Gupta and Shepherd, 1990), which comprise a multigene family, including 30–40 genes in hexaploid wheat (Cassidy et al., 1998; Sabelli and Shewry, 1991). LMW-GS represent about one-third of total seed storage proteins and 60% of glutenins (Bietz and Wall, 1973), and they also have significant effects on wheat quality through both additive and epistatic interactions, particularly with HMW-GS (Gupta et al., 1994; He et al., 2005; Luo et al., 2001; Maruyama-Funatsuki et al., 2004).

Understanding the role of single LMW-GS is very important for wheat quality breeding programs. LMW-GS alleles are usually scored using SDS-PAGE (Singh and Shepherd, 1988). In a survey of

Abbreviations: 2-DE, two-dimensional gel electrophoresis; CTAB, cetyl trimethyl ammonium bromide; HMW-GS, high-molecular-weight glutenin subunits; InDel, insertion/deletion; LMW-GS, low-molecular-weight glutenin subunits; NIL, near-isogenic line; ORF, open reading frame; SDS-PAGE, sodium-dodecyl-sulphate polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; STS, sequence tagged site.

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222 wheat accessions from 32 countries, 20 different protein alleles were identified, including six 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*) (Gupta and Shepherd, 1990). In recent years, other LMW-GS alleles have been identified (McIntosh et al., 2008) such as *Glu-A3 g* (Jackson et al., 1996; Zhang et al., 2004). However, due to the complexity of the banding patterns observed and the overlap of mobilities of the LMW-GS and the gliadins, the study of the functionality of the LMW-GS and the effect of allelic variation of these proteins on the quality attributes are much more difficult than those for the HMW-GS (Gupta et al., 1994; Maruyama-Funatsuki et al., 2004; Masci et al., 1998). Therefore, it is important to characterize LMW-GS genes and develop functional markers to identify different LMW-GS alleles (Andersen and Lübberstedt, 2003; Zhang et al., 2004). Previously, we characterized six *Glu-D3* genes and four *Glu-B3* genes and developed a number of STS markers for the discrimination of the *Glu-D3* haplotypes and *Glu-B3* protein alleles, and the relationship between *Glu-B3* or *Glu-D3* haplotypes and the protein alleles were also documented (Wang et al., 2009; Zhao et al., 2006, 2007). For the *Glu-A3* locus, Zhang et al. (2004) identified one gene, including seven allelic forms cloned from different varieties, and developed seven PCR markers (*GluA3a*, *GluA3abc*, *GluA3ac*, *GluA3d*, *GluA3e*, *GluA3f* and *GluA3g*) to discriminate the protein alleles *Glu-A3a*, *b*, *c*, *d*, *e*, *f* and *g*. However, it is very likely that there are also some other genes at the *Glu-A3* locus in comparison to *Glu-B3* and *Glu-D3* loci where four and six genes were identified, respectively (Wang et al., 2009; Zhao et al., 2006, 2007), and the relationship between *Glu-A3* haplotypes and the *Glu-A3* protein alleles has remained unclear. It is, therefore, necessary to characterize more *Glu-A3* genes and their association with protein alleles. In addition, several alleles were present at the *Glu-A3* locus as mentioned above, and it may be time-consuming and expensive to run many markers. Thus it is essential to establish multiplex PCRs with *Glu-A3* markers for application in wheat breeding.

In this paper, we report the isolation of genes at the *Glu-A3* locus, development of STS markers, and establishment of multiplex PCR with the STS markers for *Glu-A3* protein alleles. These results will greatly benefit wheat quality improvement through marker-assisted selection.

2. Materials and methods

2.1. Plant materials

Aroona (*Glu-A3c*) and its five near-isogenic lines (NILs), Aroona-A3a (*Glu-A3a*), Aroona-A3b (*Glu-A3b*), Aroona-A3d (*Glu-A3d*), Aroona-A3e (*Glu-A3e*), Aroona-A3f (*Glu-A3f*), and Glenlea (*Glu-A3g*), with different *Glu-A3* alleles defined by protein mobility were used to isolate *Glu-A3* genes. Chinese Spring and its nulli-tetrasomic lines N1A-T1D (nullisomic 1A-tetrasomic 1D), N1B-T1A, N1B-T1D, and N1D-T1B, were used to confirm the chromosomal locations of identified genes. One hundred and forty one CIMMYT wheat varieties and advanced lines with different *Glu-A3* alleles were used for the validation of the STS markers and multiplex PCRs.

2.2. DNA isolation and PCR amplification

Genomic DNA was extracted from seedlings or seeds according to the CTAB (cetyl trimethyl ammonium bromide) procedure (Gale et al., 2001) with minor modification. PCR was performed using 1.0 unit *Taq* DNA polymerase (TaKaRa, Biotechnology Co., Ltd., Dalian, China) in 20 μ l reaction volumes containing approximately 50 ng of genomic DNA, 1 $\times$ PCR buffer with 1.5 mM MgCl₂, 7.5 pmol of each PCR primer and 100 μ M of each of dNTPs. PCR cycling conditions for

locus-specific primers were 94 °C for 5 min followed by 38 cycles of 94 °C for 35 s, 57–58 °C for 35 s, 72 °C for 90 s, and a final extension at 72 °C for 8 min (Appendix Table A1).

