Theor Appl Genet (2006) 113:1247–1259
DOI 10.1007/s00122-006-0379-y

ORIGINAL PAPER

Characterization of three low-molecular-weight Glu-D3 subunit genes in common wheat

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Received: 17 October 2005 / Accepted: 25 July 2006 / Published online: 29 August 2006

Abstract Low-molecular-weight glutenins (LMW-GS) in common wheat (*Triticum aestivum* L.) are of great importance for processing quality of pan bread and noodles. The objectives of this study are to identify LMW-GS coding genes at *GluD3* locus on chromosome 1D and to establish relationships between these genes and *GluD3* alleles (*a*, *b*, *c*, *d*, and *e*) defined by protein electrophoretic mobility. Specific primer sets were designed to amplify each of the three LMW-GS chromosome 1D gene regions including upstream, coding and downstream regions of eight wheat cultivars containing *GluD3 a*, *b*, *c*, *d* and *e* alleles. Three LMW-GS genes, designated as *GluD3-1*, *GluD3-2* and *GluD3-3*, were amplified from the eight wheat cultivars. The allelic variants of these three genes were analysed at the DNA and protein level. *GluD3-1* showed two allelic variants or haplotypes, one common to cultivars containing protein alleles *a*, *d* and *e* (designated *GluD3-11*) and the other was present in cultivars with alleles *b* and *c* (designated *GluD3-12*). Comparing with *GluD3-12*, a 3-bp deletion was found in the coding region of the N-terminal repetitive domain of *GluD3-11*, leading to a glutamine deletion at the 116th position. *GluD3-2* had three variants at the DNA level in the eight cultivars, which were designated as *GluD3-21*, *GluD3-22* and *GluD3-23*. In comparison to *GluD3-21*, a single nucleotide polymorphism (SNP) was detected for *GluD3-22* in the signal peptide region, resulting in an amino acid change from alanine to threonine at the 11th position; and 11 mutations were found at *GluD3-23*, with five in upstream region, four in coding region and two in downstream region, respectively. *GluD3-3* had two haplotypes, designated as *GluD3-31* and *GluD3-32*, both belonging to LMW-s glutenin subunits though their first amino acids in N-terminal region are different. Compared with the GenBank *GluD3* genes, nucleotide sequences of *GluD3-21* and *GluD3-23* were the same as *X13306* and *AB062875*, respectively. *GluD3-22* and *GluD3-11* had only one-base difference from *U86027* and *AB062865*. *GluD3-12* was not found in the GenBank database, indicating a newly identified *GluD3* gene variation. *GluD3-3* was a new gene different from

Communicated by M. Morgante

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00122-006-0379-y and is accessible for authorized users.

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any other known *GluD3* genes. Analyses of the relationship between *Glu-D3* alleles defined by protein electrophoretic mobility and different *GluD3* gene variations at the DNA or protein level provided molecular basis for DNA based identification of glutenin alleles.

Introduction

Flour from common wheat, *Triticum aestivum* L., possesses unique processing properties in forming doughs that are viscoelastic and thus suitable for the production of breads and noodles (Shewry et al. 2002). Gluten networks provide the basis for these properties, which are developed when flour is mixed with water (Lindsay and Skerritt 1999; Shewry et al. 2002). The major functional components of the gluten network are high and low molecular weight glutenin subunit proteins (abbreviated as HMW-GS and LMW-GS, respectively), which form disulphide-bonded gluten macropolymer (Gras et al. 2001). These glutenin proteins are highly polymorphic, with different alleles varying in their contribution to the fundamental aspects of dough quality, for example, dough strength and extensibility (Payne 1987; Wesley et al. 1999, 2001; Brites and Carrillo 2001; Luo et al. 2001). HMW-GS and LMW-GS alleles are therefore important targets for marker-assisted selection in breeding for grain quality in wheat cultivars (Gupta et al. 1999; Eagles et al. 2001; Gale 2005; Ma et al. 2005). Due to their clear resolution by gel electrophoresis and low gene copy number, the allelic variation of the HMW-GS and their relationship with wheat quality has been studied extensively (Payne et al. 1981; Payne and Lawrence 1983; Anderson and Green 1989; Shewry et al. 1992), and DNA markers based on polymerase chain reaction (PCR) are available to discriminate the most important *Glu-1* alleles (Ma et al. 2003). Resolution of the LMW-GS and the scoring of alleles by direct analysis of proteins have been more difficult due to the larger number of expressed subunits and their overlapping mobility with the abundant gliadin proteins (Singh and Shepherd 1988). For these reasons, the role of individual LMW-GS in the determination of wheat quality is less clear, although some alleles or subunits are clearly beneficial or detrimental (Lee et al. 1999; D'Ovidio and Masci 2004; He et al. 2005). Selection of LMW-GS is not commonly used in breeding programs although LMW-GS often confers balanced wheat qualities (Ma et al. 2005). Compared with *Glu-A3* and *Glu-B3*, alleles at *Glu-D3* are more difficult to separate by electrophoresis and thus less information is available (Liu et al. 2005; He et al. 2005). Therefore, characterization of LMW-GS genes and their alleles at *Glu-D3* locus could potentially lead to the practical application of both HMW-GS and LMW-GS in wheat breeding programs.

LMW-GS is encoded by the complex *Glu-3* loci (*Glu-A3, Glu-B3* and *Glu-D3*) on the short arms of group 1 chromosomes (Singh and Shepherd 1988; Gupta and Shepherd 1990). Gupta and Shepherd (1990) carried out an extensive survey of LMW glutenin proteins by SDS polyacrylamide gel electrophoresis (SDS-PAGE) in bread wheat cultivars and detected 20 different banding patterns, six controlled by *Glu-A3*, nine by *Glu-B3* and five by *Glu-D3*. Consequently, six alleles existed for *Glu-A3* locus (a, b, c, d, e, f), nine for *Glu-B3* (a, b, c, d, e, f, g, h, i), and five for *Glu-D3* (a, b, c, d, e), respectively. Typically, *Glu-A3* allele contains 1–3 subunits or protein bands on the gel, *Glu-B3* allele contains 5–8 subunits, and *Glu-D3* allele contains 4–5 subunits. Furthermore, by analysing N-terminal amino acid sequences, 39 different LMW subunits/alleles were identified in one bread wheat cultivar (Lew et al. 1992). Based on the first N-terminal amino acid of the mature protein, LMW-GS was divided into three types: LMW-m, LMW-s and LMW-i, which correspond to methionine, serine and isoleucine, respectively (Kasarda et al. 1988; Tao and Kasarda 1989; Lew et al. 1992; Cloutier et al. 2001). LMW-GS was further classified into 12 groups by Ikeda et al. (2002) according to deduced amino acid sequences and in particular the number and position of cysteine residues available for inter-molecular disulphide bond formation (Shewry and Tatham 1997). More than 70 genes, or pseudogenes of the LMW-GS family have been cloned and sequenced from several common wheat cultivars (Cloutier et al. 2001; Ikeda et al. 2002; Zhang et al. 2004). D'Ovidio and Masci (2004) constructed a dendrogram comparison between the coding regions of LMW-GS genes and grouped them into five clusters containing members that are encoded by different genomes, but share more than 90% identity. Up to now, more than 20 different *Glu-D3* genes from common wheat and *Triticum tauschii* have been released in Genbank (Ikeda et al. 2002; Johal et al. 2004; Zhao et al 2004). However, the allelic relationship of these genes, and their association with glutenin subunits/alleles were still unclear. In this study, *Glu-D3* gene-specific primers were designed to amplify different *Glu-D3* genes in eight common wheat cultivars containing five common *Glu-D3* alleles (*a, b, c, d* and e; Gupta and Shepherd 1990) for the purpose of defining allelic variations and establishing the relationship between protein allele mobility variants and the *GluD3* genes at the DNA level.

