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ORIGINAL PAPER

Novel DNA variations to characterize low molecular weight glutenin *Glu-D3* genes and develop STS markers in common wheat

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Abstract Low-molecular-weight glutenin subunits (LMW-GS) play an important role in bread and noodle processing quality by influencing the viscoelasticity and extensibility of dough. The objectives of this study were to characterize *Glu-D3* subunit coding genes and to develop molecular markers for identifying *Glu-D3* gene haplotypes. Gene specific primer sets were designed to amplify eight wheat cultivars containing *Glu-D3a*, *b*, *c*, *d* and *e* alleles, defined traditionally by protein electrophoretic mobility. Three novel *Glu-D3* DNA sequences, designated as *GluD3-4*, *GluD3-5* and *GluD3-6*, were amplified from the eight wheat cultivars. *GluD3-4* showed three allelic variants or haplotypes at the DNA level in the eight cultivars, which were designated as *GluD3-41*, *GluD3-42* and *GluD3-43*. Compared with *GluD3-42*, a single nucleotide polymorphism (SNP) was detected for *GluD3-43* in the coding region, resulting in a pseudo-gene with a nonsense mutation at the 119th position of deduced peptide, and a 3-bp insertion was found in the coding region of *GluD3-41*, leading to a glutamine insertion at the 249th position of its deduced protein. The coding regions for *GluD3-5* and *GluD3-6* showed no allelic variation in the eight cultivars tested, indicating that they were relatively conservative in common wheat. Based on the 12 allelic variants of three *Glu-D3* genes identified in this study and three detected previously, seven STS markers were established to amplify the corresponding gene sequences in wheat cultivars containing five *Glu-D3* alleles (*a*, *b*, *c*, *d* and *e*). The seven primer sets *M2F12/M2R12, M2F2/M2R2, M2F3/M2R3, M3F1/M3R1, M3F2/M3R2, M4F1/M4R1* and *M4F3/M4R3* were specific to the allelic variants *GluD3-21/22*, *GluD3-22*, *GluD3-23*, *GluD3-31*, *GluD3-32*, *GluD3-41* and *GluD3-43*, respectively, which were validated by amplifying 20 Chinese wheat cultivars containing alleles *a*, *b*, *c* and *f* based on protein electrophoretic mobility. These markers will be useful to identify the *Glu-D3* gene haplotypes in wheat breeding programs.

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Introduction

The processing properties of common wheat flour are mainly affected by high and low molecular weight glutenin subunit proteins (HMW-GS and LMW-GS) that form the disulphide-bonded gluten macropolymer (Gras et al. 2001) and contribute to the fundamental aspects of dough quality such as viscoelasticity and extensibility (Payne 1987; Wesley et al. 1999, 2001; Brites and Carrillo 2001; Luo et al. 2001). HMW- and LMW-GS alleles are therefore important targets for marker-assisted selection in the development of improved wheat cultivars (Gupta et al. 1999; Eagles et al. 2001, 2002; Gale 2005). LMW-GS represents approximately one-third of total seed storage proteins and 60% of the gluten fraction (Bietz and Wall 1973). Their structural definition through nucleotide sequencing has been more problematical than for HMW-GS because they are more numerous and the respective proteins more difficult to purify.

Most of the LMW-GS are encoded by the complex *Glu-3* loci (*Glu-A3, Glu-B3* and *Glu-D3*) on the short arms of chromosomes 1A, 1B and 1D (Gupta and Shepherd 1990; Jackson et al. 1983), though other LMW-GS loci have also been reported, such as *Glu-B2* and *Glu-B4* on chromosome 1B (Jackson et al. 1985; Liu and Shepherd 1995), *Glu-D4* on chromosome 1D and *Glu-D5* on chromosome 7D (Sreeramulu and Sigh 1997). Currently, six, eleven and five alleles defined by protein electrophoretic mobility have been confirmed at *Glu-A3*, *Glu-B3* and *Glu-D3* locus, respectively, in common wheat (Branlard et al. 2003; Gianibelli et al. 2001; Gupta and Shepherd 1990). Recently, seven new alleles, *Glu-A3g*, *Glu-A3h*, *Glu-B3m*, *Glu-B3n*, *Glu-B3o*, *Glu-B3p* and *Glu-B3q* were designated (McIntosh et al. 2003). Another allele, *Glu-D3f* was also identified (CIMMYT and Japan NARC, not published) and is currently awaiting verification.

