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Characterisation and marker development for low molecular weight glutenin genes from *Glu-A3* alleles of bread wheat (*Triticum aestivum.* L)

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Abstract PCR was used to amplify low-molecular-weight (LMW) glutenin genes from the *Glu-A3* loci of hexaploid wheat cultivars containing different *Glu-A3* alleles. The complete coding sequence of one LMW glutenin gene was obtained for each of the seven alleles *Glu-A3a* to *Glu-A3g*. Chromosome assignment of PCR products using Chinese Spring nulli-tetrasomic lines confirmed the amplified products were from chromosome 1A. All sequences were classified as LMW-i-type genes based on the presence of an N-terminal isoleucine residue and eight cysteine residues located within the C-terminal domain of the predicted, mature amino acid sequence. All genes contained a single uninterrupted open reading frame, including the sequence from the *Glu-A3e* allele, for which no protein product has been identified. Comparison of LMW glutenin gene sequences obtained from different alleles showed a wide range of sequence identity between the genes, with between 1 and 37 single nucleotide polymorphisms and between one and five insertion/deletion events between genes from different alleles. Allele-specific PCR markers were designed based on the DNA polymorphisms identified between the LMW glutenin genes, and these markers were validated against a panel of cultivars containing different *Glu-A3* alleles. This collection of markers represents a valuable resource for use in marker-assisted breeding to select for specific alleles of this important quality-determining locus in bread wheat.

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Introduction

Low-molecular-weight (LMW) glutenins represent approximately 40% of the total wheat gluten fraction, and their molecular weight is estimated to range from 30 kDa to 50 kDa (Ciaffi et al. 1999). The LMW glutenins have been found to have a pronounced effect in determining the physical properties of flour during bread-making (Payne et al. 1987; Gupta et al. 1989; Gupta and McRitchie 1994) and pasta-making (Pogna et al. 1990; Ruiz and Carrillo 1995). LMW glutenins are encoded at the *Glu-A3, Glu-B3* and *Glu-D3* loci on the short arms of group 1 homeologous chromosomes (Gupta and Shepherd 1990). The investigation of the influence of allelic variation of LMW glutenins on flour quality has received less attention than the effects of variation at the *Glu-A1, Glu-B1* and *Glu-D1* high-molecular-weight (HMW) glutenin loci located on the long arms of group 1 chromosomes (Payne et al. 1987). This may be attributed largely to the difficulty in scoring alleles of this complex multigene family by protein analysis (see below).

The effects of LMW-glutenin allelic composition on dough strength and extensibility have been demonstrated for both bread and durum wheat, although these effects are largely dependent on the association of *Glu-3* and *Glu-1* loci (Payne et al. 1987; Gupta et al. 1989, 1994; Nieto-Taladriz et al. 1994; Ruiz and Carrillo 1995; Sontag-Strohm et al. 1996; Redaelli et al. 1997). Characterisation of specific alleles at the *Glu-3* loci has been conducted with respect to their effect on dough resistance and extensibility recently (Wesley et al. 1999; Brites and Carrillo 2001; Luo et al. 2001; Wesley et al. 2001). Several *Glu-3* alleles have been ranked in terms of their dough quality effects (Gupta and Shepherd 1988; Gupta et al. 1989, 1990a, 1990b, 1991, 1994; Metakovsky et al. 1990). For R_{max} (maximum dough resistance, an indicator of dough strength), the ranking of alleles at *Glu-A3* was *b>d>e>c*; the alleles of *Glu-B3*, *i>b=a>e=f=g=h>c*; the alleles of *Glu-D3*, *e>b>a>c>d*. Cornish et al. (1993) found the *Glu-3* composition *bbb* (at loci *Glu-A3*, *Glu-B3* and *Glu-D3*, respectively) gave the best extensibility.

Glu-3 composition *bbc* also had excellent extensibility. *Glu-A3e*, *Glu-B3c*, *d* and *g* alleles were associated with medium to weak dough properties.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is currently the most widely used method for determining LMW glutenin allelic composition of wheat cultivars and lines (Jackson et al. 1996). However, the difficulty of resolving the large number of LMW glutenin subunits (LMW-GS) expressed in hexaploid wheat has hindered the selection of specific LMW glutenin alleles in wheat breeding programs. Other fractionation techniques, with greater discriminating power, such as 2-D electrophoresis (Jackson et al. 1983; Skylas et al. 2000) and RP-HPLC (Burnouf and Bietz 1984; Marchylo et al. 1989; Sutton 1991; Margiotta et al. 1993) are available for LMW glutenin analysis. However, these are not routinely used in wheat breeding programs due to the complexity and the techniques and the problem of overlapping fractions (Weegels et al. 1996).

Gupta and Shepherd (1990) conducted an extensive survey of LMW glutenin proteins in hexaploid wheat cultivars and detected 20 different banding patterns: 6 controlled by *Glu-A3*, 9 by *Glu-B3* and 5 by *Glu-D3*. The complexity of the banding patterns varied widely, with some cultivars showing no LMW glutenin protein detected from the *Glu-A3* locus (*Glu-A3e*), and some *Glu-B3* banding patterns consisting of at least eight distinct bands (*Glu-B3 h* and *i*). Thirty-nine different LMW subunits were identified in one bread wheat cultivar based on N-terminal amino acid sequences (Lew et al. 1992). Cassidy et al. (1998) estimated that the copy number of LMW glutenin-like DNA sequences is 30–40 in another bread wheat cultivar based on Southern blotting analysis. Approximately 60 genes or pseudogenes of the LMW-GS family have been cloned and sequenced from seven hexaploid wheat cultivars at this time (Bartels and Thompson 1983; Pitts et al. 1988; Colot et al. 1989; Cassidy et al. 1998; Benmoussa et al. 2000; Cloutier et al. 2001; Ikeda et al. 2002). These sequences were classified into 12 groups based on the alignment of the N- and C-terminal conserved domains of the deduced amino-acid sequences (Ikeda et al. 2002). Based on the gene sequence polymorphisms between LMW glutenin genes, an LMW glutenin-specific PCR marker (D'Ovidio 1993) was developed to distinguish durum wheat cultivars according to their quality characteristics and a microsatellite marker has been developed for use in mapping of hexaploid wheat (Devos et al. 1995). The *Glu-3* LMW glutenin loci are tightly linked to the *Gli-1* loci that encode γ and ω gliadins (Brown and Flavell 1981). Linkage of a γ-gliadin allele with gluten strength in durum wheat, possibly due to the linkage of the *Gli-1* and *Glu-3* loci, has been reported (Payne et al. 1984). The gene sequences in the complex γ-gliadin gene family of bread wheat have been used to identify single nucleotide polymorphisms (SNPs) and insertion/deletions (InDels) specific for individual genes (Zhang et al. 2003). Allele-specific PCR markers based on SNPs of γ-gliadin genes were subsequently used to detect specific alleles linked to the *Glu-A3*, *Glu-B3* and *Glu-D3* LMW glutenin loci, respectively (Zhang et al. 2003). InDels have also been reported in the LMW glutenin gene family recently (Benmoussa et al. 2000; Ikeda et al. 2002). Here, we describe the isolation and characterisation of LMW glutenin genes from different *Glu-A3* alleles of bread wheat and the subsequent development of PCR markers to facilitate marker assisted selection of specific alleles of this important quality-determining locus.