Materials and methods

Wheat stocks

Eight common wheat cultivars (Tasman, Chinese Spring, Silverstar, Sunco, Aroona, Norin61, Hartog, and BT2288A) carrying five common *Glu-D3* alleles (McIntosh et al. 1998) were used to amplify *Glu-D3* genes in this study (Table 1). Chinese Spring and its nulli-tetrasomic lines N1AT1B (nullisomic 1A-tetrasomic 1B), N1BT1D (nullisomic 1B-tetrasomic 1D) and N1DT1B (nullisomic 1D-tetrasomic 1B) provided by R. A. McIntosh at the Plant Breeding Institute, University of Sydney, were used to confirm chromosomal locations of identified genes.

Development of PCR primers

Gene-specific primers were developed based on the DNA sequence alignment of Zhang et al. (2003, 2004). Eight reference *Glu-D3* genes *X13306*, *AB062851*, *AB062864*, *AB062865*, *AB062872*, *AB062873*, *AB062875* and *AY223396* available in GenBank were used for primer development (http://www.ncbi.nlm.nih.gov). Each gene was divided into three parts (upstream, coding and downstream region) to design corresponding primers in order to obtain sequence information beyond the coding region by combining three PCR products of normal length. The forward and reverse primers for coding region were first designed. The amplified coding regions were then used to design the reverse primers of upstream region. The forward primers of downstream region, and the upstream forward and downstream reverse primers were all designed based on the sequences of *X13306* (Colot et al. 1989).

Primers' screening

A total of 71 primers were designed and 334 primer sets (140 for upstream, 128 for coding, and 66 for downstream region) were tested. Primer screening was conducted in four steps: (1) confirm the chromosomal location of PCR fragments amplified with different primer sets using Chinese Spring and its nulli-tetrasomic lines of N1AT1B, N1BT1D and N1DT1B (Fig. 1a–c), (2) use the primer sets for chromosome 1D to amplify the eight wheat cultivars with protein mobility alleles *a*, *b*, *c*, *d* and *e*, (3) sequence PCR fragments amplified from these cultivars, and (4) sequence alignment of the PCR fragments with the *Glu-D3* genes available in GenBank using the software DNAMAN (http://www.lynnon.com). Based on the result of sequence alignments, nine pairs of primer sets for three *Glu-D3* genes were confirmed with annealing temperature ranging from 58 to 62°C. Primer sequences (5′–3′) and their relative locations at reference genes are shown in Table 1.

Table 1 Primers used for amplifying the three *GluD3* genes

Target gene	Primer	Sequence (5′ → 3′)	Primer location[a]	Reference gene[b]	Expected size	Ann. temp. (°C)
GluD3-1	*D3F62*	TATAGCCCAGTTCGAATTGGT	125–145	*X13306*	901[c]	62
	D3R66	CGCCGCAATGGCAAGGAGA	1,350–1,368	*GluD3–1*		
	D3F621	GCTACAACCACTCAACCACA	130–150	*GluD3-1*	850	58
	D3R661	GAAAGACGAAACTTGTGTGGA	932–952	*GluD3-1*		
	D3F3	AAGATCATCACAGGCACAATC	1,269–1,289	*GluD3-1*	941	58
	D3R3	CTGCTGACCCAATTGTTGTAG	2,182–2,202	*GluD3-1*		
	D3F71	GCAAGAACAACAACAGGGTTT	2,043–2,063	*GluD3-1*	721	58
	D3R8	AACTGGTTGTGATTGTCACTG	2,312–2,332	*X13306*		
GluD3-2	*D3F63*	GCTTGTGACTTTGAGGCCT	98–116	*X13306*	982	58
	D3R64	ATGGTCTCTCCAAACCAGGGA	926–946	*GluD3-2*		
	D3F36	GAAGGAAAAGAGGTGGTTCCT	743–763	*GluD3-2*	904	62
	D3R37	CTATCTGGTGTGGCTGCAAA	1,626–1,645	*GluD3-2*		
	D3F8	TCCTGCAAGAACAACAACAG	1,475–1,494	*GluD3-2*	702	58
	D3R9	TAGACAGTGGTACTAGTTGGT	2,292–2,311	*X13306*		
GluD3-3	*D3F67*	GGGCTGGGCTTGTGACTTT	91–109	*X13306*	989	59
	D3R62	TGGTTTCTCCAAACCAGGGAT	948–968	*GluD3-3*		
	D3F22	CCAAACGGAATAATTAAAGCTA	738–759	*GluD3-3*	974	59
	D3R31	GTTGGGGTTGGGAAACACA	1,710–1,728	*GluD3-3*		
	D3F45	CTATTTGTTCATCCATCTATC	1,422–1,442	*GluD3-3*	941	60
	D3R8	AACTGGTTGTGATTGTCACTG	2,312–2,332	*X13306*		

