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ORIGINAL PAPER

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Characterization of low-molecular-weight glutenin subunit genes and their protein products in common wheats

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Abstract To characterize the low-molecular-weight glutenin subunit (LMW-GS), we developed specific PCR primer sets to distinguish 12 groups of LMW-GS genes of Norin 61 and to decide their loci with nullisomic–tetrasomic lines of Chinese Spring. Three, two, and ten groups were assigned to *Glu-A3*, *Glu-B3*, and *Glu-D3* loci, respectively. To identify the proteins containing the corresponding amino acid sequences, we determined the N-terminal amino acid sequence of 12 spots of LMW-GSs of Norin 61 separated by two-dimensional gel electrophoresis (2DE). The N-terminal sequences of the LMW-GS spots showed that 10 of 12 groups of LMW-GSs were expressed as protein products, which included LMW-i, LMW-m, and LMW-s types. Four spots were encoded by *Glu-A3* (LMW-i). Three spots were encoded by *Glu-B3* (LMW-m and LMW-s). Five spots were encoded by *Glu-D3* (LMW-m and LMW-s). A minor spot of LMW-m seemed to be encoded by the same *Glu-B3* gene as a major spot of LMW-s, but processed at a different site. Comparing among various cultivars, there were polymorphic and non-polymorphic LMW-GSs. *Glu-A3* was highly polymorphic, i.e., the a, b, and c alleles showed one spot, the d allele showed four spots, and the e allele had no spot. Insignia used as one of the *Glu-A3* null standard cultivars had a LMW-GS encoded by *Glu-A3*. We also found that Cheyenne had a new *Glu-D3* allele. Classification of LMW-GS by a combination of PCR and 2DE will be useful to identify individual LMW-GSs and to study their contribution to flour quality.

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Introduction

Wheat seed storage proteins are composed of two major fractions, gliadin and glutenin. Glutenin consists of high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits. The HMW glutenin subunits (HMW-GSs) are encoded by *Glu-A1*, *Glu-B1*, and *Glu-D1* on the long arm of chromosomes 1A, 1B, and 1D, respectively (Payne et al. 1980). The LMW glutenin subunits (LMW-GSs) are encoded by *Glu-A3*, *Glu-B3*, and *Glu-D3* on the short arm of these chromosomes (Gupta and Shepherd 1990; Jackson et al. 1983). It has been shown that allelic variations of HMW-GSs and LMW-GSs affect the properties of dough made with various wheat cultivars (D'Ovidio and Masci 2004; Nagamine et al. 2000; Skeritt 1998). LMW-GSs are classified into three types, LMW-i, LMW-m, and LMW-s, based on the amino acid at their N-terminal end, which correspond to isoleucine, methionine, and serine residues, respectively (Cloutier et al. 2001; Lew et al. 1992). LMW-m and LMW-s types were identified as protein products (Lew et al. 1992; Masci et al. 1998). The LMW-i type was also identified as a protein product recently (Maruyama-Funatsuki et al. 2004).

In our previous study, we classified LMW-GS genes of a Japanese soft wheat, Norin 61, into six types based on the distribution of cysteine residues, and 12 groups based on the deduced amino acid sequence identity in the N- and C-terminal conserved domains (Ikeda et al. 2002) (Table 1). Among these LMW-GSs, groups 6–10 have the first cysteine residue at position 5, while groups 1, 2, and 5 have the first cysteine residue in a repetitive domain. Group 3 has the N-terminal domain of the LMW-s type with asparagine at position 3 (Masci et al. 1998). Group 4 was distinguished from group 3 by the presence of an isoleucine residue at position 1, and a lysine residue at position 11. Group 8 shared its amino acid sequence with group 9, except at the C-terminal end (Table 1). Groups 11 and 12 that were of LMW-i type (Cloutier et al. 2001; Ikeda et al. 2002) were distin-

guished by the number of glutamine repeats of the N-terminal end: groups 11 and 12 had four and five glutamine repeats, respectively (Table 1). However, individual LMW-GS loci and the level of expression as protein products were not clarified.