Based on the first amino acid present in the N-terminal sequences of the proteins, eight types of LMW-GS have been identified (D'Ovidio and Masci 2004), which are LMW-s starting with the sequence SHIPGL-, LMW-i starting with sequence ISQQQQ-, three LMW-m types with N-terminal sequences of METSHIPGL-, METSRIPGL and METSCIPGL-, respectively, and three types with N-terminal sequences resembling those of the α-, β- and γ-type gliadins (Kasarda et al. 1988; Tao and Kasarda 1989; Lew et al. 1992; Cloutier et al. 2001; Gianibelli 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 100 sequence tags of genes, partial genes and pseudo-genes of the LMW-GS family have been cloned and sequenced from several common wheat cultivars (Pitts et al. 1988; Cloutier et al. 2001; Ikeda et al. 2002; Zhang et al. 2004). Hai et al. (2005) retrieved 69 known LMW-GS genes from GenBank and classified them into nine groups based on the deduced amino acid sequence of the highly conserved N-terminal domain, and nine corresponding primer sets proved to be LMW-GS group-specific were established. Ikeda et al. (2006) also constructed ten group-specific markers according to the published nucleotide sequences. However, the relationship between different protein mobility alleles and their corresponding allelic variants at the DNA level is difficult to determine. Based on the allelic variation of one LMW-GS gene at the *Glu-A3* locus, a set of PCR markers were developed by Zhang et al. (2004), whereas, no marker sets are currently available for the identification of alleles at *Glu-B3* and *Glu-D3* loci (Gale 2005). In our previous study, three LMW-GS genes were amplified from the *Glu-D3* locus and seven haplotypes were characterized in eight common wheat cultivars (Zhao et al. 2006). In this study, we report the identification of more *Glu-D3* gene haplotypes and development of a set of STS markers for these haplotypes.

Materials and methods

Wheat stocks

Eight common wheat cultivars (Tasman, Chinese Spring, Silverstar, Sunco, Aroona, Norin61, Hartog, and BT2288A) carrying five *Glu-D3* alleles that were defined traditionally by protein electrophoretic mobility (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 Prof. R. A. McIntosh at the Plant Breeding Institute, University of Sydney, were used to confirm chromosomal locations of identified genes. Twenty Chinese wheat cultivars with protein mobility alleles *Glu-D3a*, *b*, *c* and *f* were used to validate the developed molecular markers (Table 2).

Development of PCR primers for identifying new Glu-D3 variants

Gene-specific primers were developed based on the method described by Zhang et al. (2003, 2004). Eight

Table 1 Relationship between *GluD3* mobility alleles and *GluD3* gene haplotypes

Cultivar	Protein allele[a]	*GluD3-11*[b]	*GluD3-12*	*GluD3-21/22*[c]	*GluD3-23*	*GluD3-31*	*GluD3-32*	*GluD3-41*[d]	*GluD3-42*	*GluD3-43*	*GluD3-5*	*GluD3-6*
Chinese Spring	a	+		+		+			+		+	+
BT2288A	e	+		+		+			+		+	+
Silverstar	b		+	+		+			+		+	+
Sunco	b		+	+		+			+		+	+
Aroona	c		+		+		+	+			+	+
Norin 61	d	+			+		+		+		+	+
Tasman	a	+		+		+				+	+	+
Hartog	e	+		+		+				+	+	+

The sequences of the five new variants amplified in the study have been submitted to GenBank (Accession DQ457416 to DQ457420)

[a] *GluD3* alleles were defined by protein electrophoretic mobility

[b] The gene information of *GluD3-11* to *GluD3-32* was from Zhao et al. (2006)

[c] The only SNP mutation between *GluD3-21* and *GluD3-22* occurred in signal peptide region so there was no difference between their deduced amino acid sequences

[d] The result was confirmed by amplifying 2 cultivars with allele *c* (Dagger and Halberd) using primer set *S5F54/S5R55*

"+" Means that the gene haplotype is present in the corresponding cultivar

reference *Glu-D3* genes, *X13306*, *AB062851*, *AB062872*, *AB062873*, *AB062874*, *M11077*, *U86026* and *X84961* available in GenBank were used for primer development (http://www.ncbi.nlm.nih.gov). Each gene sequence was divided into two parts to design corresponding primers and to obtain the accurate sequence results by reassembling two PCR products of normal length. The forward and reverse primers for the 5′ region of the gene were designed first. The amplified sequences were used to design the forward primers of 3′ region. A total of 48 primers were designed and 96 primer sets (48 for 5′ region and 48 for 3′ region) were tested. Primer screening was conducted according to Zhao et al. (2006). Based on the result of sequence alignments, six pairs of primer sets for three *Glu-D3* genes were confirmed with annealing temperature of 58°C. Primer sequences (5′–3′) and their locations within the reference genes are shown in Table 3.