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

[b] Reference genes refer to the genes used for marking the primer locations

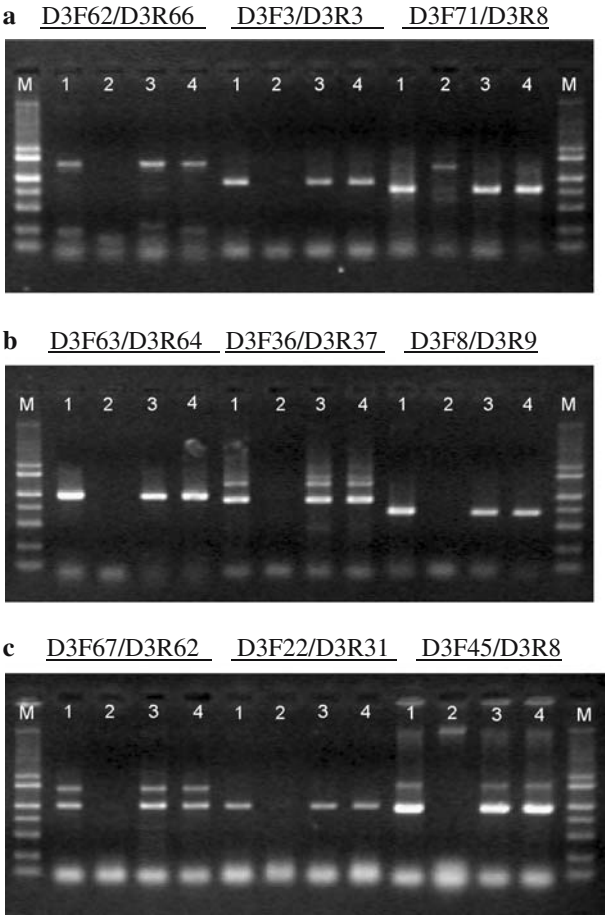

Fig. 1 Electrophoresis of PCR products amplified from Chinese Spring and its nulli-tetrasomic lines using nine specific primer sets in agarose gel. *1* Chinese Spring, *2* N1DT1B, *3* N1BT1A and *4* N1AT1B. **a** *GluD3-1* gene. **b** *GluD3-2* gene. **c** *GluD3-3* gene. *M* DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600 and 2,000 bp)

DNA extraction and PCR amplification

Genomic DNA was extracted from seedlings or seeds using modified CTAB procedure (Gale et al. 2001). PCR was performed in a total volume of 40 µl containing 1.5 mM $MgCl_2$, 60 ng of genomic DNA, 3U of *Taq* DNA polymerase, 200 µM of each of dNTPs and 10 pmol of each PCR primer. PCR cycling was 94°C for 5 min followed by 38 cycles of 94°C for 40 s, 58–62°C for 40 s, 72°C for 90 s, and a final extension at 72°C for 5 min.

Sequencing of PCR products

PCR fragments were sequenced by the Invitrogen Biotechnology Co. Ltd (Beijing). In order to eliminate sequence errors, each PCR and sequence procedure was repeated 2–4 times. Sequence analysis and characterization were performed using software DNAMAN (http://www.lynnon.com).

Results

GluD3 genes amplified from Glu-D3 locus

In all, three different *GluD3* genes including seven allelic variants at the DNA level were identified at the *Glu-D3* locus on 1D chromosome in eight common wheat cultivars. The first gene (designated as *GluD3-1*) was amplified with primer sets *D3F62/D3R66* for an upstream region, *D3F3/D3R3* for a coding region and *D3F71/D3R8* for a downstream region (Table 1), which generated 1,336, 855 and 713 bp products, respectively. The complete sequence of *GluD3-1* was assembled with a total length of 2,792 bp. Likewise, the second gene, designated as *GluD3-2* with 2,144 bp, was amplified using primer sets *D3F63/D3R64* for the upstream region, *D3F36/D3R37* for the coding region and *D3F8/D3R9* for the downstream region with fragment sizes of 912, 834 and 631 bp, respectively. The third gene, *GluD3-3* in 2,326 bp, was amplified with primer sets *D3F67/D3R62* for the upstream region, *D3F22/D3R31* for the coding region and *D3F45/D3R8* for the downstream region, which resulted in 930-, 918- and 858-bp products, respectively.

GluD3-1 had 2 haplotypes or allelic variants at the DNA level in the eight wheat cultivars, one common to cultivars containing alleles *a, d* and *e* (designated *GluD3-11*) and another variant was presented in cultivars with alleles *b* and *c* (designated *GluD3-12*). *GluD3-1* was a relatively conservative gene and only one mutation was detected between its two corresponding allelic forms, of which *GluD3-11* had a 3-bp (CAA) deletion at the position 1,672–1,674 in gene coding region (Fig. 2), leading to a glutamine deletion at the 116th position in N-terminal repetitive domain of the deduced protein (Fig. 5).

GluD3-2 had three allelic forms at the DNA level in the eight wheat cultivars, designated as *GluD3-21*, *GluD3-22* and *GluD3-23*, respectively. Compared with *GluD3-21* (Fig. 3), *GluD3-22* had a single nucleotide polymorphism (SNP) with a base G–A substitution at the 880th position in the coding region, leading to an amino-acid mutation from alanine to threonine at 11th position in signal region of deduced peptide (Fig. 6). *GluD3-23* had 11 mutations, five SNPs in upstream region, two SNPs in downstream region, and two SNPs and two deletions (a 6-bp and a 3-bp, respectively) in the coding region. In comparison to *GluD3-21*, the deduced amino acid sequences of *GluD3-23* had a single glutamine deletion at 127th position and a double-glutamine deletion at position 56–57 in N-terminal repetitive domain, an amino acid mutation from valine to leucine at position 152 in C-terminal cysteine-rich