2.3. Development of locus-specific primers for the isolation of *Glu-A3* genes

After the analysis of the LMW-GS gene sequences in GenBank, four reference genes with complete coding regions (GenBank accessions EU189087, AB062868, FJ441117 and EF426565, <http://www.ncbi.nlm.nih.gov>), were used for primer development. Locus-specific primers for cloning *Glu-A3* genes were developed following the descriptions of Zhang et al. (2003, 2004) and Zhao et al. (2006).

Based on the alignment of the reference genes, 57 primers were designed and 189 primer combinations were tested with the Aroona NILs and Glenlea with different *Glu-A3* protein alleles. Primer screening was conducted as described by Zhao et al. (2006). Finally, Three *Glu-A3* genes were isolated by three pairs of primers with annealing temperatures of 57 °C or 58 °C (depending on primer sets)(Fig. A1). Primer sequences (5'–3') and their relative positions compared to reference genes are shown in Table A1.

2.4. Cloning and sequencing of PCR products

PCR products were separated on 1.2% agarose gels, and the expected fragments were purified from gels, and then cloned into the pEASY-T1 vector (TransGen, China). 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 3–6 times to avoid any technical errors. All the sequencings were performed by the Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). Sequence analysis and characterization were performed using the software DNAMAN (<http://www.lynnnon.com>).

2.5. Development of allele-specific PCR markers and establishment of multiplex PCR

Based on the allelic variants at the *Glu-A3* locus, allele-specific STS markers were designed for different protein alleles, with the SNPs (single nucleotide polymorphisms) anchored at the 3' terminal of each primer. To ensure efficient amplification, some primer-template mismatched nucleotides were also introduced. Finally, seven allele-specific STS markers were obtained (Table 1), and their PCR cycling conditions were 94 °C for 5 min, followed by 38 cycles of 94 °C for 35 s, 60 °C for 35 s, 72 °C for 90 s, and a final extension at 72 °C for 8 min. With these markers, four multiplex PCRs were established, including *Glu-A3b* + *Glu-A3f*, *Glu-A3d* + *Glu-A3f*, *Glu-A3d* + *Glu-A3g*, and *Glu-A3b* + *Glu-A3e* (Table 2). The allele-specific STS markers and multiplex-PCR systems were validated on 141 wheat varieties and advanced lines from CIMMYT with different *Glu-A3* protein mobility alleles that were characterized with SDS-PAGE in CIMMYT previously.

3. Results

3.1. Allelic variants at the *Glu-A3* locus

With the locus-specific primer sets LA1F/LA1R, LA2F/LA2R and LA3F/LA3R, three *Glu-A3* genes with 17 allelic variants were identified from the six Aroona NILs and Glenlea, designated *GluA3-1*, *GluA3-2* and *GluA3-3*, and their deduced amino acid sequences were designated AP1, AP2 and AP3, respectively.

Table 1

Allele-specific PCR markers for the discrimination of *Glu-A3* alleles defined by protein mobility in common wheat.

Marker Name	Primer set	Sequence (5' → 3') ^a	Target <i>Glu-A3</i> allele	Fragment size
<i>gluA3a</i>	LA1F	AAACAGAATTATTAAGCCGG	a	529
	SA1R	GGTTGTTGTTGTCAGCA		
<i>gluA3b</i>	LA3F	TTCAGATGCAGCCAAACAA	b	894
	SA2R	GCTGTGCTGGATGATACTCTA		
<i>gluA3ac</i>	LA1F	AAACAGAATTATTAAGCCGG	ac ^b	573
	SA3R	GTGGCTGTTGTGAAAACGA		
<i>gluA3d</i>	LA3F	TTCAGATGCAGCCAAACAA	d	967
	SA4R	TGGGGTTGGGAGACACATA		
<i>gluA3e</i>	LA1F	AAACAGAATTATTAAGCCGG	e	158
	SA5R	GGCAGACAGAGGAAGGTT		
<i>gluA3f</i>	LA1F	AAACAGAATTATTAAGCCGG	f	552
	SA6R	GCTGCTGCTGCTGTGTA		
<i>gluA3g</i>	LA1F	AAACAGAATTATTAAGCCGG	g	1345
	SA7R	AAACACCGGTGATCCAATAA		

^a Mismatched nucleotides are underlined.

^b Specific for *Glu-A3a* and *c* alleles.

GluA3-1 gene had seven allelic variants, designated as *GluA3-11*, *GluA3-12*, *GluA3-13*, *GluA3-14*, *GluA3-15*, *GluA3-16* and *GluA3-17* (GenBank accessions FJ549928, FJ549929, FJ549930, FJ549931, FJ549932, FJ549933 and FJ549934) cloned from the six NILs and Glenlea, respectively. The sizes of the coding region of the seven allelic variants ranged from 1068 bp (*GluA3-14* and *GluA3-14*) to 1167 bp (*GluA3-15*) (Fig. A2). Compared with *GluA3-11* in the coding domain, 6, 1, 27, 25, 19 and 18 SNPs were detected in *GluA3-12*, *GluA3-13*, *GluA3-14*, *GluA3-15*, *GluA3-16* and *GluA3-17*, respectively, and one or more InDels (insertions/deletions) were also found in these allelic variants except in *GluA3-13*, leading to some changes in the corresponding deduced peptide (Fig. A3).