Materials and methods

Plant materials

Wheat germplasm included in this study was maintained at CSIRO Plant Industry or obtained from the Australian Winter Cereals Collection in Tamworth, Australia. Chinese Spring euploid and its nulli-tetrasomic lines were kindly provided by Dr. E. Lagudah (CSIRO Plant Industry, Canberra) and derived from stocks described in Sears and Miller (1985).

Allelic composition at the *Glu-A3* loci by SDS-PAGE

The *Glu-A3* allelic compositions of all wheat cultivars used in this study were confirmed by SDS-PAGE as described by Zhang et al. (2003). The cultivars used as DNA template for amplification and sequencing of LMW-GS genes were Chinese Spring (*Glu-A3a*), Gabo (*Glu-A3b*), Cheyenne (*Glu-A3c*), Suneca (*Glu-A3d*), Halberd (*Glu-A3e*), Rescue (*Glu-A3f*) and Glenlea (*Glu-A3g*).

DNA isolation and PCR amplification

DNA was isolated from pools of five 3- to 5-day-old seedlings with a modified CTAB procedure (Gale et al. 2001). PCR was performed using Hotstar *Taq* polymerase (1 U, Qiagen) in 10 μL of reaction buffer (Qiagen, containing 1.5 mM $MgCl_2$) containing 50 ng genomic DNA, 100 μM of each dNTP and 5 pmol of each PCR primer. PCR was 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 1 min.

Chromosome assignment of specific PCR markers

Assignment of specific PCR markers to specific chromosomes was accomplished through PCR amplification using template DNA from each of the three Chinese Spring, group 1 nulli-tetrasomic lines: N1AT1B, N1BT1A and N1DTA.

Genome walking

The method described by Siebert et al. (1995) was applied with some modification. Genomic DNA of wheat cv. Halberd (*Glu-A3e* allele) was digested with restriction endonucleases *Dra*I, *Eco*RV, *Pvu*II or *Stu*I (New England Biolabs). The digested genomic DNA was ligated with adaptor (Universal GenomeWalker Kit, Clontech PT3042–2) and nested PCR performed using the supplied adaptor-specific primers (AP1 and AP2) in combination with the *Glu-A3e* gene-specific primers GluA3eF1 (5′-ACTCGGGCAACAACCT-CAACAACAACAG-3′) and GluA3eF2 (5′-CCTTTTTGCAGC-CACACCAGATAGCCCAA-3′), respectively. PCR amplifications were performed as described in the manufacturer's protocol using Hotstar *Taq* polymerase (Qiagen) and with the addition of incubation at 94°C for 3 min before the cycling. The PCR products were directly sequenced as described below.

Sequencing of amplification products

Individual amplification products (2 μl) were treated with 0.25 U shrimp alkaline phosphatase (USB) and 2.5 U exonuclease I (USB) at 37°C for 30 min and followed by 80°C for 15 min in PCR reaction buffer (Qiagen, 1.5 mM $MgCl_2$). The PCR products were then sequenced using the appropriate PCR primers (the same primers as used for PCR and a primer GluA3R3 (5′-GGATTG-CTCGGGGATTTGCC-3′ common to all public LMW-i-type sequences) using BigDye terminator mix (Applied Biosystems) as per the manufacturer's instructions. The cycle sequencing reactions were performed on a FTS-1 Thermal Sequencer (Corbett Research) using 25 cycles of (95°C for 30 s, 50°C for 20 s and 60°C for 1 min). The extension products were precipitated by addition of 1/10 vol. of 3M sodium acetate pH 5.2 and 2 vol. of ethanol and sequenced on an ABI PRISM 377XL Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Allele-specific PCR primer design

Allele-specific PCR primers were designed manually from DNA sequence alignments as described previously (Zhang et al. 2003).

Results

Sequence alignment and *Glu-A3* locus LMW-i-type sequence specific PCR

All publicly available LMW glutenin gene sequences were used for alignment using GCG software (Web-ANGIS). Based on this sequence alignment, five LMW glutenin gene sequences were found to cluster, and these genes were predicted to encode LMW glutenins with an isoleucine amino acid residue at the N-terminus of the mature peptide. The hexaploid wheat cultivars from which these genes were obtained were: cv. Yamhill (X07747, Pitts et al. 1988), cv. Cheyenne (U86030, Cassidy et al. 1998), cv. 1CW (AB008497, Maruyama et al. 1998), cv. Glenlea (pGH3.1, Cloutier et al. 2001) and cv. Norin 61 (AB062876 and AB062878, Ikeda et al. 2002). Two of these genes, pGH3.1 and AB062878, were previously shown to be located at the *Glu-A3* locus on chromosome 1A (Cloutier et al. 2001; Ikeda et al. 2002).