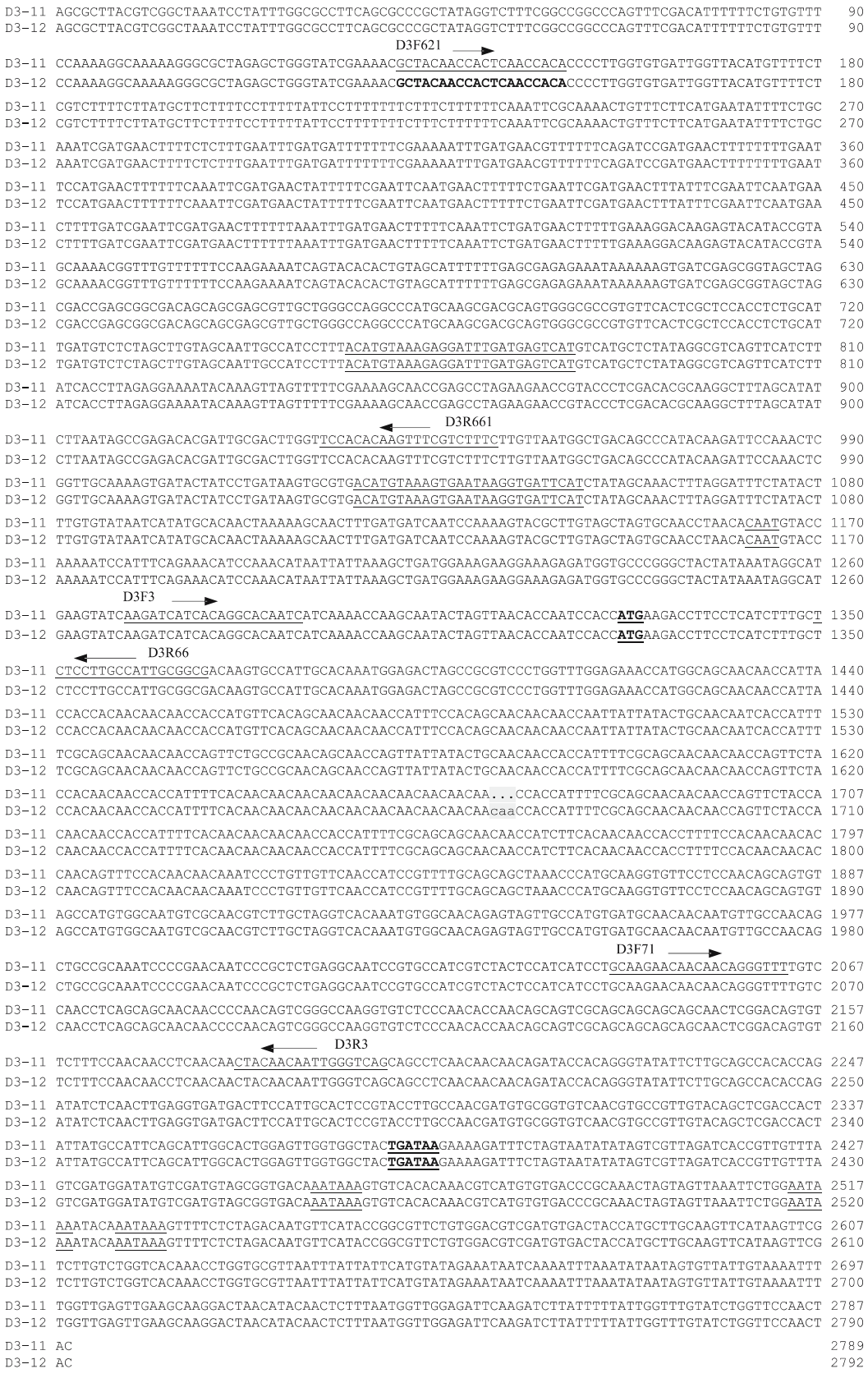

Fig. 2 Alignment of two *GluD3-1* gene haplotypes. The nucleotide mutation locus is *shadowed*. The endosperm boxes, CAAT box, TATA motif and AATAAA polyadenylation signals are *underlined*. The initiation codon and terminator codon are *bold and underlined*. The primer positions are indicated with *arrows*

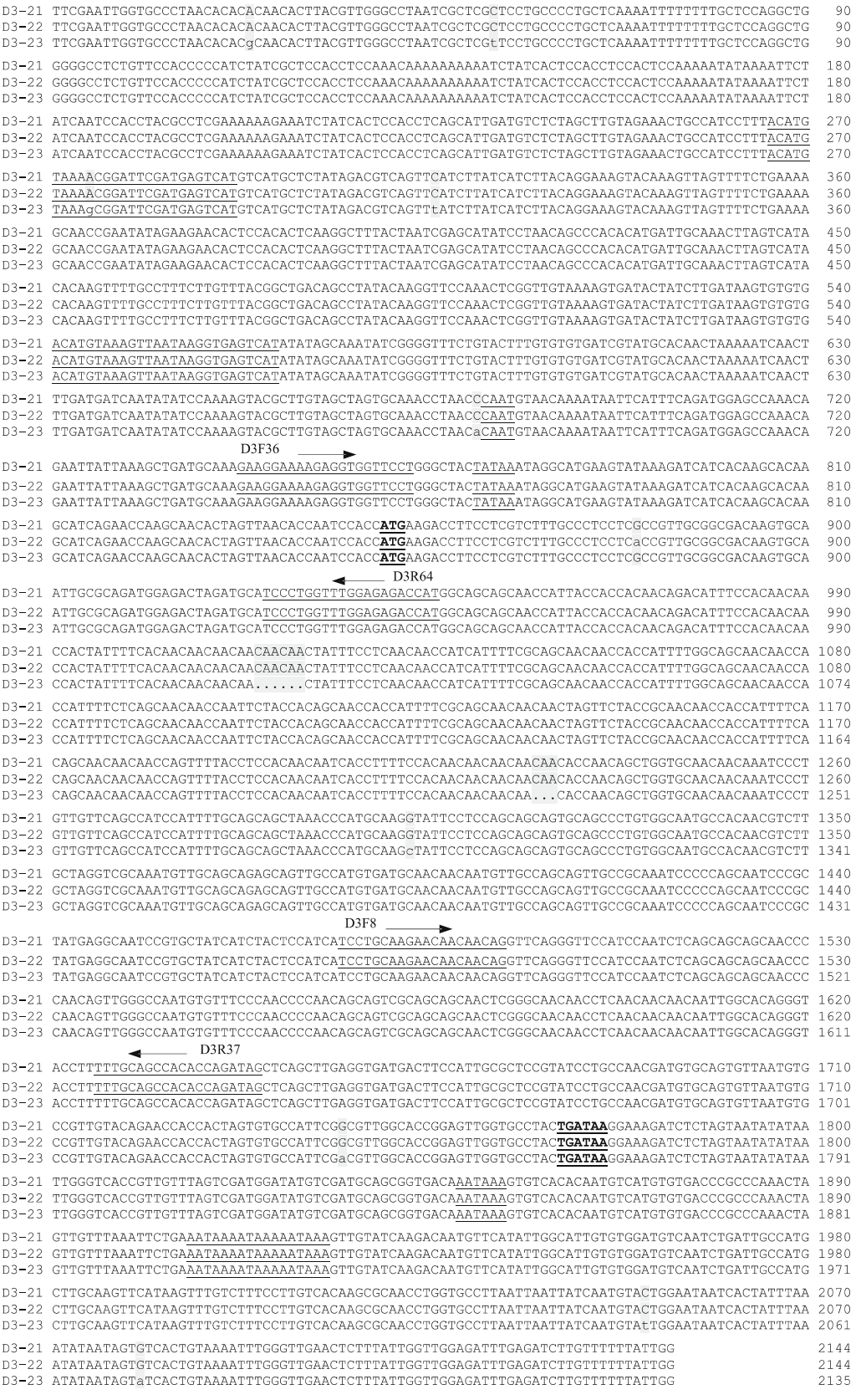

Fig. 3 Alignment of three *GluD3-2* haplotypes. The nucleotide mutation loci are *shadowed*. The endosperm boxes, CAAT box, TATA motif and AATAAA polyadenylation signals are *underlined*. The initiation codon and terminator codon are *bold and underlined*. The primer positions are indicated with *arrows*

region and a mutation from glycine to asparaginic acid at the position 299 in the final conserved part of the protein (Fig. 6).