The *GluA3-2* gene had four allelic variants, designated as *GluA3-21*, *GluA3-22*, *GluA3-23* and *GluA3-24* (GenBank accessions FJ549935, FJ549936, FJ549937 and FJ549938), respectively. The sizes of the complete coding region were 912 bp in *GluA3-21* and *GluA3-23*, 909 bp in *GluA3-22*, and 918 bp in *GluA3-24* (Fig. A4). *GluA3-21*, *GluA3-23* and *GluA3-24* were cloned from the varieties Aroona-A3a, Aroona-A3d and Glenlea, respectively, and *GluA3-22* was cloned in the varieties with the protein alleles *Glu-A3b*, *c*, *e* and *f*. Compared with *GluA3-21* in the coding domain, a 3-bp (CAA) deletion in *GluA3-22*, eight SNPs in *GluA3-23*, seven SNPs and a 6-bp insertion in *GluA3-24* were detected, respectively. Particularly, the transitions (A→G) at the 223rd position in *GluA3-23* and *GluA3-24* resulted in the changes from tyrosine (Y) to cysteine (C) in the deduced amino acid sequences AP2-3 and AP2-4 respectively (Fig. A5). In addition, both *GluA3-21* and *GluA3-22* had an in-frame stop codon (TAA) at the 306th position, resulting in the termination of the gene translation ahead of completion.

GluA3-3 gene had six allelic variants, designated as *GluA3-31*, *GluA3-32*, *GluA3-33*, *GluA3-34*, *GluA3-35* and *GluA3-36* (GenBank

accessions FJ549939, FJ549940, FJ549941, FJ549942, FJ549943 and FJ549944) with the coding sequences ranging from 996 bp (*GluA3-31*) to 1011 bp (*GluA3-36*) (Fig. A6). *GluA3-31* was cloned in the varieties containing protein mobility alleles *a* and *c*, and the others were amplified from the varieties with *GluA3b*, *d*, *e*, *f* and *g*, respectively. Compared with *GluA3-31* in the coding region, 6, 32, 19, 5 and 18 SNPs, and 1, 3, 3, 1 and 3 InDels were detected in *GluA3-32*, *GluA3-33*, *GluA3-34*, *GluA3-35* and *GluA3-36*, respectively. Particularly, the mutation at the 658th position of *GluA3-32* resulted in the change from cysteine (C) to tyrosine (Y) in the deduced peptide AP3-2 (Fig. A7). The SNP at positions 870, 1011 and 337 in *GluA3-32*, *GluA3-33* and *GluA3-34*, respectively, and two SNPs at the 337th and 729th position in *GluA3-36* were nonsense mutations. In addition, the sequences at the positions 297–299 in *GluA3-31*, *GluA3-32* and *GluA3-35* were stop codons, which might result in the termination of the gene translation.

3.2. Characterization of *GluA3-1*, *GluA3-2* and *GluA3-3* genes and their deduced amino acids

All the three *Glu-A3* genes identified contain TATA motif, initiation codon ATG and termination signal with double codons TGA-TAA, and the alleles of *GluA3-1* and *GluA3-2* also have AATAAA polyadenylation in the downstream domain. Sequence alignments showed that the nucleotide identity among the three genes was 80.9–93.4%, and 96.3–99.9% among the allelic variants of each gene.

The deduced amino acid sequences of the 17 allelic variants showed that the seven allelic variants of *GluA3-1* and two of *GluA3-2* (*GluA3-23* and *GluA3-24*) contained an uninterrupted open reading frame (ORF), and the others belonged to pseudogenes with one or two stop codons in the ORF. Each deduced amino acid sequence had a highly conserved signal peptide of 20 amino acids and a short N-terminal conserved domain, which was followed by an N-terminal repetitive domain and a C-terminal conserved domain including three sub-regions of cysteine-rich, glutamine-rich and final conserved domain. All the deduced amino acid sequences included a typical of eight cysteines, except nine cysteines found in AP1-5 with an extra in signal domain and seven in AP2-1, AP2-2 and AP3-2. Based on the N-terminal domain, *GluA3-1* and *GluA3-3* were classified into group 1 and *GluA3-2* into group 2 (Long et al., 2005), respectively.

3.3. Gene-specific PCR markers for different *Glu-A3* protein alleles

To discriminate the materials containing different *Glu-A3* alleles *a*, *b*, *c*, *d*, *e*, *f* and *g*, seven STS markers were developed based on the SNPs detected among the allelic variants of *Glu-A3* genes. In the six NILs and Glenlea, the marker *gluA3a* amplified a 529-bp specific PCR product in Aroona-A3a with the *Glu-A3a* protein allele (Fig. 1a).

Table 2

PCR conditions for different multiplex PCRs.