DNA extraction and PCR amplification

Genomic DNA was extracted from seedlings or seeds using modified CTAB procedure (Gale et al. 2001). PCR was performed using 3U of TaKaRa *Taq* polymerase in 40 µl of reaction buffer (1.5 mM $MgCl_2$) containing 60 ng of genomic DNA, 200 µM of each of dNTPs and 10 pmoles of each PCR primer. PCR cycling was 94°C for 5 min followed by 38 cycles of 94°C for 40 s, 58°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 errors in sequencing, PCR reaction and sequencing procedure were repeated two to four times. Some PCR products that were difficult to sequence directly were purified, cloned, and sequenced using pGEM®-T Easy Vector cloning System (Promega). Sequence analysis and characterization were performed using software DNAMAN (http://www.lynnon.com).

Marker development and validation

STS marker primers were developed based on the sequence alignments of 12 *Glu-D3* gene haplotypes detected in this and previous studies (Zhao et al. 2006) and screened by amplifying the eight wheat cultivars with known alleles. The PCR products of gene-specific primer sets were sequenced and compared with their target gene haplotypes. The STS markers were then

Table 2 Validation of the 7 STS markers with 20 Chinese common wheat cultivars

No.	Cultivar	Protein allele[a]	*M2F12/M2R12*[b]	*M2F2/M2R2*	*M2F3/M2R3*	*M3F1/M3R1*	*M3F2/M3R2*	*M4F1/M4R1*	*M4F3/M4R3*
1	Gaocheng 8901	f	–	–	+	–	+	–	–
2	Yumai 63	f	–	–	+	–	+	–	–
3	PH1521	f	–	–	+	–	+	–	–
4	Jing 411	f	–	–	+	–	+	–	–
5	Linfen 137	c	–	–	+	–	+	–	–
6	Zhongyou 9701	c	–	–	+	–	+	–	–
7	CA9722	c	–	–	+	–	+	–	–
8	Xiaoyan 54	c	–	–	+	–	+	–	–
9	Zhengzhou 81-1	c	–	–	+	–	+	–	–
10	Yumai 47	c	–	–	+	–	+	–	–
11	Wanmai 33	b	+	–	–	+	–	–	–
12	Yumai 70	b	+	–	–	+	–	–	–
13	Yannong 15	b	+	–	–	+	–	–	–
14	Shaan 229	b	+	+	–	+	–	–	–
15	CA9550	b	+	+	–	+	–	–	–
16	Yunmai 42	a	+	–	–	+	–	–	–
17	Shaanyou 225	a	+	–	–	+	–	–	–
18	Zhengzhou 9023	a	+	+	–	+	–	–	–
19	Yumai 34	a	+	–	–	+	–	–	–
20	Jingdong 8	a	+	+	–	+	–	–	–

[a] The information of allele at protein electrophoretic mobility came from CIMMYT. "f" was newly named allele and needed to be validated further

"+" and "–" mean presence or absence of PCR products, respectively

Table 3 Primers used for amplifying the three newly described *Glu-D3* genes

Target gene	Primer	Sequence (5′→ 3′)	Primer location[a]	Reference gene[b]	Expected size (bp)	Ann.tem. (°C)
GluD3-4	*S2F21*	TGT ACC AAA AAA TCA TTT CT	36-55	*AB062872*	701	58
	S2R21	GGA TTG TTC GGG GAT TTG CT	548–567	*GluD3-4*		
	S5F54	AAC AAC AAC TTG TGC AAC AG	462–481	*GluD3-4*	959	58
	S5R55	GAT CTC AAA TCT CCA ACC AT	1,322–1,341	*M11077*		
GluD3-5	*S1F11*	ATC AAT CCA AAA GTA CGC GTA	−8 to 13[c]	AB062851	880	58
	S1R11	CAT GGC AAC TGC TCT GCC A	807–825	*GluD3-5*		
	S4F41	CAA CAA CGA CCA CCA TTT TCT	546–566	*GluD3-5*	770	58
	S4R43	TTG TGT GAC ACT TTA TTT GTC	1,035–1,055	*M11077*		
GluD3-6	*S3F31*	ATG ATC AAT CCA AAA GTA CCG	11–31	*U86026*	855	58
	S3R33	GGG TTG GTA GAC ACC TTG AA	803–822	*GluD3-6*		
	S6F61	CAT TTT CGC AGC AAC AAC AAA	440–460	*GluD3-6*	987	58
	S6R64	CAC CAG GTT GAG GTT GTG AT	1,350–1,369	*U86026*		

[a] The location is numbered from the first nucleotide of the available gene fragments

[b] Reference genes indicate the gene sequences that were used to determine the primer locations

[c] The first 8 bp of the primer *S1F11* was based on *AB062872*, because there was insufficient upstream sequence for *AB062851*

validated by amplifying 20 Chinese wheat cultivars with different protein mobility alleles (Table 2).