GluD3-3 gene also had two allelic forms at the DNA level, one common to cultivars with protein mobility alleles *a*, *b* and *e* and the other from cultivars with *c* and *d* (designated as *GluD3-31* and *GluD3-32*, respectively). Sixteen SNPs were detected between the two haplotypes with seven in the upstream region, eight in the coding region and one in the downstream region (Fig. 4). The deduced amino acid sequences showed that the two variants of *GluD3-3* belong to LMW-s glutenin subunits even though the N-terminal amino acids of *GluD3-32* has been mutated from methionine to isoleucine (Fig. 7) (Ikeda et al 2002). Between the two variants, there were seven amino-acid substitutions, and a synonymous mutation at the 52nd position in the gene-coding region (Fig. 7).

Characterization of GluD3-1, GluD3-2 and GluD3-3 genes and their deduced amino acid sequences

The seven allelic variants of the three *GluD3* sequences identified in this study all contain a complete gene-coding sequence, including promoter sequences with the endosperm boxes, CAAT boxes, TATA motif, start codon, terminator sequence with double-stop codons, and AATAAA polyadenylation signals (Figs. 2, 3, 4). Sequence alignments indicated that the homology of DNA sequences was 81.5–88.5% among the three gene sequences, and 99.3–99.9% among different allelic forms within each of the three gene sequences (Table 2, below diagonal). In addition, the upstream sequence of *GluD3-1* was about 500 bp longer than those of *GluD3-2* and *GluD3-3*.

The deduced amino-acid sequences of the three genes showed that they all had a single open reading frame (ORF) (Figs. 5, 6, 7). Each allele encodes a highly conserved signal peptide of 20 amino acids and a short N-terminal conserved region with 13 amino acids, which was followed by an N-terminal repetitive domain and a C-terminal conserved domain involving three sub-regions of cysteine-rich, glutamine-rich and final conserved domain. All deduced LMW glutenin subunits showed a typical set of eight conserved cysteine (Cys) residues. Based on the position of the first and the seventh cysteine (Ikeda et al. 2002), the *GluD3-1*, *GluD3-2* and *GluD3-3* deduced subunits could be classified into types III, IV and I, respectively. Multiple sequence alignments showed that the amino acid identity was 77.5–88.9% among three genes and 98.0–99.9% among allelic forms of each gene sequences (Table 2). The results indicated that *GluD3-1*, *GluD3-2* and *GluD3-3* are typical *Glu-D3* genes.

The relationship between GluD3 gene haplotypes and Glu-D3 mobility alleles

PCR amplification of *GluD3* genes indicated that each of the eight cultivars with five different LMW-GS electrophoretic mobility alleles at *GluD3* locus contained at least three different *GluD3* genes, all varying across the traditionally recognized alleles defined by protein electrophoretic mobility, i.e., *a*, *b*, *c*, *d*, *e* (Table 3). The cultivars Tasman and Chinese Spring (*Glu-D3a* allele) possessed the allelic forms *GluD3-11*, *GluD3-21*, and *GluD3-31*, while Silverstar and Sunco (*Glu-D3b* allele) was associated with haplotypes of *GluD3-12*, *GluD3-22*, and *GluD3-31*. Hartog and Bt2288A (*Glu-D3e* allele) contained common allelic forms of *GluD3-11*, *GluD3-31*, and *GluD3-21* or *GluD3-22* (the latter two should be same at the protein level). Aroona with allele *c* and Norin 61 with allele *d* carried two common alleles *GluD3-23* and *GluD3-32*, and, displayed some difference for the gene *GluD3-1* with *GluD3-12* and *GluD3-11*, respectively. The results indicated that the cultivars with same protein mobility allele always display the same alleles at protein level.

Discussion

Complexity of primer development

Bread wheat is an allohexaploid species, consisting of three subgenomes (A, B, and D) with high sequence identity (Gu et al. 2004), which makes it difficult to design gene-specific primers. In the development of PCR primers, the homology and specificity among different LMW-GS genes were considered simultaneously. For the 71 primers designed, both the forward primers of the upstream region and reverse primers of the downstream region of the three genes were all designed based on *X13306*, indicating the high nucleotide identity among LMW-GS genes (Ikeda et al. 2002; D'Ovidio and Masci 2004). Though other primers were developed according to the specific SNPs of relevant genes, the rate of useful primers were much less than expected. In the 334 pairs of primer set, over 86% was not specific to *Glu-D3* locus or could not amplify PCR products that could be interpreted. Only 46 pairs (13.8%) were clearly assigned to the *Glu-D3* locus, but most of them could not produce acceptable PCR products from the eight cultivars for sequencing. Even for the primer sets with successful sequencing results, there were also some exceptions where several primer sets designed according to SNPs of one gene also amplified another gene product. A typical example was