Multiplex PCR	Marker ^a	Target alleles	Amount of primer (pmol) ^b	PCR condition
<i>Glu-A3b</i> + <i>Glu-A3f</i>	<i>gluA3b</i>	<i>Glu-A3b</i> , <i>f</i>	4	94 °C/35s–60 °C/45s–72 °C/90s
	<i>gluA3f</i>		4	
<i>Glu-A3d</i> + <i>Glu-A3f</i>	<i>gluA3d</i>	<i>Glu-A3d</i> , <i>f</i>	4	94 °C/35s–60 °C/45s–72 °C/90s
	<i>gluA3f</i>		4	
<i>Glu-A3d</i> + <i>Glu-A3g</i>	<i>gluA3d</i>	<i>Glu-A3d</i> , <i>g</i>	3	94 °C/35s–58 °C/45s–72 °C/90s
	<i>gluA3g</i>		5	
<i>Glu-A3b</i> + <i>Glu-A3e</i>	<i>gluA3b</i>	<i>Glu-A3b</i> , <i>e</i>	3	94 °C/35s–58 °C/45s–72 °C/90s
	<i>gluA3e</i>		4	

^a The sequences of the primers are in Table 1.

^b The PCR volume is 20 µl.

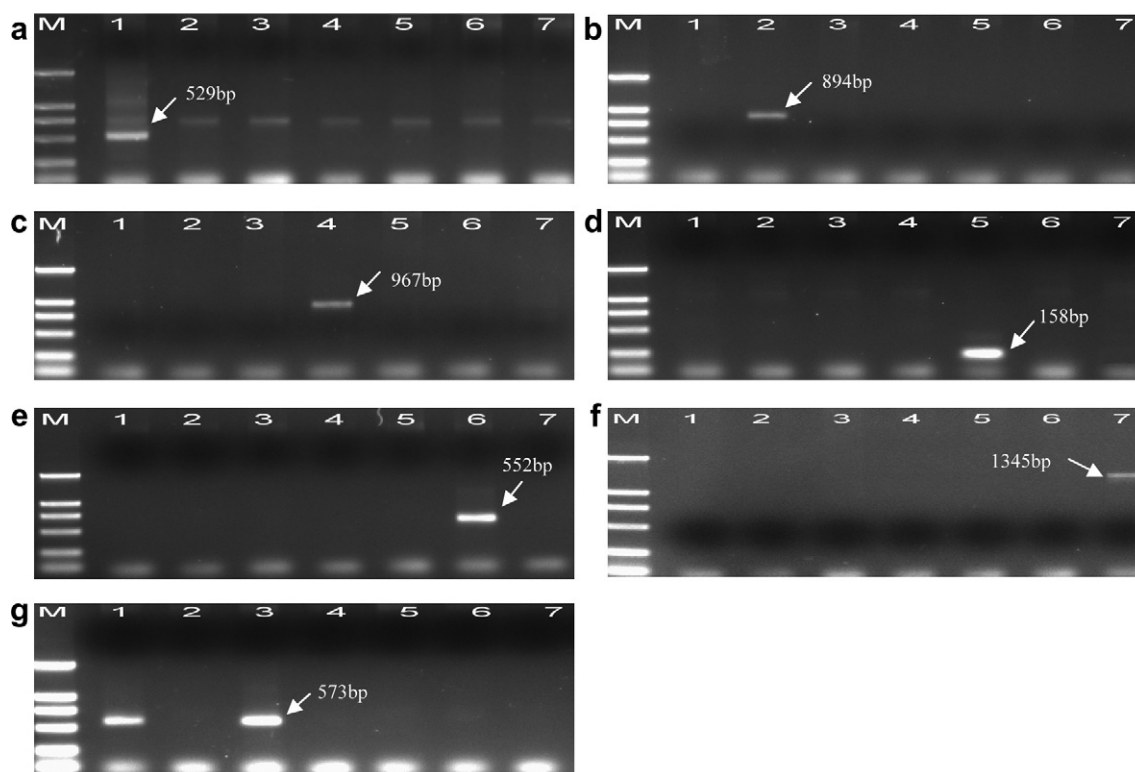


Fig. 1. Electrophoresis of PCR products of the six NILs and Glenlea on agarose gels using seven allele-specific markers. **a** *gluA3a*, **b** *gluA3b*, **c** *gluA3d*, **d** *gluA3e*, **e** *gluA3f*, **f** *gluA3g*, **g** *gluA3ac*. Materials used as PCR templates were: 1 Aroona-A3a (*Glu-A3a*), 2 Aroona-A3b (*b*), 3 Aroona (*c*), 4 Aroona-A3d (*d*), 5 Aroona-A3e (*e*), 6 Aroona-A3f (*f*), 7 Glenlea (*g*). M DNA Ladder 2000 (100, 250, 500, 750, 1000 and 2000 bp).