Results

New DNA variations at the Glu-D3 locus

In addition to the three *Glu-D3* genes reported in our previous study (Zhao et al. 2006), three additional LMW-GS genes including five allelic variants were identified at the *Glu-D3* locus on chromosome 1D in eight common wheat cultivars. The first gene, designated as *GluD3-4*, was amplified with a primer set *S2F21/S2R21* for the 5′ region and *S5F54/S5R55* for the 3′ region of the gene (Table 3), which generated 701- and 959-bp products, respectively (Fig. 1). The complete sequence of *GluD3-4* was assembled with a size of 1,384 bp. Likewise, the second gene, designated as *GluD3-5* with 1,292 bp, was amplified using primer sets *S1F11/S1R11* for the upstream region and *S4F41/S4R43* for the downstream region with fragment sizes of 880 and 770 bp,

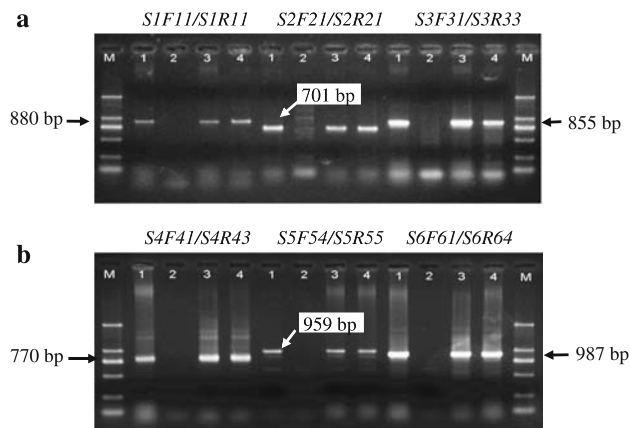

Fig. 1 Electrophoresis of PCR products amplified from Chinese Spring and its nulli-tetrasomic lines using 6 specific primer sets in agarose gel. *1* Chinese Spring; *2* N1DT1B; *3* N1BT1A; *4* N1AT1B. M, DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600, 2,000 bp). **a** The upstream region, and b the downstream region of *GluD3-4*, *GluD3-5* and *GluD3-6* genes

respectively (Fig. 1). The third one, designated as *GluD3-6* in 1,300 bp, was amplified with primer sets *S3F31/S3R33* for the upstream region, and *S6F61/S6R64* for the downstream region, which resulted in 855- and 987-bp fragments, respectively (Fig. 1).

GluD3-4 showed three haplotypes or allelic variants at the DNA level in the eight wheat cultivars (Table 1). The first allele, designated *GluD3-41*, was found in the cultivar Aroona. The second allele, designated *GluD3-42*, was present in cultivars Chinese Spring, BT2288A, Silverstar, Sunco and Norin 61. The third allele, designated *GluD3-43*, was presented in cultivars Tasman and Hartog. Compared with *GluD3-42*, *GluD3-41* had a 3-bp insertion at the 857–859 position in gene coding region (Sup-Fig. 1), leading to a glutamine insertion at the 249th position of the C-terminal glutamine-rich region (Sup-Fig. 4); *GluD3-43* had a single nucleotide polymorphism (SNP) in the coding region, resulting in an nonsense mutation at the 119th position of deduced peptide, which made the haplotype a pseudogene.

Both *GluD3-5* and *GluD3-6* had no allelic variation in the eight wheat cultivars (Table 1; Sup-Fig. 2, 3, 5, 6), indicating they were relatively conservative in common wheat.

Development of STS markers for identifying different *GluD3* gene haplotypes

In the previous (Zhao et al. 2006) and present studies, we characterized six LMW-GS genes at *Glu-D3* locus of common wheat. Among them, *GluD3-1*, *GluD3-2*, *GluD3-3* and *GluD3-4* had two, three, two and three allelic variants or haplotypes, respectively, which were used to design gene specific primers. In total, seven STS markers for the haplotypes of *GluD3-2*, *GluD3-3* and *GluD3-4* were confirmed by amplifying the eight wheat cultivars containing mobility alleles *GluD3a*, *b*, *c*, *d* and *e*. The primer sequences and their locations in reference genes were listed in Table 6. *GluD3-5* and *GluD3-6* did not show any allelic variation in all the eight cultivars tested, and thus no gene-specific markers were developed for them. For *GluD3-1*, the only mutation between its two allelic forms was a CAA indel that occurred in the high repetitive region with 11 CAA repeats. Though both forward and reverse primers were developed based on the indel locus, none of them was specific and useful.

Based on the SNPs between the three haplotypes of *GluD3-2*, three specific primer sets were selected. Primer pair *M2F12/M2R12* generated an 884-bp fragment and was specific to allelic forms *GluD3-21/22*. *M2F2/M2R2* amplified a fragment of 958 bp that was specific to *GluD3-22*. *M2F3/M2R3* amplified a fragment of 725 bp specific to *GluD3-23* (Figs. 2, 3).