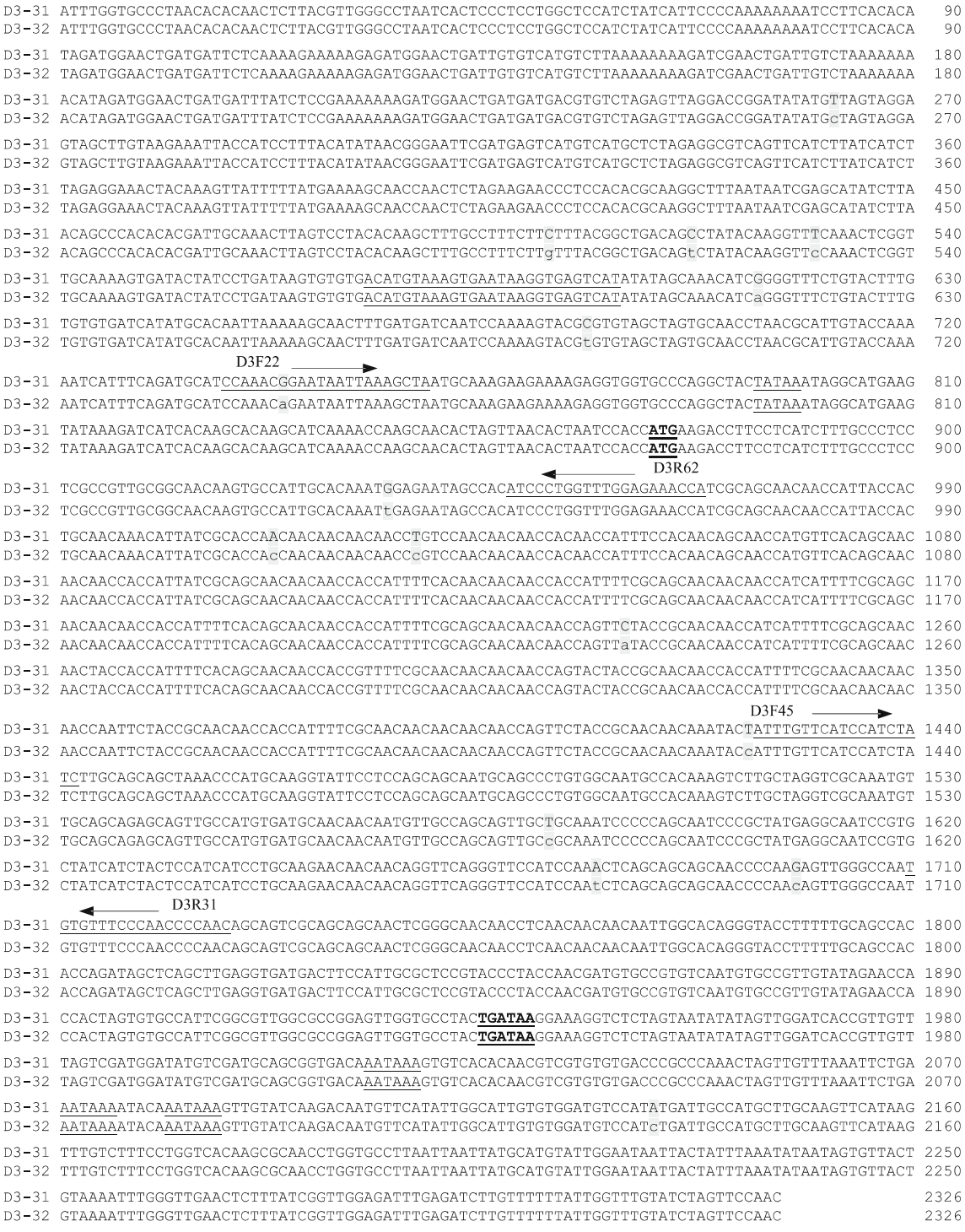

Fig. 4 Alignment of two *GluD3-3* haplotypes. The nucleotide mutation loci are *shadowed*. The endosperm boxes, CAAT box, TATA motif and AATAAA polyadenylation signals are *underlined*. The initiation codon and terminator codon are *bold and underlined*. The primer positions are indicated with *arrows*

the upstream reverse primer *D3R61* (TGGCACTTGTCGCCGCAAT), designed for *GluD3-1* gene, but the primer set *D3F68/D3F61* amplified the same nucleotide sequence of *GluD3-2* as *D3F63/D3R64*, though the second base (G → T) and last base (T → C) of *D3R61* could not match to the *GluD3-2* gene. These results indicated that the so-called conserved sequences of 3′-terminal were relative and the gene-specific primers were difficult to obtain (Ikeda et al. 2002; Zhang et al. 2004). In the present study, the primers

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P-11 MKTFLIFALLAIAATSAIAQMETSRVPGLEKPWQQQPLPPQQQPPCSQQQQPFPQQQQPI  60
P-12 MKTFLIFALLAIAATSAIAQMETSRVPGLEKPWQQQPLPPQQQPPCSQQQQPFPQQQQPI  60

P-11 IILQQSPFSQQQQPVLPQQQPVIILQQPPFSQQQQPVLPQQPPFSQQQQQQQQQQ.PPFS 119
P-12 IILQQSPFSQQQQPVLPQQQPVIILQQPPFSQQQQPVLPQQPPFSQQQQQQQQQQqPPFS 120

P-11 QQQQPVLPQQPPFSQQQQPPFSQQQQPSSQQPPFPQQHQQFPQQQIPVVQPSVLQQLNPC 179
P-12 QQQQPVLPQQPPFSQQQQPPFSQQQQPSSQQPPFPQQHQQFPQQQIPVVQPSVLQQLNPC 180

P-11 KVFLQQQCSHVAMSQRLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL 239
P-12 KVFLQQQCSHVAMSQRLARSQMWQQSSCHVMQQQCCQQLPQIPEQSRSEAIRAIVYSIIL 240

P-11 QEQQQGFVQPQQQQPQQSGQGVSQHQQQSQQQQQLGQCSFQQPQQLQQLGQQPQQQQIPQ 299
P-12 QEQQQGFVQPQQQQPQQSGQGVSQHQQQSQQQQQLGQCSFQQPQQLQQLGQQPQQQQIPQ 300

P-11 GIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGY          350
P-12 GIFLQPHQISQLEVMTSIALRTLPTMCGVNVPLYSSTTIMPFSIGTGVGGY          351
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Fig. 5 Alignment of deduced amino-acid sequences of two *GluD3-1* haplotypes. The positions of the cysteine residues are *bold and underlined*, and a mutation locus is *shadowed*. *Filled nabla* the beginning of short N-terminal conserved region; *open nabla* the beginning of N-terminal repetitive domain; *down arrow* the beginning of the three subregions of C-terminal part of the protein which indicate a cysteine-rich region, a glutamine-rich region and the final conserved part of the protein, respectively

Fig. 6 Alignment of deduced amino-acid sequences of three *GluD3-2* haplotypes. The positions of the cysteine residues are *bold and underlined*, and five mutation loci are shadowed. *Filled nabla* the beginning of short N-terminal conserved region; *open nabla* the beginning of N-terminal repetitive domain; *down arrow* the beginning of the three subregions of C-terminal part of the protein which indicate a cysteine-rich region, a glutamine-rich region and the final conserved part of the protein, respectively

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P-21 MKTFLVFALLAVAATSAIAQMETRCIPGLERPWQQQPLPPQQTFPQQPLFSQQQQQQLFP  60
P-22 MKTFLVFALLtVAATSAIAQMETRCIPGLERPWQQQPLPPQQTFPQQPLFSQQQQQQLFP  60
P-23 MKTFLVFALLAVAATSAIAQMETRCIPGLERPWQQQPLPPQQTFPQQPLFSQQQQ..LFP  58

P-21 QQPSFSQQQPPFWQQQPPFSQQQPILPQQPPFSQQQQLVLPQQPPFSQQQQPVLPPQQSP 120
P-22 QQPSFSQQQPPFWQQQPPFSQQQPILPQQPPFSQQQQLVLPQQPPFSQQQQPVLPPQQSP 120
P-23 QQPSFSQQQPPFWQQQPPFSQQQPILPQQPPFSQQQQLVLPQQPPFSQQQQPVLPPQQSP 118

P-21 FPQQQQQHQQLVQQQIPVVQPSILQQLNPCKVFLQQQCSPVAMPQRLARSQMLQQSSCHV 180
P-22 FPQQQQQHQQLVQQQIPVVQPSILQQLNPCKVFLQQQCSPVAMPQRLARSQMLQQSSCHV 180
P-23 FPQQQQ.HQQLVQQQIPVVQPSILQQLNPCKlFLQQQCSPVAMPQRLARSQMLQQSSCHV 177