The marker *gluA3b* was designed for the *Glu-A3b* allele in Aroona-A3b, generating an 894-bp unique band (Fig. 1b). The marker *gluA3d* was used to identify the *Glu-A3d* allele in Aroona-A3d, producing a 967-bp PCR product (Fig. 1c). For the *Glu-A3e* allele in Aroona-A3e, the marker *gluA3e* generated a 158-bp band (Fig. 1d). To discriminate the *Glu-A3f* allele from the others, the marker *gluA3f* was developed, generating a 552-bp band in Aroona-A3f (Fig. 1e). In Glenlea with *Glu-A3g* allele, a 1345-bp specific PCR product was amplified with the marker *gluA3g* (Fig. 1f). Since it was difficult to design a specific primer set for *Glu-A3c*, the marker *gluA3ac* was developed to amplify both *Glu-A3a* and *Glu-A3c* in Aroona-A3a and Aroona (Fig. 1g), and this marker can be used to identify *Glu-A3c* in combination with the marker *gluA3a*.

3.4. Validation of the *Glu-A3* allele-specific markers and multiplex-PCR systems

Six Aroona NILs and Glenlea used in the cloning of *Glu-A3* genes, and additional 141 CIMMYT wheat varieties and advanced lines were used to validate the seven *Glu-A3* allele-specific markers (Table A2). The results showed that the same *Glu-A3* alleles were identified with the seven markers as those with SDS-PAGE in 121 varieties and lines. Nine genotypes with *Glu-A3e* allele in SDS-PAGE were detected to be *Glu-A3f*, and two *Glu-A3b* genotypes were assumed to be *Glu-A3g* by molecular markers, and the inconsistent results were also found in the other nine lines related to the *Glu-A3c* allele.

For the sake of more rapid and economical PCR-based assays, four multiplex PCRs were established, including *Glu-A3b* + *Glu-A3f*, *Glu-A3d* + *Glu-A3f*, *Glu-A3d* + *Glu-A3g*, and *Glu-A3b* + *Glu-A3e* (Table 2) (Fig. 2). According to the length heterogeneity of PCR fragments in detecting different alleles by these multiplex-PCR

systems, the PCR products of *Glu-A3d* + *Glu-A3f* and *Glu-A3b* + *Glu-A3e*, or those of *Glu-A3b* + *Glu-A3f* and *Glu-A3d* + *Glu-A3g* can be mixed together for electrophoresis on an agarose gel (Fig. 3). These multiplex-PCR systems were validated on 141 CIMMYT wheat varieties and advanced lines. The results showed that the multiplex PCRs worked stably, and could efficiently discriminate the targeting alleles in these materials.

4. Discussion

4.1. Comparison of *GluA3-1*, *GluA3-2* and *GluA3-3* with other *Glu-A3* genes in GenBank

To date, about 40 *Glu-A3* genes or mRNA sequences have been registered in GenBank (An et al., 2006; Gao et al., 2007; Huang and Cloutier, 2008; Ikeda et al., 2002; Li et al., 2008; Van Campenhout et al., 1995; Zhang et al., 2007, 2004). The sequences with complete coding region were selected to compare with the three *Glu-A3* genes isolated in this study. The alignment indicated that the sequences of *GluA3-1* were highly similar to the gene cloned by Zhang et al. (2004); especially, *GluA3-11* and AY453154, *GluA3-12* and AY453155, *GluA3-13* and AY453156 had the identical coding sequences. The gene *GluA3-2* was 97.7–99.5% identical to the sequences of AB062868, AB062869, AB062870, AB062871 and AJ293099 in GenBank, and the highest similarity was present between *GluA3-23* and AB062868. *GluA3-3* shared 96.4–99.9% sequence identity with EF426565, and *GluA3-36* had only two SNP differences from EF426565 in the upstream non-coding domain. The alignment also showed that most of the *Glu-A3* gene sequences from common wheat in GenBank could be clustered into three groups, and each group corresponded to certain genes cloned in this study with the similarity over 96.0%. Compared with the