Two primer sets were specific for *GluD3-3*. The first primer set *M3F1/M3R1* amplified a fragment of 528 bp

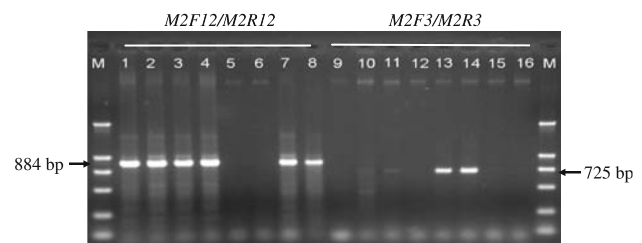

Fig. 2 Haplotype-specific PCR for *GluD3-21/22* and *GluD3-23* using primer sets *M2F12/M2R12* and *M2F3/M2R3*. 1,9 Tasman (a); 2,10 Chinese Spring (a); 3,11 Silverstar (b); 4,12 Sunco (b), 5,13 Aroona (c); 6,14 Norin61 (d); 7,15 Hartog (e); 8,16 BT2288A (e); M DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600, 2,000 bp)

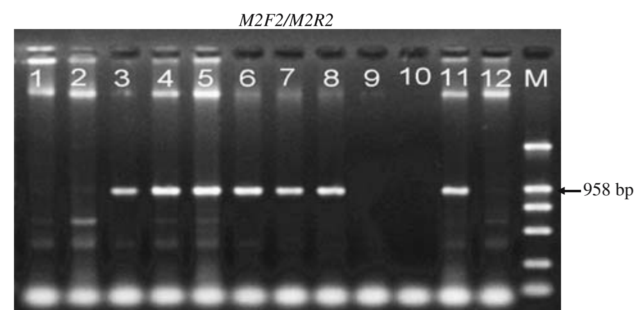

Fig. 3 Haplotype-specific PCR for *GluD3-22* using primer sets *M2F2/M2R2*. 1 Tasman (a); 2 Chinese Spring (a); 3 Silverstar (b); 4 Amery (b); 5 Sunco(b), 6 Leichhardt (b); 7 Baxter (b); Cunningham (b); 9 Aroona (c); 10 Norin61 (d); 11 Hartog (e); 12 BT2288A (e); M DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600, 2,000 bp)

specific to *GluD3-31* and the second set *M3F2/M3R2* amplified a fragment of 334 bp specific to *GluD3-32* (Fig. 4). For *GluD3-4*, only two gene specific markers were verified, of which *M4F1/M4R1* was specific to *GluD3-41* and *M4F3/M4R3* specific to *GluD3-43*, with 773- and 413-bp PCR products, respectively (Fig. 5).

Validation of the seven *GluD3* STS markers

In order to verify the accuracy of the developed markers, the PCR products of each primer set were sequenced and compared with its corresponding gene haplotype. DNA sequence analysis indicated that all the seven markers were completely matched to their target gene haplotypes. Validation with 20 Chinese wheat cultivars (Table 2; Sup-Fig. 7–13) indicated that the 10 cultivars containing alleles *a* and *b* were positive to both *M2F12/M2R12* and *M3F1/M3R1*, indicating that they have haplotypes of *GluD3-21* or *GluD3-22* and *GluD3-31*; the 10 cultivars containing alleles *c* and *f* were positive to both *M2F3/M2R3* and *M3F2/M3R2*, exhibiting that they contain the *GluD3-23* and *GluD3-32* haplotypes; and all cultivars were negative to both *M4F1/M4R1* and *M4F3/M4R3*, suggesting that they may contain another haplotype of this gene *GluD3-42* (Note, STS marker for *GluD3-42* was unsuccessful).

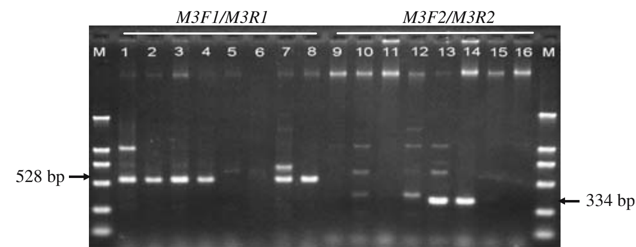

Fig. 4 Haplotype-specific PCR for *GluD3-31* and *GluD3-32* using primer sets *M3F1/M3R1* and *M3F2/M3R2*. 1,9 Tasman (a); 2,10 Chinese Spring (a); 3,11 Silverstar (b); 4,12 Sunco (b), 5,13 Aroona (c); 6,14 Norin61 (d); 7,15 Hartog (e); 8,16 BT2288A (e); M DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600, 2,000 bp)