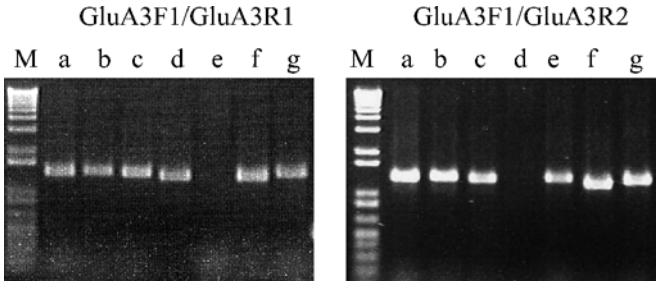


Fig. 1 Locus-specific PCR for low-molecular-weight (LMW)-i-type genes from different *Glu-A3* alleles using primer sets GluA3F1/GluA3R1 and GluA3F1/GluA3R2. Template DNAs were as follows: *a* Chinese Spring (*Glu-A3a*), *b* Gabo (*Glu-A3b*), *c* Cheyenne (*Glu-A3c*), *d* Suneca (*Glu-A3d*), *e* Halberd (*Glu-A3e*), *f* Rescue (*Glu-A3f*), *g* Glenlea (*Glu-A3 g*). The marker used was the 1-kb Plus DNA Ladder (Invitrogen)

Using this information, a primer set (GluA3F1/GluA3R1, Table 1) was designed to facilitate the PCR amplification of the entire open reading frame (ORF) from seven wheat varieties with different *Glu-A3* alleles. Using this primer pair, a single band was amplified from cv. Chinese Spring (*Glu-A3a*), cv. Gabo (*Glu-A3b*), cv. Cheyenne (*Glu-A3c*), cv. Suneca (*Glu-A3d*), cv. Rescue (*Glu-A3f*) and cv. Glenlea (*Glu-A3 g*), but not from cv. Halberd that contains the *Glu-A3e* allele (Fig. 1). A second reverse primer, GluA3R2 (Table 1) located near the 3′ end of the ORF was therefore designed. The primer set GluA3F1/GluA3R2 was used for amplification from the same seven wheat varieties described above. A single band was amplified from the six varieties containing the *Glu-A3 a, b, c, e, f* and *g* alleles, respectively, although no amplification product was obtained from cv. Suneca (*Glu-A3d* allele, Fig. 1).

Table 1 Bread wheat LMW-GS gene locus or allele-specific PCR markers. *Bolded* Allele-specific SNPs, *underlined* additional mismatched nucleotide, # specific for *Glu-A3 a, b, c* and *g* alleles, * specific for *Glu-A3 a, b, c* and *f* alleles. All primer sequences are 5′ to 3′

Marker name	Primers	Primer sequence (5′-3′)	Product length (bp)	Specific loci or alleles
GluA3-1	GluA3F1 GluA3R1	GTACGCTTTTGTAGCTTGTGC TCCATCGACTAAACAACGGAGA	1,414	*Glu-A3*
GluA3-2	GluA3F1 GluA3R2	As above GATGCCAACGCCTAATGGCACAC	1,346	*Glu-A3*
GluA3a	GluA3F1 GluA3aR	As above TGGTGGTTGTTGTTGTTGCTAC**A**	596	*Glu-A3a*
GluA3abc	GluA3bF GluA3bR	CACAATTTTCACAGCAACAGCA**G** # ggcacattgacactacacatt**g** *	823	*Glu-A3a, b, c*
GluA3ac	GluA3bF GluA3cR	As above TTGGTGGCTGTTGTGAAGACG**A**	196	*Glu-A3a, c*
GluA3d	GluA3dF GluA3dR	ACCAGTTATTCATCCATCTGCT**C** GTGGTTTCGTACAACGGCTC**G**	488	*Glu-A3d*
GluA3e	GluA3eF GluA3R2	CAATGAA**A**ACCTTCCTCGTCT**G** As above	1,151	*Glu-A3e*
GluA3f	GluA3F1 GluA3fR	As above GTTGCTGCTACAACTGCTGT**A**	1,101	*Glu-A3f*
GluA3g	GluA3gF GluA3R2	CAGCAGCCACCACATTCGCA**A** As above	861	*Glu-A3g*

Table 2 Numbers of SNPs between different LMW-GS gene sequences obtained from various alleles at the *Glu-A3* locus

Allele	*Glu-A3a*	*Glu-A3b*	*Glu-A3c*	*Glu-A3d*	*Glu-A3e*	*Glu-A3f*	*Glu-A3 g*
Glu-A3a	0	-	-	-	-	-	-
Glu-A3b	6	0	-	-	-	-	-
Glu-A3c	1	5	0	-	-	-	-
Glu-A3d	28	26	27	0	-	-	-
Glu-A3e	25	21	24	37	0	-	-
Glu-A3f	19	19	19	36	29	0	-
Glu-A3 g	18	16	17	34	9	27	0

Table 3 DNA sequence deletions or insertions of LMW-GS gene sequences obtained from various alleles at the *Glu-A3* locus. Positions refer to the consensus sequence of the seven aligned DNA sequences

Positions (bp)	73–96	166–168	394–459	406–486	484–486	481–483	850–861	859–867	1007–1027
Glu-A3a	Del	-	-	-	-	-	-	Del	-
Glu-A3b	-	-	-	-	-	-	-	Del	-
Glu-A3c	Del	-	-	-	-	-	-	Del	-
Glu-A3d	-	Del	-	Del	-	-	Del	-	-
Glu-A3e	-	-	-	-	Ins	-	-	-	-
Glu-A3f	-	-	Del	-	-	Del	-	-	Del
Glu-A3g	-	-	-	-	Ins	-	-	-	-

Chromosome assignment of PCR products

Two primer sets (GluA3F1/GluA3R1 and GluA3F1/GluA3R2) were used to amplify products from Chinese Spring and its group 1 nulli-tetrasomic lines. A single band was amplified with Chinese Spring, N1BT1A and N1DT1A, but no PCR products were amplified with N1AT1B (Fig. 2). These results confirmed that the products were amplified from chromosome 1A, the location of the *Glu-A3* locus.