In this study, we constructed LMW-GS group-specific PCR primer sets to determine their loci, and identified the proteins corresponding to these groups by determining the N-terminal amino acid sequence of all LMW-GSs of Norin 61 separated by two-dimensional gel electrophoresis (2DE).

Materials and methods

Plant material

Norin 61 was used for protein analysis by 2DE. Nullisomic–tetrasomic lines of Chinese Spring lacking group 1 chromosomes (N1AT1B, N1BT1D, and N1DT1B) were used for the determination of individual LMW-GS groups. These cultivars and lines were obtained from the Genebank of the Ministry of Agriculture, Forestry and Fisheries. We also used wheat cultivars, Cheyenne, Gabo, Glenlea, Insignia, Orca and Rescue, to compare LMW-GS composition. These cultivars were obtained from the National Small Grains Collection of the United States Department of Agriculture.

Methods

Group-specific PCR primer sets were constructed based on LMW-GS sequences of Norin 61 (Ikeda et al. 2002) using a primer analysis software, Oligo ver. 6 (Molecular Biology Insights, USA). The total DNA of Norin 61 and nullisomic–tetrasomic lines of Chinese Spring lacking group 1 chromosomes were prepared according to Ikeda et al. (2002). PCRs were performed in a total volume of 50 µl containing 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, USA), PCR Gold buffer (Applied Biosystems, USA), and 100 ng of the total DNA. Reactions were performed according to the following protocol using GeneAmp PCR System 9700 (Applied Biosystems, USA): denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s; and a final extension at 72°C for 5 min. PCR products were electrophoresed in 1% agarose gel in a TAE buffer and cloned into pGEM-T or pGEM-T Easy vectors (Promega, USA), and sequenced, respectively.

Glutenin protein fraction was prepared according to Singh et al. (1990). The glutenin fraction was precipitated with 80% acetone, and the resulting pellets were dissolved in 250 µl of IEF sample buffer for very basic proteins (Gorg et al. 1997; Ikeda et al. 2005) containing 16% isopropanol, 8.5 M urea, 4% CHAPS, 25 mM DTT, and 0.5% IEF buffer pH 6–11 (Amersham Biosciences, USA). After incubation for 30 min at room temperature, samples were applied to Immobiline DryStrip pH 6–11: 13 cm (Amersham Biosciences, USA) was cut on the acidic side to 11 cm to fit the Protean IEF Cell (Bio-Rad Lab, USA). After in-gel rehydration for about 12 h, IEF was carried out for a total of 18 kVh. The gels were equilibrated with 6 M urea, 2% SDS, 20% glycerol, 10 mM DTT, and 50 mM Tris–Cl pH 6.8, and stored at −25°C. For the second dimension, SDS-PAGE was performed using a 10% gel (14 cm×14 cm×1 mm, acrylamide:bis-acrylamide = 29.6:0.4). Electrophoresis was carried out at 30 mA for 2.5 h. The gels were stained with Coomassie Brilliant Blue G-250 according to the method of Neuhoff et al. (1988). For the N-terminal amino acid sequence analysis, the gels were blotted onto a Sequi-Blot PVDF Membrane (Bio-Rad Lab, USA) with a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Lab, USA). The N-terminal amino acid sequences of individual spots were determined with a PPSQ-21A protein sequencer (Shimadzu, Japan).