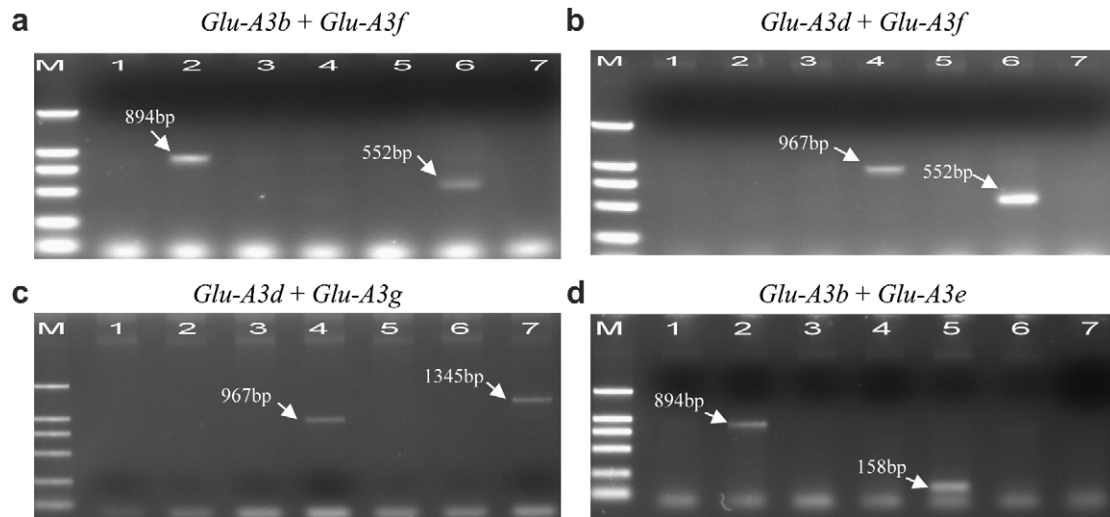


Fig. 2. Electrophoresis of PCR products of the four multiplex-PCR products (*Glu-A3b + Glu-A3f*, *Glu-A3d + Glu-A3f*, *Glu-A3d + Glu-A3g* and *Glu-A3b + Glu-A3e*). The PCR templates are as follows: 1 Aroona-A3a (*Glu-A3a*), 2 Aroona-A3b (*b*), 3 Aroona (*c*), 4 Aroona-A3d (*d*), 5 Aroona-A3e (*e*), 6 Aroona-A3f (*f*), 7 Glenlea (*g*). M DNA Ladder 2000 (100, 250, 500, 750, 1000 and 2000 bp).

nucleotide identity of the allelic variants of each gene at *Glu-B3* (98.3–100%) and *Glu-D3* (99.3–100%) loci (Wang et al., 2009; Zhao et al., 2006, 2007), it seems more divergences occurred in the allelic variants at the *Glu-A3* locus (96.3–99.9%).

4.2. Characterization of the deduced amino acid sequences of *GluA3-1*, *GluA3-2* and *GluA3-3*

Based on the first N-terminal amino acid of the mature protein, LMW-GS were divided into three types of LMW-m, LMW-s and LMW-i, corresponding to methionine, serine and isoleucine, respectively (Lew et al., 1992). The LMW-i type lacks the N-terminal

region, starting directly with the repetitive domain after the signal sequence, with ISQQQ- being the deduced N-terminal sequence (D'Ovidio and Masci, 2004). In this study, the deduced proteins of gene *GluA3-1* could be classified as LMW-i type subunits. For gene *GluA3-2*, the deduced amino acid sequences AP2-3 and AP2-4 started with MDTSCIP-. The genes with the same N-terminus in the deduced polypeptide were first identified by D'Ovidio et al. (1992), and these were also observed by Lee et al. (1999) and Ikeda et al. (2002). However, it's still not clear whether they were LMW-m or LMW-s type subunits because no LMW-s with such sequence has been reported so far. AP2-1 and AP2-2 were deduced from pseudogenes, and started with MDTSYIP- in the N-terminal region. AP3

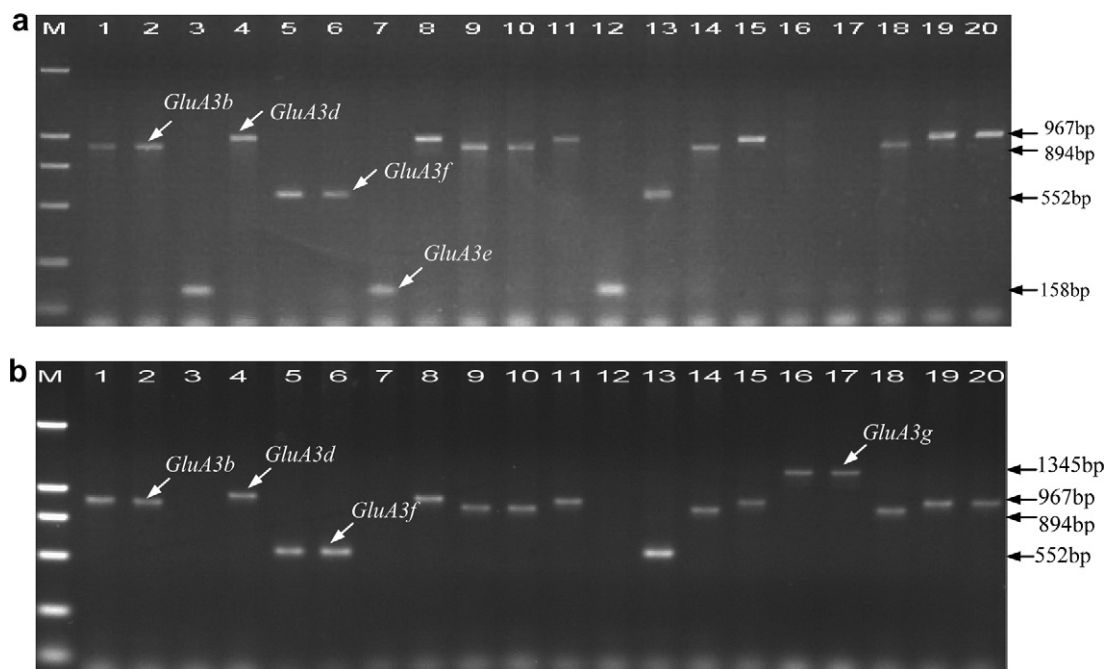


Fig. 3. Electrophoresis of PCR products on agarose gels by mixing PCR products from two multiplex PCRs. **a** *Glu-A3d + Glu-A3f* plus *Glu-A3b + Glu-A3e*, **b** *Glu-A3b + Glu-A3f* plus *Glu-A3d + Glu-A3g*. 1 entry No. 10 in Table A2, 2 No. 11, 3 No. 12, 4 No. 17, 5 No. 40, 6 No. 41, 7 No. 89, 8 No. 90, 9 No. 93, 10 No. 94, 11 No. 98, 12 No. 104, 13 No. 105, 14 No. 107, 15 No. 118, 16 No. 120, 17 No. 121, 18 No. 131, 19 No. 135, 20 No. 141, M DNA Ladder 2000 (100, 250, 500, 750, 1000 and 2000 bp).