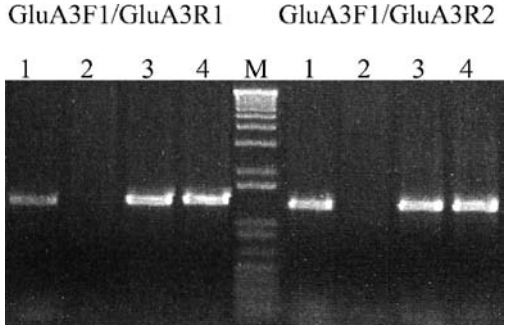


Fig. 2 Determination of LMW-i-type gene location using locus-specific PCR using primer sets GluA3F1/GluA3R1 and GluA3F1/GluA3R2. Template DNAs were as follows: *1* Chinese Spring, *2* CSN1AT1B (Chinese Spring nullisomic 1A, tetrasomic 1B), *3* CSN1BT1A, *4* CSN1DT1A, *M* 1-kb Plus DNA Ladder (Invitrogen)

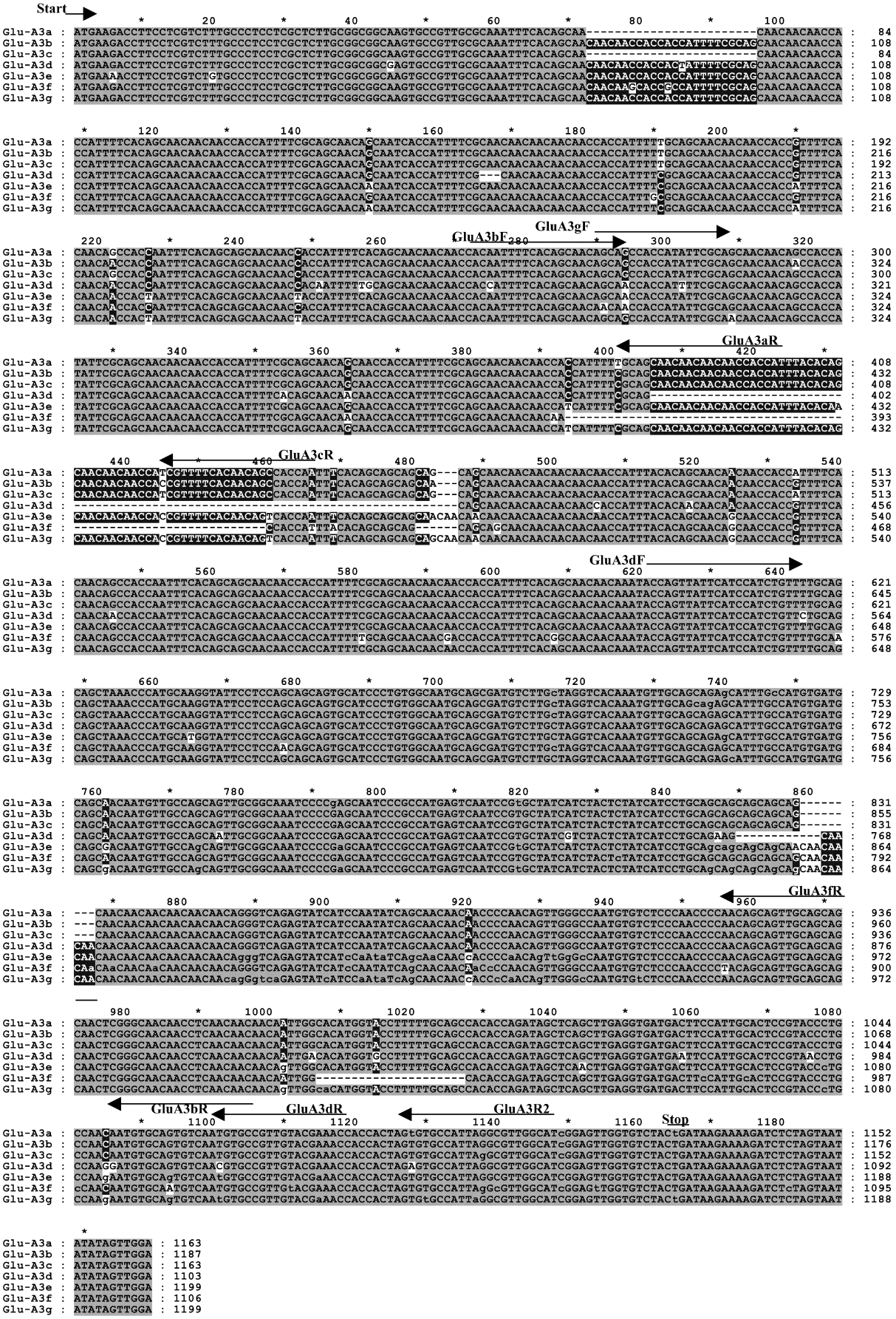
Level of sequence polymorphism between *Glu-A3* genes and relationships to previously characterised genes

The PCR products obtained using the GluA3F1/GluA3R1 and GluA3F1/GluA3R2 primer sets were directly sequenced. The PCR primers were used for sequencing with one additional primer, GluA3R3, required to obtain full DNA sequence from the PCR product obtained from cv. Suneca (*Glu-A3d*, see Materials and methods). DNA sequences of the PCR products were assembled using GCG software.

To obtain the whole ORF sequence from cv. Halberd (*Glu-A3e*), a genomic DNA walking technique was applied as described in Materials and methods. GenBank accession numbers for the genes characterised from Glu-A3a through to Glu-A3g are AY453155 to AY453160, respectively.

The seven DNA sequences derived from cultivars containing different *Glu-A3* alleles in the current study were used to construct a 1,199-bp alignment (Fig. 3). Numerous SNPs and several InDels were found between the LMW glutenin gene sequences obtained in this study (Tables 2 and 3, respectively). The sequences obtained from the cultivars containing the *Glu-A3 a, b* and *c* alleles showed the highest level of sequence identity (Tables 2, 3). Two single-nucleotide differences were identified between the sequence from cv. Glenlea (*Glu-A3 g*) and pGH3.1 (also from Glenlea, Cloutier et al. 2001). The sequence from *Glu-A3e* showed a high level of sequence identity with GenBank sequence AB008497 (Maruyama et al. 1998), with three SNPs and one three-base pair InDel difference. The sequence obtained from cv. Rescue (*Glu-A3f*) showed a high level of sequence identity with GenBank sequence X07747 (Pitts et al. 1988) with one SNP and one three-base pair InDel difference. The sequence from cv. Suneca (*Glu-A3d*) was most similar to AB062878 (Ikeda et al. 2002), with 27 SNPs and four InDels difference.