Results

Construction of LMW-GS group-specific PCR primer sets and determination of their loci

To identify the loci of individual LMW-GS groups, group-specific primer sets were constructed based on the published nucleotide sequences of these groups (Ikeda

Table 1 Classification of LMW-GSs based on the distribution of cysteine residues (type) and the deduced N- and C-terminal amino acid sequence identity (group)

Type	Group	Predicted N-terminal amino acid sequence	Predicted C-terminal amino acid sequence
I	1	METSHIPGLEKPS	SVPFGVGTQVGAY
	2	METSHIPSLEKPL	IVPFGVGTRVGAY
II	3	MENSHIPGLERPS	RVPFGVGTGVGGY
	4	IENSHIPGLEKPS	SVPFGVGAGVGAY
III	5	METSRVPGLEKPW	IMPFSIGTGVGGY
IV	6	MDTSCIPGLERPW	SVPFGVGTGVGAY
	7	METSCISGLERPW	SVPFGVGTGVGAY
	8	METSCIPGLERPW	SAPLGVGSRVGAY
	9	METSCIPGLERPW	SVPFGVGTQVGAY
V	10	METRCIPGLERPW	SVPFDVGTGVGAY
VI	11	ISQQQQPPLFSQQ	SVPLGVGIGVGVY
	12	ISQQQQQPPFSQQ	SVPLGIGIGVGVY

et al. 2002). The relative positions and sequences of these primers are shown in Fig. 1 and Table 2. We could not construct a primer set to distinguish between groups 7 and 8, or groups 11 and 12, which share almost identical amino acid sequences (Table 1). All group-specific primer sets gave a single PCR product, except those for groups 11 and 12 which produced smeared bands (Fig. 2). These PCR products were sequenced to confirm that these products encode the expected groups. Their loci of individual groups were determined using nullisomic–tetrasomic lines of Chinese Spring lacking group 1 chromosomes (Fig. 3). Three, two, and seven LMW-GS groups were encoded by *Glu-A3*, *Glu-B3*, and *Glu-D3* loci, respectively (Table 2).

Identification of the LMW-GS proteins corresponding to LMW-GS genes

To identify proteins corresponding to the LMW-GS groups, we separated a glutenin fraction of Norin 61 by 2DE (Fig. 4). Nine spots (spots 1, 2, 3, 4, 5, 7, 8, 9, and 10) of B subunits and two spots (spots 11 and 14) of C subunits had the N-terminal amino acid sequences of LMW-GSs, while the remaining spots of C subunits were α/β and γ-gliadins, or a mixture of both gliadins (Table 3). The N-terminal sequences of the 12 spots matched with 10 of 12 groups of LMW-GSs (Table 3). Four, three, and five spots were encoded by *Glu-A3*, *Glu-B3*, and *Glu-D3*, respectively. We could not detect

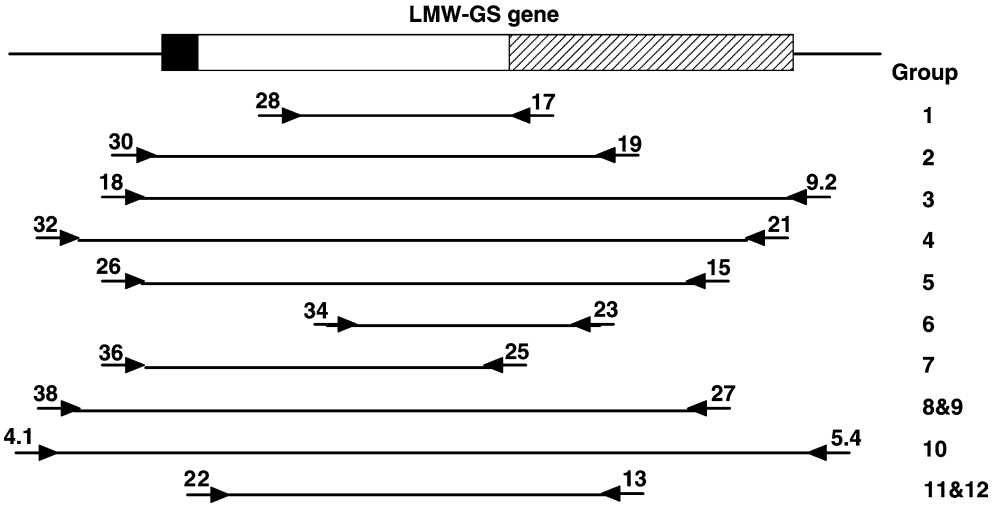

Fig. 1 Relative positions of group-specific primer sets. The LMW-GS gene coding region is shown as a *box*. *Black box*: the region encoding the N-terminal conserved domain. *Hatched box*: the region encoding the C-terminal conserved domain