contains six sequences, and all of them were deduced from the pseudogene *GluA3-3* with ISQQQ- at the N-terminus.

It is well known that the typical LMW-GS sequences have eight cysteine residues, which are present in the glutenin subunits contributing to the formation of intra- or inter-molecular disulfide bonds. In the deduced mature protein of AP1, all the eight cysteines located in the C-terminus, which differ from LMW-m and LMW-s subunits with a cysteine residue at N-terminus or in repetitive domain (Wang et al., 2009; Zhao et al., 2006, 2007). In general, the first and the seventh cysteines form the inter-molecular disulfide bond while the remaining form three intra-molecular disulfide bonds (Lew et al., 1992; Masci et al., 1998). Thus, the secondary and three-dimensional structures of the mature protein of AP1 would be different from those of LMW-m and LMW-s subunits, and such a structure may have an impact on the visco-elastic properties of protein (Cloutier et al., 2001).

Though just a few cysteine mutants of LMW-GS with α -, γ - and ω -gliadin like sequences were published in GenBank (Anderson et al., 2001; D'Ovidio et al., 1991), it seems more cases occurred in *Glu-A3* genes. An et al. (2006) reported a LMW-i type subunit gene cloned from cultivated einkorn (*Triticum monococcum* L., A^mA^m), containing nine cysteines with an extra one in the C-terminus. In this study, the first cysteine in AP2-1, AP2-2 and AP3-2 changed into tyrosine compared with other allelic variants; an extra cysteine located in the conserved signal peptide domain of AP1-5 was also found. Interestingly, AP1-5 was the deduced protein of *GluA3-15* isolated from Aroona-A3e with *Glu-A3e* allele. However, no protein product was identified on SDS-PAGE (Zhang et al., 2004). The explanation can be that the extra cysteine affects the processing of the premature protein, or prevents the signal peptides from constructing a functional structure.

The LMW-glutenins were classified as B- and C-subunits based on their electrophoretic mobilities on SDS-PAGE (D'Ovidio and Masci, 2004), and their molecular weights were 40–50 and 30–40 kDa, respectively (Payne and Corfield, 1979). For the nine allelic variants with uninterrupted ORFs at the *Glu-A3* locus, the predicted molecular weights of the seven deduced peptides of *GluA3-1* were AP1-5 (42.7 kDa) = AP1-7 (42.7 kDa) > AP1-2 (42.2 kDa) > AP1-1 (41.3 kDa) > AP1-3 (41.2 kDa) > AP1-6 (39.0 kDa) > AP1-4 (38.9 kDa). Their corresponding subunits may be ranked in $e = g > b > a > c > f > d$ on SDS-PAGE, and belong to B-subunits. AP2-3 and AP2-4 were 32.2 and 32.5 kDa, respectively, and they can be classified into C-subunits. However, the molecular weight may not predict the electrophoretic mobility of subunits precisely, because it was affected by the peptide structure, and often overestimated by SDS-PAGE (Zhang et al., 2004). In fact, *Glu-A3e* is a null allele (Gupta and Shepherd, 1990), and Gupta and MacRitchie (1994) also showed that this allele in the bread wheat variety Halberd didn't produce any major LMW subunits. This indicated that the allelic variant *GluA3-15* amplified from Aroona-A3e (*Glu-A3e*) might have some mutations in the promoter region, and thus can't encode a peptide as expected. The corresponding relationships between the deduced peptides of genes and the subunits still need to be studied.

4.3. The gene compositions of the *Glu-A3* protein mobility alleles

Previously, we characterized 4 and 6 genes at *Glu-B3* and *Glu-D3* loci respectively (Wang et al., 2009; Zhao et al., 2006, 2007), and found each LMW-GS protein allele was corresponding to 2–6 genes. Similar results were also obtained at the *Glu-A3* locus as shown in Table 3, and an isogenic line with one protein allele may have more than one coding genes. Initially, the LMW-GSs were defined by protein electrophoretic mobility on SDS-PAGE for the recognized bands (Gupta and MacRitchie, 1991; Gupta and

Table 3
Relationship between *Glu-A3* protein mobility alleles and *Glu-A3* haplotypes.