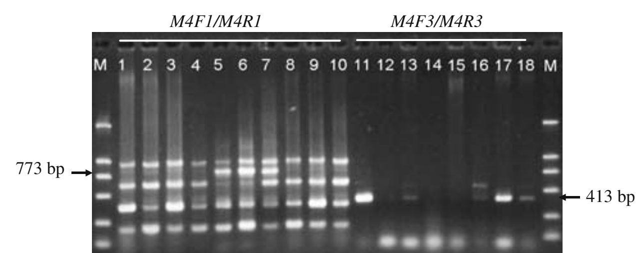

Fig. 5 Haplotype-specific PCR for *GluD3-41* and *GluD3-43* using primer sets *M4F1/M4R1* and *M4F3/M4R4*. 1,11 Tasman (a); 2,12 Chinese Spring (a); 3,13 Silverstar (b); 4,14 Sunco (b), 5,15 Aroona (c); 6 Dagger (c); 7 Halberd; 8,16 Norin61 (d); 9,17 Hartog (e); 10,18 BT2288A (e); M DNA Ladder 2000 (100, 250, 500, 750, 1,000, 1,600, 2,000 bp)

For the primer set *M2F2/M2R2*, two cultivars with protein allele *b* (Shaan 229 and CA9550) and two cultivars with allele *a* (Zhengzhou 9023 and Jingdong 8) gave the 528 bp band (Sup-Fig. 8), indicating that they contain haplotype *GluD3-22*.

Discussion

The relationship between the *GluD3* gene haplotypes and *GluD3* mobility alleles

The differences in electrophoretic patterns for LMW-GS that were observed between the cultivars with different mobility alleles (Lew et al. 1992; Gupta and Shepherd 1990) were conditioned by several *Glu-3* genes (Zhao et al. 2006; D'Ovidio and Masci 2004). In this and previous studies (Zhao et al. 2006), six different *Glu-D3* genes were identified from *Glu-D3* locus on chromosome 1D of common wheat. Among these, four showed allelic variations in the wheat cultivars tested in this study. In most cases, the cultivars with the same mobility allele (*a*, *b*, *c*, *d* or *e*) always had the same allelic variant at DNA level (Table 1) (Zhao et al. 2006). For example, cultivars with allele *b* contain gene haplotypes *GluD3-12*, *-21/22*, *-31*, *-42*, *-5* and *-6*. However, there were also exceptions in that cultivars Chinese Spring with allele *a* and BT2288A with allele *e* had the same haplotype of *GluD3-42*, and Tasman with allele *a* and Hartog with allele *e* had the same haplotype of *GluD3-43*. This may due to the presence of gamma-gliadins in the LMW glutenin fraction, which link Glu-D3 locus and are having similar molecular weights with LMW-GS. Meanwhile, the LMW-GS encoded at *Glu-D3* locus may be complicated by post-translational modification leading to changes at protein mobility level that is not related to differences in the gene sequence (Liu et al. 2005; He et al. 2005). Based on the deduced amino acid sequences of the 12 haplotypes of 6 genes, 5 types of allelic combinations (i.e. 5 alleles at DNA level) were found across the 8 wheat cultivars (Table 1), although they were not completely consistent with the traditional *Glu-D3* alleles defined by protein electrophoretic mobility (McIntosh et al. 1998). The results indicated that the so-called alleles (protein electrophoretic patterns) were, in fact, controlled jointly by the combinations of haplotypes of six *Glu-D3* genes at least. This is consistent with Ikeda et al. (2006).

Relationship between the *GluD3* genes identified and those registered in GenBank

So far, more than 100 entries related to LMW-GS genes in common wheat have been registered in

GenBank, including complete genes, partial genes and pseudo-genes (Okita et al. 1985; Colot et al. 1989; Van et al. 1995; Masci et al. 1998; Ikeda et al. 2002; Ozdemir and Cloutier 2005; Zhao et al. 2006). In this study, 18 *GluD3* and 16 undefined LMW-GS gene sequences were selected to compare with the 6 genes identified. Results indicated that *GluD3-4* was highly similar to *AB062872* and *M11077*; *GluD3-41* had 3 bp difference from *M11077* at position 79, 106 and 1,010 with the former two being in start codon region and the third in encoding region, respectively; *GluD3-42* had only one base difference from *AB062872* (G to A) at position 1,051 but was 300 bp longer in the downstream region than the later; *GluD3-5* is highly identical to *AB062851* and *X84961*, with only 3 bp difference from *AB062851* at position 843 (G to A), 1,002 (G to T) and 1,076 (C to T); and *GluD3-6* shared 99.4–99.7% identity with *AB062873*, *AB062874* and *U86029*. The identity with the other 11 *Glu-D3* genes from GenBank was all under 90%. Further analysis showed that all the 18 *Glu-D3* and seven undefined genes in GenBank could also be classified into six groups that matched the haplotypes of the six *Glu-D3* genes studied in this manuscript even though one or few bp difference existed between each groups (Table 7). Overall these results indicate that the *Glu-D3* locus is a multiple gene locus consisting of at least six different LMW-GS genes that all have allelic variants among different genotypes.