Table 2 LMW-GS group-specific primers and their loci

Group	Group-specific primer set	Position	Locus
1	Glu-28: CACCAACAGCAACCGA	139 to 154	*Glu-D3*
	Glu-17: CAAGATAGATGGCTGAACAT	551 to 570	
2	Glu-30: TCAAAACCAAGCAACACTAT	−36 to −17	*Glu-B3*
	Glu-19: GCTGCTGAGGTTGGTTC	729 to 745	
3	Glu-18: CATCACAAGCACAAGCATCAA	−53 to −33	*Glu-B3*
	Glu-9.2: ACTAGAGATCTTTCCTTATTAG	1179 to 1200	
4	Glu-32: GCTAGTGCAACCTAACGCAT	−142 to −161	*Glu-D3*
	Glu-21: ACGGCACATCGTTGGTA	935 to 951	
5	Glu-26: AAGATCATCACAGGCACAATC	−58 to −38	*Glu-D3*
	Glu-15: CTGCTGACCCAATTGTTGTAG	853 to 873	
6	Glu-34: TTTCACAGCAAACACAACCT	260 to 279	*Glu-A3*
	Glu-23: TTGTAGGATGATGGAGTAGG	572 to 591	
7	Glu-36: CATCAAAACCAAGCAAAAG	−38 to −20	*Glu-D3*
	Glu-25: GGATTTGCTGTTGCACAAGT	342 to 361	
8	Glu-38: TGCAACCTACCACAATGTCC	−177 to −158	*Glu-D3*
	Glu-27: GGGTTGGTAGACACCTTGAA	629 to 648	
9	Glu-38: TGCAACCTACCACAATGTCC	−176 to −157	*Glu-D3*
	Glu-27: GGGTTGGTAGACACCTTGAA	628 to 647	
10	Glu-50: TAATTCATTTCAGATGGAGC	−155 to −136	*Glu-D3*
	Glu-47: GGGATTTGTTGTTGCACC	384 to 400	
11	Glu-22: CGTCTTTGCCCTCCTCGCTC	15 to 34	*Glu-A3*
	Glu-13: TTGGGGCTGTTGTTGCTGATA	379 to 399	
12	Glu-22: CGTCTTTGCCCTCCTCGCTC	15 to 34	*Glu-A3*
	Glu-13: TTGGGGCTGTTGTTGCTGATA	916 to 936	