Fig. 3 Alignment of the seven LMW-i-type gene sequences from *Glu-A3* alleles *a–g*. The positions of allele-specific PCR primers and the start and stop codons are indicated on the alignment. The location for GluAeF lies outside of the alignment shown (−20 to −2 bp)


Start
GluA3bF
GluA3gF
GluA3aR
GluA3cR
GluA3dF
GluA3fR
GluA3bR
GluA3dR
GluA3R2
Stop
Glu-A3a
Glu-A3b
Glu-A3c
Glu-A3d
Glu-A3e
Glu-A3f
Glu-A3g

Design of LMW-GS, allele-specific PCR markers

Based on the SNPs between sequences obtained from different *Glu-A3* alleles, sets of primer pairs (Table 1) were designed to specifically amplify each *Glu-A3* gene where possible. It was not possible to design allele-specific primers from the sequences from the cultivars containing the *Glu-A3 b* and *c* alleles, which required the use of a combination of two primer sets for scoring (see below). Primer set GluA3F1/GluA3aR was used to specifically amplify the 586-bp expected product from cv. Chinese Spring containing the *Glu-A3a* allele (Fig. 4a). Primer set GluA3bF/GluA3bR was shown to amplify the expected product (823 bp) from cvs. Chinese Spring (*Glu-A3a*), Gabo (*Glu-A3b*) and Cheyenne (*Glu-A3c*, Fig. 4b). Primer set GluA3bF/GluA3cR was shown to amplify the expected product (196 bp) from cvs. Chinese Spring (*Glu-A3a*) and Cheyenne (*Glu-A3c*, Fig. 4c). Primer set GluA3dF/GluA3dR was shown to amplify the expected product (488 bp) from cv. Suneca (*Glu-A3d*, Fig. 4d). Primer set GluA3eF/GluA3R2 was shown to amplify the expected product (1,151 bp) from cv. Halberd (*Glu-A3e*, Fig. 4e), Primer set GluA3F1/GluA3fR was shown to specifically amplify a 1,101-bp product from cv. Rescue (*Glu-A3f*, Fig. 4f) and GluA3gF/GluA3R2 produced an 861 bp product from cv. Glenlea (*Glu-A3 g*, Fig. 4g). The seven cultivars above mentioned for gene isolation and ten Australian wheat cultivars containing the *Glu-A3b* allele, ten cultivars with *Glu-A3c*, seven cultivars with *Glu-A3d* and two cultivars with *Glu-A3e* were selected to check the seven allele-specific markers. All the PCR results matched the corresponding *Glu-A3* allele data (Table 4). These results further confirmed that the markers developed here are *Glu-A3* allele specific.

Deduced amino acid sequences of genes from cultivars containing different *Glu-A3* alleles

An alignment of the deduced protein sequences from the seven different genes isolated from cultivars containing *Glu-A3* alleles *a–g* is shown in Fig. 5. All genes contain a single, uninterrupted ORF, including the gene isolated from the cultivar containing the *Glu-A3e* allele, from which no gene product has been identified (Singh and Shepherd 1988). No introns were observed, consistent with the general absence of introns from glutenin genes. Six of the deduced proteins contain an identical, hydrophobic 20 amino acid predicted signal peptide, typical of LMW-GS genes. The predicted protein from the cultivar containing the *Glu-A3e* allele, however, contains a cysteine residue at position 7 in the predicted signal sequence. All predicted mature proteins have an isoleucine at the truncated N-terminus, eight cysteine residues located within the C-terminal domain and two polyglutamine repeats of up to 15 residues, as observed for LMW-i-type proteins reported previously (Cloutier et al. 2001; Ikeda et al. 2002).

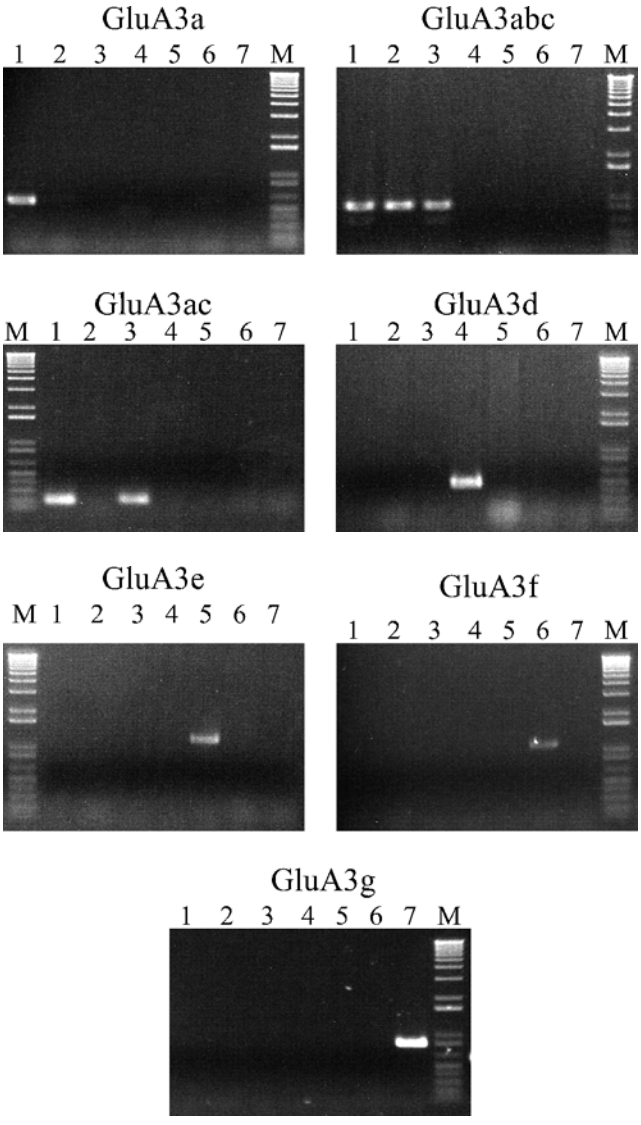


Fig. 4 Allele-specific PCR markers based on SNPs between different *Glu-A3* alleles. Allele specificity is labelled above each gel. *Glu-A3abc* refers to specificity for the three alleles, *a*, *b* and *c*. Wheat varieties used as PCR templates were Chinese Spring (*Glu-A3a*), Gabo (*Glu-A3b*), Cheyenne (*Glu-A3c*), Suneca (*Glu-A3d*), Halberd (*Glu-A3e*), Rescue (*Glu-A3f*) and Glenlea (*Glu-A3g*). PCR product sizes are as listed in Table 1. The marker used was the 1-kb Plus DNA Ladder (Invitrogen)

Comparison of predicted and observed i-type LMW-GS from cultivars containing different *Glu-A3* alleles by SDS-PAGE

Reduced and alkylated glutenin subunits from the cultivars containing *Glu-A3* alleles *a–g* were extracted and fractionated by one-step SDS-PAGE as described previously (Zhang et al. 2003). Molecular mass standards were used to compare mobility of the *Glu-A3* B-type LMW-GS expressed by the different cultivars (Fig. 6). Values obtained are shown in Table 5.