GluD3 gene variation and STS marker development

Within the six LMW-GS genes, including the 12 allelic variants or haplotypes identified at the *Glu-D3* locus, two types of mutations were detected. These mutations were either base substitutions or indels. Here we also found an indel of the triplet code CAA in the repeat region of *GluD3-4*, the same as in *GluD3-1* and *GluD3-2*, and which may influence protein feature (Zhao et al. 2006). It was interesting to note that although the base sequences among the six *Glu-D3* genes varied significantly (with identity of 80.3–92.4%, Table 4), the differences between the allelic forms or haplotypes of each gene were relatively small (with similarity of 99.3–100%). In addition, no allelic variation for *GluD3-5* and *GluD3-6* was found in the eight wheat cultivars tested. The results indicated that the LMW-GS genes at *Glu-D3* locus were relatively conservative compared with HMW-GS genes (Lei et al. 2006; Ma et al. 2003; Gianibelli et al. 2001).

Allelic variation at LMW-GS loci is related to differences in dough quality in common wheat (Gupta et al. 1989; Gupta and MacRitchie 1994) and durum wheat (Pogna et al. 1990; Ruiz and Carrillo 1993). Some allelic forms of LMW-GS show even greater effects on dough strength and extensibility than HMW-GS (Payne 1987). Pogna et al. (1996) reported that the durum genotypes with the *Gli-D1/GluD3* translocation showed increase in dough strength and extensibility and decrease in tenacity compared with its wild-type durum wheats. Ma et al. (2005) showed that the *Glu-D3* locus played multifaceted effects on dough physical

Table 5 the N-terminal amino acid sequences of the six deduced *Glu-D3* proteins

Glu-D3 gene	N-terminal amino acid sequence	Type based on the first amino acid	Type (Group) based on Ikeda et al.
GluD3-1	METSRVPGL-	LMW-m	III (5)
GluD3-2	METRCIPGL-	LMW-m	V (10)
GluD3-3	(M/IEN)SHIPGL-	LMW-s	II (4)
GluD3-4	METSCISGL-	LMW-m	IV (7)
GluD3-5	METSHIPGL	LMW-m	I (1)
GluD3-6	METSCIPGL	LMW-m	IV (8 & 9)

Table 4 Similarity comparison of 12 haplotypes of six *GluD3* genes (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*	*GluD3-41*	*GluD3-42*	*GluD3-43*	*GluD3-5*	*GluD3-6*
GluD3-11	**100**	99.7	79.0	78.7	79.5	77.5	77.8	81.4	81.4	81.3	68.5	78.2
GluD3-12	99.9	**100**	79.0	78.7	79.5	77.6	77.9	81.4	81.4	81.3	68.5	78.2
GluD3-21	81.6	81.6	**100**	99.7	99.3	88.2	88.9	87.2	87.2	87.2	74.1	81.0
GluD3-22	81.5	81.5	100	**100**	99.0	87.9	88.5	86.9	86.9	86.8	73.8	81.6
GluD3-23	81.6	81.6	99.6	99.5	**100**	88.1	88.8	86.8	86.8	86.7	73.5	81.2
GluD3-31	80.3	80.3	88.4	88.4	88.4	**100**	98.0	83.4	83.4	83.3	80.6	74.3
GluD3-32	80.4	80.4	88.5	88.5	88.5	99.3	**100**	83.4	83.4	83.3	81.2	75.0
GluD3-41	89.0	89.0	88.8	88.7	88.8	87.5	87.5	**100**	100	100	72.6	85.5
GluD3-42	89.0	89.0	88.8	88.7	88.8	87.5	87.5	100	**100**	100	72.6	85.5
GluD3-43	88.9	88.9	88.7	88.7	88.7	87.4	87.5	99.9	99.9	**100**	72.5	84.9
GluD3-5	78.8	78.8	81.8	81.8	81.6	87.9	88.2	80.7	80.6	80.5	**100**	69.1
GluD3-6	85.2	85.2	85.6	85.6	85.4	81.3	81.6	92.4	92.4	92.3	81.1	**100**

The gene information of *GluD3-11–GluD3-32* were from Zhao et al. (2006)

Table 6 Seven pairs of PCR primers for the identification of different *GluD3* gene haplotypes