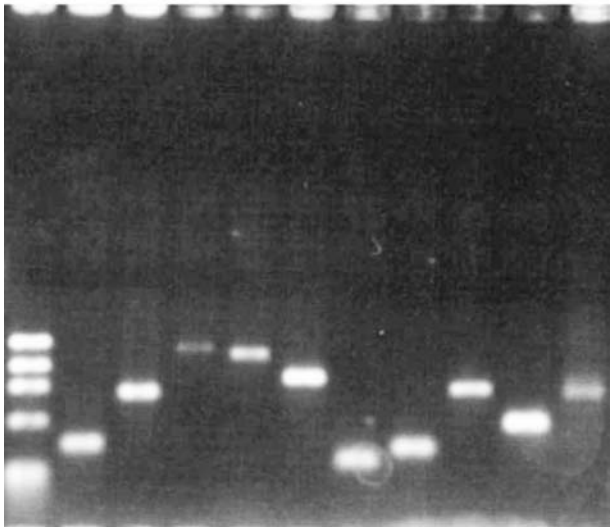

Fig. 2 PCR products obtained using group-specific primer sets. Group-specific PCR products were electrophoresed on 1% agarose gel in TAE buffer. *M* ϕX174/*Hae*III digest. Group-specific primer sets are as follows: *1*, primers 28 and 17 (group 1-specific); *2*, primers 30 and 19 (group 2-specific); *3*, primers 18 and 9.2 (group 3-specific); *4*, primers 32 and 21 (group 4-specific); *5*, primers 26 and 15 (group 5-specific); *6*, primers 34 and 23 (group 6-specific); *7*, primers 36 and 25 (groups 7-specific); *8*, primers 38 and 27 (groups 8- and 9-specific); *9*, primers 50 and 47 (group 10-specific); *10*, primers 22 and 13 (groups 11- and 12-specific). Primer sequences for amplifying these groups are shown in Table 2

groups 6 and 7 of LMW-GS genes encoded by *Glu-A3* and *Glu-D3*, respectively.

Among the largest LMW-GSs, a major spot was group 3 encoded by the *Glu-B3* locus showing the N-terminal amino acid sequence starting with a serine residue (LMW-s type, Table 3). However, a minor spot (spot 2) appearing slightly more acidic and larger than the major spot (spot 1) was also group 3, but showing a methionine residue at the N-terminal end (LMW-m type, Table 3). Another protein spot (spot 8) starting from a serine residue was also identified, and matched the N-terminal amino acid sequence of group 4 encoded by the *Glu-D3* locus (Table 3). We found four protein spots having N-terminal amino acid sequences corresponding to the LMW-i type. One spot (spot 9) and three spots (spots 3, 4, and 5) matched group 11 and group 12, respectively (Table 3). From these results, we could assign all LMW-GSs of Norin 61 into ten groups.

To compare LMW-GS composition with that of Norin 61, we separated LMW-GSs and determined their N-terminal amino acid sequences of other common wheat cultivars including Cheyenne, Chinese Spring, Gabo, Glenlea, Insignia, and Orca, whose *Glu-3* alleles were partly classified by other groups (Gupta and Shepherd 1990; Zhang et al. 2004). The wheats showed no variation in the spots corresponding to groups 1, 2, 5, and 8/9 (Fig. 5). For the spots encoded by *Glu-A3*, Norin 61 showed spots corresponding to groups 11 and 12 at the same positions as those of Orca (*Glu-A3d*), which had one spot of group 11 and three spots of group 12. On the other hand, Cheyenne (*Glu-A3c*), Chinese Spring (*Glu-A3a*), Gabo (*Glu-A3b*), and Rescue (*Glu-A3f*) showed only one spot of group 11 or 12. Although the N-terminal amino acid sequences (ten amino acids) of other groups showed the same sequences among these cultivars, group 11 showed different sequences after the glutamine repeats (Table 4). Rescue (*Glu-A3f*) had an alanine residue after the glutamine repeats instead of a proline residue. Surprisingly, we also found one spot of group 11 in Insignia, which was classified as *Glu-A3e* (null allele) in the same position as that of Rescue.

For the spots encoded by *Glu-B3*, Orca (*Glu-B3d*) had two spots of group 3 as found in Norin 61, but these spots were slightly smaller than those of Norin 61 based on SDS-PAGE analysis (data not shown). Although Rescue (*Glu-B3 h*) had spots of group 3 of similar molecular weight to Norin 61, it had three spots instead of two. Insignia (*Glu-B3c*) showed two spots of group 3, but these had a smaller molecular weight, and were less abundant than those of Norin 61. Gabo (*Glu-B3b*) showed three spots of group 3, but a major spot had a much smaller molecular weight than in other cultivars having *Glu-B3c*, *Glu-B3 h*, or *Glu-B3i*. Although Glenlea (*Glu-B3 g*) had three spots of which a major spot and a minor spot (3*) showed the same position as that of Gabo, the other spot that was more basic pI showed smaller molecular weight than the other two spots (Fig. 5).