P-21 MQQQCCQQLPQIPQQSRYEAIRAIIYSIILQEQQQVQGSIQSQQQQPQQLGQCVSQPQQQ 240
P-22 MQQQCCQQLPQIPQQSRYEAIRAIIYSIILQEQQQVQGSIQSQQQQPQQLGQCVSQPQQQ 240
P-23 MQQQCCQQLPQIPQQSRYEAIRAIIYSIILQEQQQVQGSIQSQQQQPQQLGQCVSQPQQQ 237

P-21 SQQQLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFGV 300
P-22 SQQQLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFGV 300
P-23 SQQQLGQQPQQQQLAQGTFLQPHQIAQLEVMTSIALRILPTMCSVNVPLYRTTTSVPFdV 297

P-21 GTGVGAY                                                      307
P-22 GTGVGAY                                                      307
P-23 GTGVGAY                                                      304
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P-31 MKTFLIFALLAVAATSAIAQMENSHIPGLEKPSQQQPLPLQQTLSHQQQQQPVQQQPQPF  60
P-32 MKTFLIFALLAVAATSAIAQiENSHIPGLEKPSQQQPLPLQQTLSHhQQQQPVQQQPQPF  60

P-31 PQQQPCSQQQQPPLSQQQQPPFSQQQPPFSQQQQPSFSQQQQPPFSQQQPPFSQQQQPVL 120
P-32 PQQQPCSQQQQPPLSQQQQPPFSQQQPPFSQQQQPSFSQQQQPPFSQQQPPFSQQQQPVi 120

P-31 PQQPSFSQQQLPPFSQQQPPFSQQQQPVLPQQPPFSQQQQPILPQQPPFSQQQQQPVLPQ 180
P-32 PQQPSFSQQQLPPFSQQQPPFSQQQQPVLPQQPPFSQQQQPILPQQPPFSQQQQQPVLPQ 180

P-31 QQILFVHPSILQQLNPCKVFLQQQCSPVAMPQSLARSQMLQQSSCHVMQQQCCQQLLQIP 240
P-32 QQIpFVHPSILQQLNPCKVFLQQQCSPVAMPQSLARSQMLQQSSCHVMQQQCCQQLpQIP 240

P-31 QQSRYEAIRAIIYSIILQEQQQVQGSIQTQQQQPQELGQCVSQPQQQSQQQLGQQPQQQQ 300
P-32 QQSRYEAIRAIIYSIILQEQQQVQGSIQsQQQQPQqLGQCVSQPQQQSQQQLGQQPQQQQ 300

P-31 LAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTSVPFGVGAGVGAY        354
P-32 LAQGTFLQPHQIAQLEVMTSIALRTLPTMCRVNVPLYRTTTSVPFGVGAGVGAY        354
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Fig. 7 Alignment of deduced amino-acid sequences of two *GluD3-3* haplotypes. The positions of the eight cysteine residues are marked with *bold letter and underlined*. Seven mutation loci and a synonymous mutation locus are *shadowed*. *Filled nabla* the beginning of short N-terminal conserved region; *open nabla* the beginning of N-terminal repetitive domain; *down arrow* the beginning of the three subregions of C-terminal part of the protein which indicate a cysteine-rich region, a glutamine-rich region and the final conserved part of the protein, respectively

Table 2 Homology comparison of the seven LMW-GS haplotypes (below diagonal) and their deduced amino-acid sequences (above diagonal) (%, irrespective of the sequence length)

Gene	*GluD3-11*	*GluD3-12*	*GluD3-21*	*GluD3-22*	*GluD3-23*	*GluD3-31*	*GluD3-32*
GluD3-11	**100**	99.7	79.0	78.7	79.5	77.5	77.8
GluD3-12	99.9	**100**	79.0	78.7	79.5	77.6	77.9
GluD3-21	81.6	81.6	**100**	99.7	99.3	88.2	88.9
GluD3-22	81.5	81.5	100	**100**	99.0	87.9	88.5
GluD3-23	81.6	81.6	99.6	99.5	**100**	88.1	88.8
GluD3-31	80.3	80.3	88.4	88.4	88.4	**100**	98.0
GluD3-32	80.4	80.4	88.5	88.5	88.5	99.3	**100**

Table 3 Relationship between *Glu-D3* mobility alleles and *GluD3* gene haplotypes

Cultivar	*Glu-D3* allele[a]	*GluD3-11*[b]	*GluD3-12*[b]	*GluD3-21*[b]	*GluD3-22*[b]	*GluD3-23*[b]	*GluD3-31*[b]	*GluD3-32*[b]
Tasman	*a*	+		+			+	
Chinese Sp.	*a*	+		+			+	
Silverstar	*b*		+		+		+	
Sunco	*b*		+		+		+	
Aroona	*c*		+			+		+
Norin 61	*d*	+				+		+
Hartog	*e*	+			+		+	
BT2288A	*e*	+		+			+	

These new gene sequences have been submitted to Genbank (The accession numbers of GluD3-11, GluD3-12, GluD3-21, GluD3-22, GluD3-23, GluD3-31, and GluD3-32 are DQ357052, DQ357053, DQ357054, DQ357055, DQ357056, DQ357057, and DQ357058, respectively)

[a] *Glu-D3* alleles were defined by protein electrophoretic mobility

[b] "+" means the gene haplotype is present in the corresponding cultivars

were selected firstly using Chinese Spring nulli-tetrasomic lines, so their specificity of amplification could be confirmed.

Nucleotide mutations of the three genes

Mutations contributing to different *Glu-D3* alleles include substitution (transition and transversion), deletion, or insertion of base pairs (Heidenreich 2005). For the DNA sequences of the three genes identified in this study, a total of 29 mutations were detected, including three deletions/insertions and 26 SNPs. In the 26 SNPs, 18 were transitions and eight transversions, indicating that the former mutation type occurs more frequently than the latter at the *Glu-D3* locus. It was interesting to note that all three deletions were complete triplet code (two three-base: CAA and one six-base: CAACAA) deletion. This kind of deletion was also found in triticale (Li et al. 2005) and in other common wheat cultivars (Zhao et al. 2004). It was further observed from the multiple alignments of different *Glu-3* genes that almost all deletions/insertions were on the basis of the triplet code, i.e. one or multiple triplets except pseudogenes (Ikeda et al. 2002; Johal et al. 2004; Zhang et al. 2004). The triplet deletions/insertions did not cause frame shift and resulted in less variation to its amino acid sequence than frame shift mutation. Furthermore, CAA was the codon of glutamine that is the most abundant amino acid in LMW-GS (Figs. 5, 6), so its deletion might have the least effect on protein properties compared with other amino acid deletion. The average mutation rate of the three genes was about 4.0‰, a little higher than that observed in human genome (approximately one SNP per kilobase, Cooper et al. 1985), but lower than that occurred at *Glu-A3* locus (Zhang et al. 2004).