Table 4 Correlation between wheat *Glu-A3* allele-specific PCR markers and LMW-glutenin subunit alleles. + PCR product present, – PCR product absent

Wheat variety	*Glu-A3*	*GluA3a*	*GluA3abc*	*GluA3ac*	*GluA3d*	*GluA3e*	*GluA3f*	*GluA3g*
Banks	*b*	–	+	–	–	–	–	–
Barunga	*c*	–	+	+	–	–	–	–
Batavia	*c*	–	+	+	–	–	–	–
Calingiri	*d*	–	–	–	+	–	–	–
Chinese Spring	*a*	+	+	+	–	–	–	–
Cheyenne	*c*	–	+	+	–	–	–	–
Cunningham	*c*	–	+	+	–	–	–	–
Diamondbird	*b*	–	+	–	–	–	–	–
Frame	*c*	–	+	+	–	–	–	–
Gabo	*b*	–	+	–	–	–	–	–
Glenlea	*g*	–	–	–	–	–	–	+
Goldmark	*c*	–	+	+	–	–	–	–
Gordon	*c*	–	+	+	–	–	–	–
Grebe	*c*	–	+	+	–	–	–	–
Halberd	*e*	–	–	–	–	+	–	–
Harrier	*d*	–	–	–	+	–	–	–
Janz	*b*	–	+	–	–	–	–	–
Kellalac	*c*	–	+	+	–	–	–	–
Kite	*e*	–	–	–	–	+	–	–
Lark	*d*	–	–	–	+	–	–	–
Molineux	*c*	–	+	+	–	–	–	–
Nyabing	*d*	–	–	–	+	–	–	–
Osprey	*c*	–	+	+	–	–	–	–
Pelsart	*b*	–	+	–	–	–	–	–
Perenjori	*d*	–	–	–	+	–	–	–
Rescue	*f*	–	–	–	–	–	+	–
Sunbri	*b*	–	+	–	–	–	–	–
Sunbrook	*b*	–	+	–	–	–	–	–
Sunco	*b*	–	+	–	–	–	–	–
Suneca	*d*	–	–	–	+	–	–	–
Sunelg	*e*	–	–	–	–	+	–	–
Sunkota	*b*	–	+	–	–	–	–	–
Sunland	*b*	–	+	–	–	–	–	–
Sunlin	*d*	–	–	–	+	–	–	–
Sunmist	*b*	–	+	–	–	–	–	–
Wilgoyne	*d*	–	–	–	+	–	–	–

Table 5 Physical characteristics of the predicted mature peptides encoded by the seven *Glu-A3* i-type LMW glutenin genes characterised in this study. Also shown are approximate sizes of the observed B-type LMW-GS linked to each *Glu-A3* allele, calculated from relative mobility on SDS-PAGE (Fig. 6). The estimated molecular weights of both protein bands that comprise the *Glu-A3d* allele are shown. *matP* Deduced mature peptide

Origin (*Glu-A3* allele)	No. of residues (matP)	Predicted molecular weight (Da)	Predicted pI	Observed molecular weight (approx., Da)
a	356	41,267	8.05	53,000
b	364	42,161	8.05	55,000
c	356	41,241	8.05	54,000
d	336	38,951	8.63	50,000, 57,500
e	368	42,715	8.25	–
f	337	39,130	8.43	50,000
g	368	42,712	8.43	56,000

Discussion

Approximately 60 LMW-GS genes or pseudogenes have been isolated from genomic or cDNA libraries (Bartels and Thompson 1983; Pitts et al. 1988; Colot et al. 1989; Cassidy et al. 1998; Ikeda et al. 2002) and via gene-specific PCR (Masci et al. 1998; Benmoussa et al. 2000; Cloutier et al. 2001; Ikeda et al. 2002). LMW-GS genes have been divided into three broad classes based on the first amino acid residue of the predicted mature protein: s-type (serine), m-type (methionine) and i-type (isoleucine, Cloutier et al. 2001). LMW-GS genes have been further classified into 12 groups based on alignment of the conserved N- and C-terminal domains of the deduced amino acid sequences (Ikeda et al. 2002). Whereas most previous studies have isolated LMW-GS genes from one specific hexaploid wheat cultivar, here we report the isolation and characterisation of one LMW-i-type gene from seven hexaploid wheat cultivars, each with a different *Glu-A3* allele.

Sequences of the LMW-i-type gene from *Glu-A3g* (this study) and pGH3.1 (Cloutier et al. 2001) are both derived from cv. Glenlea. Two single SNPs were identified over a 1,199 bp alignment of these sequences.