For the spots encoded by *Glu-D3*, each group 4, 8/9, and 10 showed one spot among cultivars. The pattern of the spots of groups 8/9 and 10 of Norin 61 matched those of Orca (*Glu-D3e*) and Insignia (*Glu-D3c*), but the spot of group 4 was slightly smaller than that of Orca and Insignia (Fig. 5). Cheyenne, Chinese Spring (*Glu-D3a*), Gabo (*Glu-D3b*), and Rescue (*Glu-D3a*) shared the same pattern, but spot 4 of Cheyenne was more acidic than the others (Fig. 5).

The loci of these groups were confirmed by the analysis of glutenin fractions of the nullisomic–tetrasomic lines of Chinese Spring lacking group 1 chromosomes (Fig. 6). Chinese Spring having N1AT1B showed no spot of group 11, and a larger amount of spots of groups 2 and 3. On the other hand, Chinese Spring having N1BT1D showed no spot of groups 2 and 3, and a larger amount of spots of groups 1, 4, 5, 8/9, and 10. Chinese Spring having N1DT1B showed no spots of groups 1, 4, 5, 8/9, and 10, and a larger amount of spots of groups 2 and 3. These results matched our chromosome assignment of these groups.

Discussion

We developed specific PCR primer sets to distinguish all 12 groups of LMW-GSs of Norin 61. PCR with the 11- and 12-specific primer sets gave smeared PCR products (Fig. 2), which might include PCR products encoding the spot of group 11 and three spots of group 12. Using

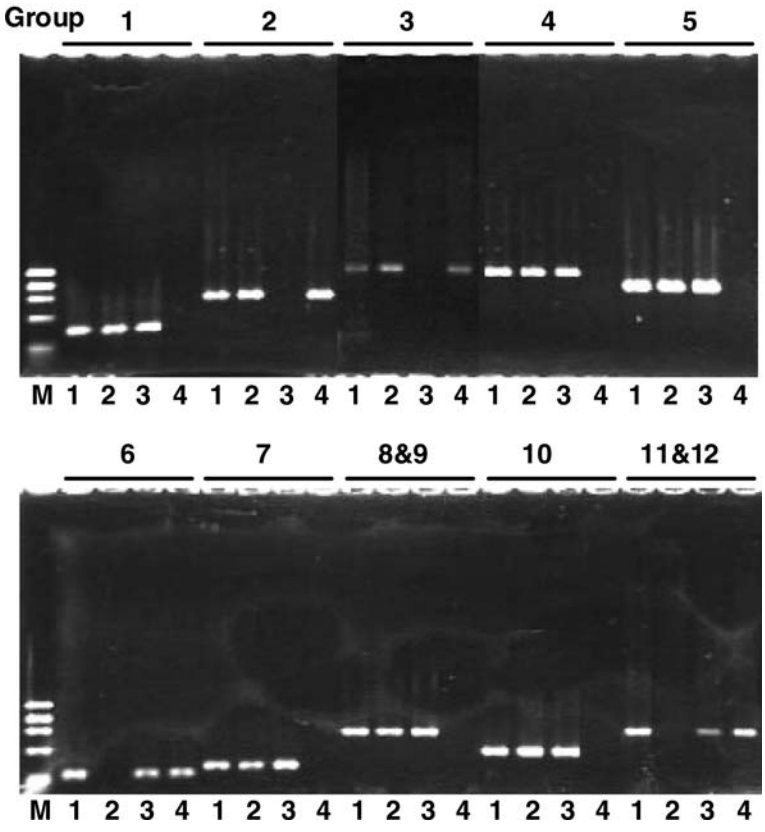

Fig. 3 Nullisomic–tetrasomic analysis of LMW-GS genes. Group-specific PCR products were electrophoresed on 1% agarose gel in TAE buffer. Each group is indicated above the images. *M*, molecular weight marker (ϕX174/*Hae*III digest); *1*, Chinese spring; *2*, N1AT1B; *3*, N1BT1D; *4*, N1DT1B