Target gene	Marker primer	Sequence (5′→ 3′)	Primer location[a]	Expectedsize (bp)	Conditions[b]
GluD3-21/22	*M2F12*	TTGGGCCTAATCGCTCGC	36–53	884	94°C/40 s–60°C/40 s–72°C/90 s
	M2R12	TAGTCTCCATCTGCGCAATT	900–919		
GluD3-22	*M2F2*	CTCGTCTTTGCCCTCCTCA	862–880	958	94°C/40 s–60°C/40s–72°C/60 s
	M2R2	CTAAACAACGGTGACCCAAT	1,800–1,819		
GluD3-23	*M2F3*	TCTGTACTTTGTGTGTGATCG	588–608	725	94°C/40 s–59°C/40 s–72°C/60 s
	M2R3	ACTGCTGCTGGAGGAATAG	1,284–1,312		
GluD3-31	*M3F1*	ACAAGTGCCATTGCACAAATG	915–935	528	94°C/45 s–56°C/45 s–72°C/80 s
	M3R1	GATAGATGGATGAACAAATA	1,423–1,442		
GluD3-32	*M3F2*	CAAGTGCCATTGCACAAATT	916–935	334	94°C/30 s–59°C/30 s–72°C/60 s
	M3R2	AATGATGGTTGTTGCGGTAT	1,230–1,249		
GluD3-41	*M4F1*	AAGTAGTTAGCACCAATCCAT	106–126	773	94°C/45 s–59°C/45 s–72°C/90 s
	M4R1	CCTGTTGTTGTTGTTGTTGTT	858–878		
GluD3-43	*M4F3*	GCATCAAAACCAAGCAAAAG	89–108	413	94°C/30 s–61°C/30 s–72°C/60 s
	M4R3	GGCTGAACAATAGGGATTTA	482–501		

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

[b] PCR cycling was all carried out for 38 cycles, in addition to a beginning at 94°C for 5 min and a final extension at 72°C for 5 min

Table 7 The relationship between *GluD3* haplotypes identified in this study and *GluD3* genes from Genbank

GluD3 gene	GluD3 *haplotype*	*GluD3* genes from GenBank[a]	Identity (%)[b]
GluD3-1	*GluD3-11*	*AB062865*, *AB062866*, *AB062867*, *AY214450**	99.3–100
	GluD3-12		
GluD3-2	*GluD3-21*	*X13306*	100
	GluD3-22	*U86027*, *U86029*	100
	GluD3-23	*AB062875*, *AY223396*, *AY299485*, *AJ519835**, *AY542897**	99.7–100
GluD3-3	*GluD3-31*		
	GluD3-32	*AB062863*, *AB062864*, *AY542898**	99.1–100
GluD3-4	*GluD3-41*	*M11077*	99.8
	GluD3-42 [c]	*AB062872*, *AY296753**, *AY994364**	99.8–99.9
	GluD3-43		
GluD3-5	*GluD3-5*	*AB062851*, *X84961*	99.2–99.8
GluD3-6	*GluD3-6*	*AB062873*, *AB062874*, *U86026*, *AY695380**	99.4–99.9

[a] The classification mainly depend on their indels

[b] Without considering the sequence length and deletion

[c] *AB062872*, *AY296753* and *AY994364* each had a long deletion

*Genbank sequences for which loci were previously unknown but are likely to be at the *Glu-D3* locus, based on their sequence homology with other *Glu-D3* genes

properties. Ikeda et al. (2006) also reported that the abundance of LMW-GS encoded by *Glu-D3* might contribute more to the gluten viscoelasticity of common wheat.

Based on the present study, the effects of *Glu-D3* subunits on quality could not be unambiguously traced among different genotypes using the currently established mobility allele system. For example, the results of *M2F2*/*M2R2* and *M4F1*/*M4R1* with 20 wheat cultivars was not consistent with the expected results, even though a consistent result was obtained using the 10–12 Australian cultivars (Figs. 3, 5). For *M2F2*/*M2R2*, the three cultivars with protein mobility allele *b* (Wanmai 33, Yumai 70 and Yannong 15) were expected to have the 958 bp band missing, but in fact a product was detected. For the two cultivars containing allele *a* (Zhengzhou 9023 and Jingdong 8), a PCR band of 958 bp was present, which is contrary to the expectation that they should be missing. For *M4F1*/*M4R1* (a marker for *GluD3-41*), all the six Chinese cultivars with allele *c* did not contain the specific haplotype PCR band but the three Australian cultivars with *c* allele showed this PCR product. This may be due to the complexity of relations between protein alleles and their coding haplotypes, or the ambiguity in identification of the *Glu-D3* protein subunits by SDS-PAGE. Due to the difficulty in directly and correctly identifying LMW-GS proteins, it is of great importance to clarify the gene composition of the *Glu-D3* locus and to develop markers for these genes. Until now, no molecular

marker for distinguishing *Glu-D3* alleles has been available. In this study, we developed and validated seven STS markers for different *Glu-D3* gene haplotypes. Our markers will be useful in accurately dissecting the effects of the LMW *Glu-D3* locus on wheat quality at the gene level, and make it possible to utilize this information in wheat breeding.

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