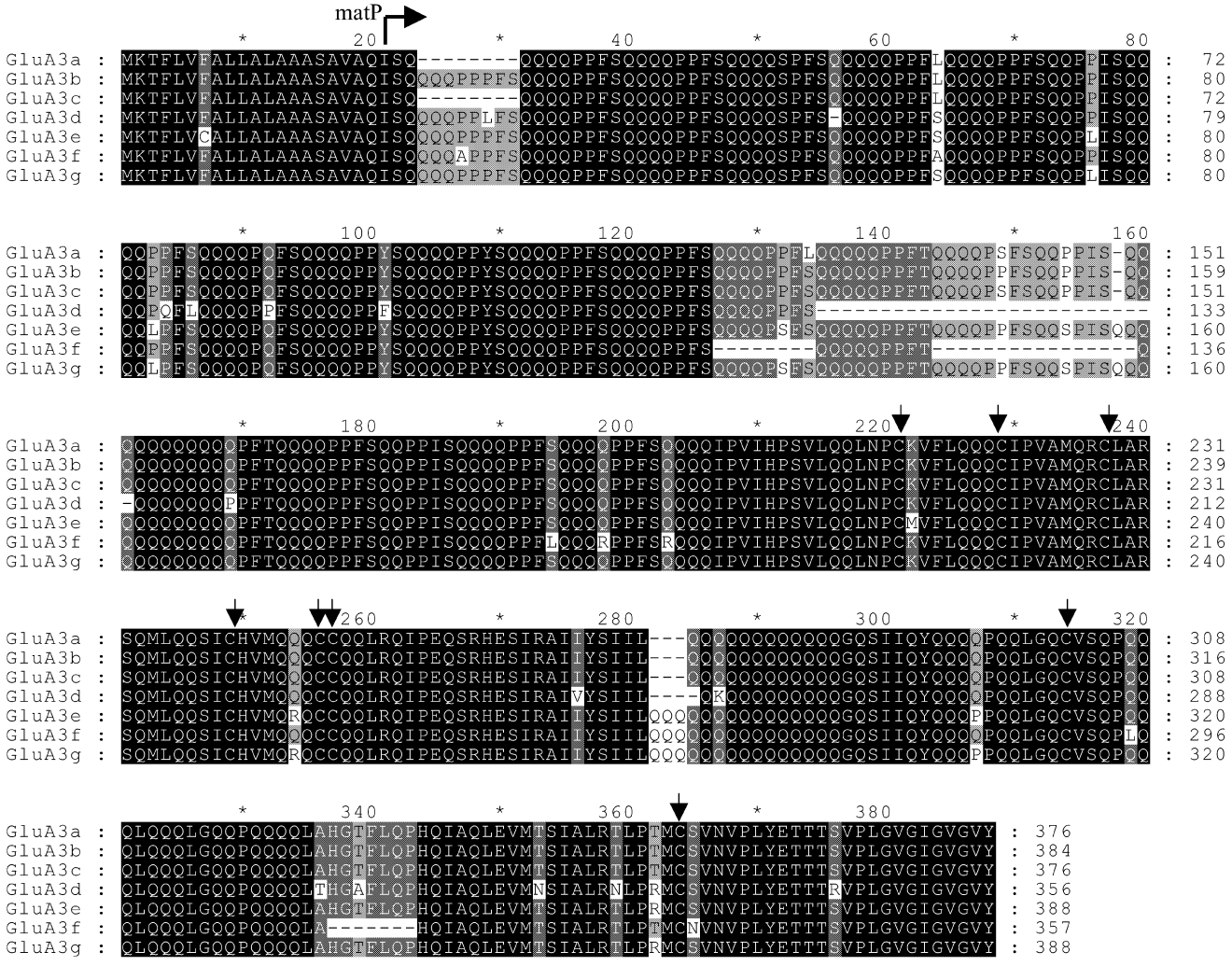


Fig. 5 Alignment of deduced protein sequences from seven genes amplified from seven cultivars containing *Glu-A3* alleles *a–g*. The predicted 20-amino acid signal peptide is also included in the alignment. The start of the deduced mature peptide (*matP*) is indicated. Conserved cysteine residues are indicated by *arrows*. *Dashes* indicate gaps introduced into the alignment

These SNPs may indicate these are two very similar but non-identical genes, or may be attributable to sequencing errors or errors generated during propagation in *Escherichia coli* (Cooper and Krawczak 1993). Sequences from *Glu-A3c* (this study) and U86030 (Cassidy et al. 1998) are both derived from cv. Cheyenne. However, 18 SNPs and three InDels were identified between these two sequences, indicating that these are probably paralogous genes. In addition, no gene with a high level of sequence identity with AB062878 (Ikeda et al. 2002) was observed for any of the *Glu-A3*-derived sequences in the current study. These results are consistent with the observation of Ikeda et al. (2002) that cultivars containing some *Glu-A3* alleles (as classified according to the observed protein profile) may contain more than one LMW-i-type gene or pseudogene.

The polymorphism levels between LMW-i-type gene sequences obtained in the current study for cultivars containing different *Glu-A3* alleles are much higher than expected for orthologous genes. The SNP rate in wheat low copy sequences (Bryan et al. 1999) and multi-copy-gene family γ-gliadin sequences (Zhang et al. 2003) was approximately one SNP per kilobase. A similar SNP level was also observed in the human genome (Cooper et al. 1985). In the current study, only comparison of the LMW-i-type genes derived from *Glu-A3a* and *Glu-A3c* shows a level of polymorphism consistent with this SNP level. Five to 35 times this SNP rate was observed between the other LMW-i-type gene sequences from different *Glu-A3* alleles. Therefore, it appears unlikely that all the LMW-i-type genes sequenced from different *Glu-A3* alleles in this study are from the same origin, as confirmed for two other low-copy wheat genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase (Huang et al. 2002). The LMW-i-type genes from *Glu-A3a*, *Glu-A3b* and *Glu-A3c* probably represent orthologues, the LMW-i-type genes from *Glu-A3e* and *Glu-A3 g* may have the same origin, whereas LMW-i-type genes from *Glu-A3d*

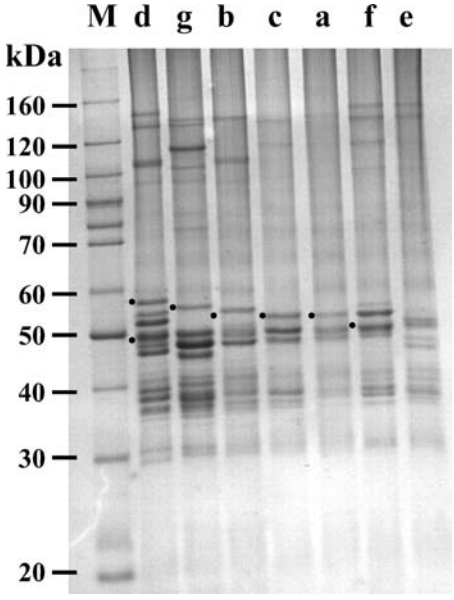


Fig. 6 SDS-PAGE analysis of proteins expressed from cultivars containing different *Glu-A3* alleles. *M* Molecular weight standard (Benchmark Protein Ladder, Invitrogen), *d* cv. Suneca (*Glu-A3d* allele), *g* Glenlea (*Glu-A3 g*), *b* Gabo (*Glu-A3b*), *c* Cheyenne (*Glu-A3c*), *a* Chinese Spring (*Glu-A3a*), *f* Rescue (*Glu-A3f*), e Halberd (*Glu-A3e*). Bands corresponding to the B-type low-molecular-weight-glutenin subunits linked to each allele are *highlighted* on the *left* of each gel lane

and *Glu-A3f* appear to be from separate origins. Therefore, there appear to be at least four different origins for the *Glu-A3* loci LMW-i-type genes of hexaploid wheat. Another possible explanation is that a monomorphic progenitor *Glu-A3* locus underwent sequence rearrangement during allopolyploidy formation as described by Ozkan et al. (2001) and Shaked et al. (2001).