these primer sets and nullisomic–tetrasomic lines lacking group 1 chromosomes, the loci of these groups were determined. PCR products obtained using these group-specific primer sets were assigned to individual loci; i.e., three, two, and ten groups were assigned to *Glu-A3*, *Glu-B3*, and *Glu-D3* loci, respectively. The chromosome 1D genome encoded more *Glu-3* genes than chromosomes 1A and 1B.

At the protein level, we identified the N-terminal amino acid sequences of LMW-GSs separated by 2DE. Analyses of LMW-GSs by 2DE were reported previously (Jackson et al. 1983, 1996; Maruyama-Funatsuki et al. 2004; Payne et al. 1985); however no comprehensive analysis of LMW-GSs was carried out by identifying their N-terminal amino acid sequences and their chromosomal loci. Based on the groupings of the de-

Table 3 N-terminal amino acid sequence of LMW-GSs of Norin 61 and their loci

Spot number	N-terminal amino acid sequence	Group	Locus	Type
1	SHIPGLERPS	3	*Glu-B3*	LMW-s
2	MENSHIPGLE	3	*Glu-B3*	LMW-mn3h5
3	ISQQQQQP	12	*Glu-A3*	LMW-i
4	ISQQQQQP	12	*Glu-A3*	LMW-i
5	ISQQQQQP	12	*Glu-A3*	LMW-i
6	METSHIPGLEKPSQQ	1	*Glu-D3*	LMW-mh5
7	NMQVDPSGQV	γ-gliadin		
8	METSRVPGLEKPWQQ	5	*Glu-D3*	LMW-mr5
9	SHIPGLEKPSQQQP	4	*Glu-D3*	LMW-s
10	ISQQQQPPQSFQQQQ	11	*Glu-A3*	LMW-i
11	METSHIPSLEKPLQQ	2	*Glu-B3*	LMW-mh5
12	METRXIPGLERPWQQ	10	*Glu-D3*	LMW-mc5
13	NMQVDPSGQV	γ-gliadin		
14	XMQVDPSGQV	γ-gliadin		
15	METSXIPGLERPWQQ	8 or 9	*Glu-D3*	LMW-mc5
16	NMQVDPSGQVQWPQQ	γ-gliadin		
17	V(R/M)(V/Q)PDP(Q/S)L/G)Q(V/P)	α/β-gliadin? γ-gliadin		
18	NMQVDPGYQVQWPQQ	γ-gliadin		
19	VMQVDPGYQVQNPQQ	γ-gliadin		
20	VRVPVPQLQP	α/β-gliadin		