Comparison of GluD3-1, GluD3-2 and GluD3-3 with other Glu-D3 genes registered in Genbank

Currently, approximately 100 entries related to LMW-GS genes have been registered in Genbank, which include complete genes, partial genes and pseudogenes in common wheat, durum and *Ae. tauschii* (Johal et al. 2004). In total, 63 wheat genes with complete coding sequence including 16 *Glu-A3*, 13 *Glu-B3*, 18 *Glu-D3* and 16 undefined genes (Okita et al. 1985; Pitts et al. 1988; Colot et al. 1989; Van et al. 1995; Masci et al. 1998; Ikeda et al. 2002; D'Ovidio and Masci 2004; Zhao et al. 2004; Ozdemir and Cloutier 2005) were selected to compare with the genes identified in this study. The sequence alignments of the seven allelic forms of three

genes found in this study with 18 *Glu-D3* genes (Table 4) from Genbank indicate that *GluD3-1* is highly similar to *AB062865*, *AB062866* and *AB062867*, of which *GluD3-11* has only 1 bp difference (T to C) at the 1,784th position compared with *AB062865* (Table 4). *GluD3-2* is highly identical to *X13306*, *U86027*, *U86029*, *AB062875*, *AY223396* and *AY299485*, among which the sequences of *GluD3-21* and *GluD3-23* were the same as *X13306* and *AB062875*, respectively, and shared 99.9% identity with the rest relevant entries (Table 4). *GluD3-32* had, in fact, significant differences with *AB062863* and *AB062864* although they share 99.9–100% identity in the common sequence (Table 4). Compared with *GluD3-32*, *AB062863* had a base substitution from A to G at the 1,416th position and a 42-base deletion from the 1,339th to 1,380th position in its coding region, leading to an amino acid mutation from glutamine to arginine at the181th position and 14-amino-acid deletion from the 160th to 173rd position, respectively (data not shown); *AB062864* also revealed a 171-base deletion from the 1,214th to 1,384th position in its coding region, resulting in a deletion of 57-amino-acids from the 117th to 173th position. The above results indicated that *GluD3-3* represents a new gene different from any reported *Glu-D3* genes.

It is obvious that there are more than three genes at the *Glu-D3* locus on 1D chromosome because several *Glu-D3* entries from Genbank do not match the three genes (*GluD3-1*, *GluD3-2* and *GluD3-3*) reported in this study. The three genes characterized in this study have two, three, and two allelic variations at the DNA level, respectively. Theoretically, each gene defined in this study encodes a protein subunit and the allelic variation of these genes will result in different protein subunits. Previous studies have all considered *Glu-3* allele defined by protein mobility as a unit in studying their genetic effects. There are numerous reports that different *Glu-A3*, *Glu-B3* and *Glu-D3* alleles have different effects on wheat quality (Gupta et al. 1991; Morel 1994; Morel et al. 1994; Redaelli et al. 1997; Branlard et al. 2001; He et al. 2005). Due to difficulties in distinguishing *Glu-D3* alleles by SDS-PAGE, the genetic study of *Glu-D3* alleles is seldom reported (Liu et al. 2005; He et al. 2005). However, Ma et al (2005) demonstrated that different *Glu-D3* alleles have different genetic effects on dough quality. These allelic effects are actually combinations of different genes and proteins. The genes and their allelic variations reported in this study make it possible to further dissect genetic effects of *Glu-D3* alleles at the DNA or protein level. This could have potential use in practical breeding program.

Identifying *Glu-D3* alleles defined by protein mobility is more difficult than identifying *Glu-A3* and *Glu-B3* alleles by direct protein electrophoresis (Liu et al.

Table 4 Identity comparison with *Glu-D3* genes in Genbank (%, without considering the sequence length and deletions)

Gene	*GluD3-11*	*GluD3-12*	*GluD3-21*	*GluD3-22*	*GluD3-23*	*GluD3-31*	*GluD3-32*
AB062865	100[a]	99.9	86.6	86.5	86.6	85.0	85.0
AB062866	99.3	99.3	85.9	85.9	86.0	84.5	84.5
AB062867	99.7	99.7	86.8	86.8	86.9	85.1	85.2
X13306	78.1	78.1	100[b]	99.9	99.6	88.4	88.5
U86027	86.7	86.7	99.7	100[c]	99.4	92.8	93.0
U86029	86.7	86.7	99.9	100[c]	99.6	92.8	93.0
AB062875	86.6	86.6	99.7	99.6	100[d]	92.3	92.5
AY223396	86.2	86.2	99.6	99.5	99.9	92.2	92.4
AY299485	86.8	86.8	99.6	99.5	99.9	92.2	92.2
AB062863	83.0	83.0	91.8	91.7	91.8	99.1	99.9
AB062864	85.1	85.1	91.9	91.8	91.7	99.2	100[e]
U86026	85.1	85.1	88.5	88.4	88.4	86.9	87.1
AB062873	89.1	89.1	90.5	90.4	90.4	86.1	86.2
AB062874	85.7	85.7	89.9	89.8	89.8	88.0	88.2
AB062872	85.1	85.1	91.7	91.7	91.6	88.6	88.6
M11077	83.0	83.0	88.5	88.4	88.3	85.6	85.6
AB062851	86.7	86.7	86.7	86.6	86.9	86.6	86.8
X84961	77.5	77.5	82.1	82.1	82.3	83.5	83.8

[a] *GluD3-11* had 1-bp difference from *AB062865* at the 1,784th position (T → C)

[b] *GluD3-21* is the same as *X13306*

[c] Based on *GluD3-22*, *U86027* has 1-base (A) deletion at the fourth position backwards; U86029 has 1-base (A) deletion at the 45th position and three-base (AGC) deletion at the 667–669th position

[d] *GluD3-23* is the same as *AB062875*

[e] Compared with *GluD3-32*, *AB062863* has a 42-base deletion in the mid-encoding region and a base substitution from A to G at the 503rd position, and *AB062864* has 171-base deletion in the mid-coding region

2005; He et al. 2005). PCR based markers are necessary to readily identify *Glu-D3* alleles and study their genetic effects. Zhang et al (2004) developed a series of PCR markers for discriminating *Glu-A3* alleles based on sequence variations of one single gene among alleles. We thoroughly characterized three genes and their variations across the cultivars with five *Glu-D3* alleles and established the relationship between them. Table 3 indicates that different genes have different polymorphism distribution patterns across cultivars, thus providing molecular bases for designing independent PCR based markers. The reported information provides the molecular basis for developing new markers for not only discriminating *Glu-D3* alleles but also for dissecting *Glu-D3* alleles' effects at protein subunit level.

Acknowledgments This project was funded by the National Basic Research Program (2002CB11300), International Collaboration Project on Wheat Improvement from Ministry Of Agriculture (2003-Q01), and Natural Sciences Foundation of the Beijing Municipal Government (5041001).

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