It is estimated that several dozen genes or pseudogenes comprise the *Glu-3* loci in hexaploid wheat (Cassidy et al. 1998). Alleles *Glu-A3 a, b, c* and *f* appear to encode a single, B-type LMW-GS, whereas the *d* allele encodes two B-type subunits and no product has been detected for the *e* allele (Gupta and Shepherd 1990). Just one gene was isolated from each cultivar containing a different *Glu-A3* allele in this study. All seven LMW-GS genes characterised in this study contain a single, predicted ORF that encodes an LMW-GS i-type protein (Cloutier et al. 2001), also described as 'type VI' LMW-GS (Ikeda et al. 2002) and reported previously by Pitts et al. (1988), Cassidy et al. (1998) and Maruyama et al. (1998). Where known, the location of genes encoding i-type LMW-GS is chromosome 1A [the gene of Cloutier et al. (2001), two genes of Ikeda et al. (2002), seven genes in this study].

The i-type LMW-GS characterised by Cloutier et al. (2001) from cv. Glenlea ('LMW-50', encoded by clone pGH3.1) is almost identical to the sequence we obtained from the same cultivar (*Glu-A3g*, one amino acid difference) and these authors demonstrated that this gene encodes a protein of approximately 50 kDa observed by SDS-PAGE. This contrasted with a molecular mass of 42.6 kDa for the predicted mature protein. We observed differences of between 11 kDa and 13 kDa between the predicted proteins encoded by the genes from cultivars containing *Glu-A3* alleles *a–g* and the SDS-PAGE-estimated molecular masses of the proteins encoded by these alleles (Fig. 6; Table 5), with an estimated molecular mass of 56 kDa for the *Glu-A3g*-encoded LMW-GS of cv. Glenlea. It is interesting to note that the ranking of the mobilities of the SDS-PAGE-observed proteins and the predicted proteins from the cultivars containing the corresponding *Glu-A3* alleles are similar (predicted: *g>b>c~a>d~f*, observed: *g>b>c>a>d~f*, Table 5). It is known that HMW-GS do not migrate according to their predicted molecular masses, with the molecular mass estimated by SDS-PAGE being significantly higher than that predicted by amino acid composition (Bunce et al. 1985; Anderson et al. 1989; Shewry et al. 1989). Bunce et al. (1985) have attributed the overestimation of the molecular weight of HMW-GS in SDS-PAGE to their particular structure and specifically to the central repetitive domain that may not be completely denatured under the conditions of SDS-PAGE (Goldsbrough et al. 1989). It is likely that the structurally similar repetitive domain of the LMW-GS is involved in the anomalous mobility of these proteins.

The deduced amino acid sequence of the gene isolated from the *Glu-A3e*-containing cultivar contains a single ORF despite no protein being observed for this allele. It is interesting to note that this predicted protein contains a cysteine in the signal sequence not found in any other LMW-GS signal sequence.

LMW glutenins have been shown to significantly effect wheat dough properties (Payne et al. 1987; Gupta et al. 1989, 1994; Pogna et al. 1990). The effects of individual *Glu-3* alleles are largely additive with those of *Glu-1* alleles (Gupta et al. 1989; Pogna et al. 1990; Sontag-Strohm et al. 1996; Redaelli et al. 1997; Wesley et al. 1999). Interactions between these loci also have significant effects (Gupta et al. 1994; Luo et al. 2001). The absence of a cysteine residue in the N-terminal or repetitive domains of the mature protein in i-type LMW-GS may have significant effects on gluten polymer formation. This could be due to the exclusion of the repetitive domain of i-type LMW-GS from the disulphide-linked network frame or by this LMW-GS type acting as a chain terminator (Ikeda et al. 2002). For the cv. Gleanlea (*GluA3g*), Cloutier et al. (2001) have reported positive effects for the *Glu-A3* locus on dough strength. In Australia, specific LMW-GS alleles have been actively selected against is certain breeding programs, for example *Glu-B3e* (Lawrence 1986) and *Glu-A3e* (Eagles et al. 2001).

In order to obtain DNA markers for all of the *Glu-A3* alleles, we designed a series of allele-specific primers according to the principles that we have successfully used for wheat γ-gliadin gene detection (Zhang et al. 2003). Five allele-specific primer sets were successfully designed for *Glu-A3a*, *Glu-A3d*, *Glu-A3e*, *Glu-A3f* and *Glu-A3g* detection. No *Glu-A3b* allele-specific primer could

be designed because the only *Glu-A3b*-specific SNP is located in the repetitive sequence region of the gene. Detection of this allele therefore requires the use of the GluA3abc marker in combination with either the GluA3a or GluA3ac marker (depending on the cross). No *Glu-A3c*-allele specific primer could be designed as no allele-specific SNP could be identified relative to the sequences obtained from the other six *Glu-A3* alleles. Detection of this allele therefore requires the use of the GluA3ac marker in combination with the GluA3a marker. Although the allele-specific markers described in this study are dominant, the use of pairs of markers together would provide the capacity to detect heterozygous plants, with the exception of the crosses *Glu-A3* $a \times b$, $b \times c$ and $a \times c$.

A similar approach to that used in the current study may now be used to identify genes and markers linked to alleles of the *Glu-B3* and *Glu-D3* loci to provide a comprehensive set of LMW glutenin markers for use in marker-assisted breeding.

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