NIL/Variety	<i>Glu-A3</i> allele ^a	Haplotype ^b																
		<i>GluA3-11</i>	<i>GluA3-12</i>	<i>GluA3-13</i>	<i>GluA3-14</i>	<i>GluA3-15</i>	<i>GluA3-16</i>	<i>GluA3-17</i>	<i>GluA3-21</i> ^c	<i>GluA3-22</i> ^c	<i>GluA3-23</i>	<i>GluA3-24</i>	<i>GluA3-31</i> ^c	<i>GluA3-32</i> ^c	<i>GluA3-33</i> ^c	<i>GluA3-34</i> ^c	<i>GluA3-35</i> ^c	<i>GluA3-36</i> ^c
Aroona-A3a	a	+							+									
Aroona-A3b	b		+							+				+				
Aroona	c			+						+			+					
Aroona-A3d	d				+						+				+			
Aroona-A3e	e					+				+						+		
Aroona-A3f	f						+			+							+	
Aroona-A3g	g							+				+						+

^a *Glu-A3* alleles defined by protein electrophoretic mobility in SDS-PAGE.

^b "+" means the allelic variant is present in the corresponding NIL or variety.

^c The allelic variants belong to pseudogenes.

Shepherd, 1990). One LMW glutenin allele is corresponding to one or more protein bands, which are in agreement with our results that a line with one protein allele has more than one coding gene, as indicated in this and previous studies (Wang et al., 2009; Zhao et al., 2006, 2007). Although each *Glu-A3* protein allele corresponded to three allelic variants, only 1 or 2 genes might encode peptides, and 8 of 17 allelic variants were pseudogenes (Table 3), which may explain why fewer bands were involved in *Glu-A3* protein alleles, compared with *Glu-B3* and *Glu-D3* alleles (Gupta and MacRitchie, 1991; Gupta and Shepherd, 1990).

In the present study, *Glu-A3d* and *Glu-A3g* genotypes comprise two functional genes, respectively, and the other alleles have only one. Ikeda et al. (2006) also found four spots for *Glu-A3d* allele in Norin 61, but only one for the alleles *Glu-A3a*, *b*, *c* and *f* using two-dimensional gel electrophoresis (2-DE), indicating that more genes are associated with the *Glu-A3d* protein allele. Such gene composition may be associated with the different role of the alleles on wheat quality. Previous studies demonstrated that the wheat lines with the *Glu-A3d* allele had good SDS-sedimentation value and extensibility (Branlard et al., 2001; Gupta and MacRitchie, 1991). He et al. (2005) indicated *Glu-A3d* was better than other alleles on dry white Chinese noodle quality and dough strength. For *Glu-A3g*, though two functional genes were detected too, little knowledge was available about its contribution to wheat quality, but Glenlea (*Glu-A3g*) was a bread wheat with extra strong dough extensibility (Lukow and Townley-Smith, 1995).

4.4. Functional markers for the discrimination of *Glu-A3* protein mobility alleles

In many breeding programs, laboratory testing or bioassay was required for the selection of breeding lines with enhanced performance, and traits such as LMW-GS are laborious and costly to be evaluated. Functional markers, derived from polymorphic sites within genes that causally affect phenotypic trait variation and are independent of breeding stages, would be ideal tools for marker-assisted breeding (Bagge et al., 2007; Gale, 2005). Nevertheless, the practicality and precision of many of these markers still require additional verification (Bagge et al., 2007). Zhang et al. (2004) reported a set of markers that can be used to discriminate the protein alleles *Glu-A3a*, *b*, *c*, *d*, *e*, *f* and *g*. In this study, we developed an alternative set of STS markers for different *Glu-A3* alleles including an allele-specific marker for *Glu-A3b*, which was not involved in Zhang et al. (2004), and all of them can be performed easily in PCR. The validation test showed that all the markers can be used easily with stable results, and the same results as SDS-PAGE can be obtained in most of the materials. Ikeda et al. (2006) indicated that the standard variety Insignia for *Glu-A3e* should be classified as *Glu-A3f*. This was confirmed by the fact that the nine lines with *Glu-A3e* allele in SDS-PAGE were detected to be *Glu-A3f* with the STS markers in this study. In addition, some varieties also showed inconsistencies in different studies. For example, Champlein, a French variety, was assumed to have *Glu-A3c* in SDS-PAGE by Gupta et al. (1994), but it was determined as *Glu-A3a* by Branlard et al. (2003); Bluesky, a Canadian variety, was detected to contain *Glu-A3b* allele in Gupta et al. (1994), but defined as *Glu-A3g* (Békés et al., 2008). In this study, two lines with *Glu-A3b* allele in SDS-PAGE were detected to be *Glu-A3g* genotype by molecular markers, and nine inconsistent cases related to *Glu-A3c* were also found. Therefore, the *Glu-A3* alleles in some materials defined by SDS-PAGE should be reassigned by the STS markers.

Despite the important role of the LMW-GS in wheat quality, the methods for discriminating LMW-GS were the bottleneck for investigating the effect of allelic variants. In our previous study, ten STS markers for *Glu-B3* alleles were developed (Wang et al., 2009),

and seven STS markers for *Glu-A3* alleles were also designed in this study. These markers can provide an efficient tool to study the effect of LMW-GS. Especially, the four multiplex PCRs established and validated may be helpful for marker-assisted breeding to screen the *Glu-A3* alleles in a great mass of individuals or lines.

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Appendix. Supplementary material

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