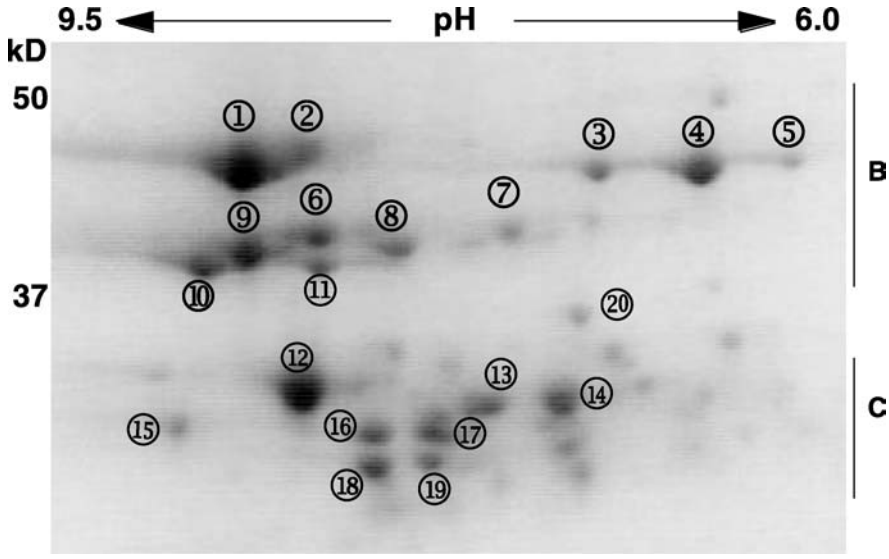

Fig. 4 2D gel electrophoresis of the LMW-GS fraction of Norin 61. Individual spots are numbered. The N-terminal amino acid sequences of these spots are shown in Table 3

duced amino acid sequence of LMW-GS genes, there were 10 groups of LMW-GS genes among 12 groups that matched the N-terminal amino acid sequence of LMW-GSs. Three, three, and five spots were encoded by *Glu-A3*, *Glu-B3*, and *Glu-D3*, respectively. The abundance of LMW-GS encoded by *Glu-D3* might contribute more to the gluten viscoelasticity of common wheat than that of durum wheat.

It is interesting that protein spots corresponding to groups 6 and 7 were not present. The genes encoding these groups did not have a nonsense mutation within their coding regions (Ikeda et al. 2002). Zhang et al. (2004) also reported that a null allele of *Glu-A3e* encoded LMW-GS without any inframe stop codons. They found that the deduced amino acid sequence contained a cysteine residue in a signal sequence. In groups 6 and 7 in Norin 61, no cysteine residues were found in signal sequences (Ikeda et al. 2002). It is necessary to study if these genes had mutations in a promoter region.

The LMW-GS spots included LMW-i, LMW-m, and LMW-s types. LMW-s type was encoded by two genes, groups 3 and 4 located at *Glu-B3* and *Glu-D3*, respectively. These spots were distinguished by the amino acid at position 8: group 3 had an arginine residue, and group 4 had a lysine residue (Table 1). LMW-i type was encoded by two genes, groups 11 and 12, at the same *Glu-A3*. Both groups started with an isoleucine residue followed by a serine residue. Groups 11 and 12 had four and five glutamine-repeats from position 3, respectively.

We found that a small fraction of group 3 (LMW-m type) shared the same amino acid sequence as that of a major spot of group 3 (LMW-s type). This suggests that the same gene encoded both the LMW-m and LMW-s, but was processed at different sites. Dupont et al. (2004) suggested that some omega-gliadins were processed post-translationally by an asparaginyl endoprotease (Müntz and Shutov 2002). It appears that, in this case, the majority of group 3 was processed by the asparaginyl endoprotease, but a minor fraction of group 3 was not. This is the first finding that one LMW-GS gene encoded two different types of LMW-GS. It also means that the classification of LMW-m and LMW-s types does not necessarily correspond to two different genes. The other LMW-s type, group 4, also had a very minor fraction of LMW-m type (data not shown).

In comparison with other cultivars, Norin 61 in our stock was classified as *Glu-A3d* and *Glu-B3i*. However for *Glu-D3*, the position of a spot corresponding to group 4 did not match *Glu-D3a*, *Glu-D3b*, *Glu-D3c*, or

Table 4 The N-terminal amino acid sequence comparison of the *Glu-A3* encoded LMW-GSs among cultivars

	Glu-A3 allele	N-terminal amino acid sequence	Group
Norin 61	D	ISQQQQPPQ	11
Norin 61	D	ISQQQQQP	12
Cheyenne	C	ISQQQQQPPF	12
Chinese Spring	A	ISQQQQQPPF	12
Gabo	B	ISQQQQPPPF	11
Glenlea	G	ISQQQQPPPF	11
Insignia	F	ISQQQQAPPF	11
Orca	D	ISQQQQPPQ	11
Orca	D	ISQQQQQP	12
Rescue	F	ISQQQQAPPF	11

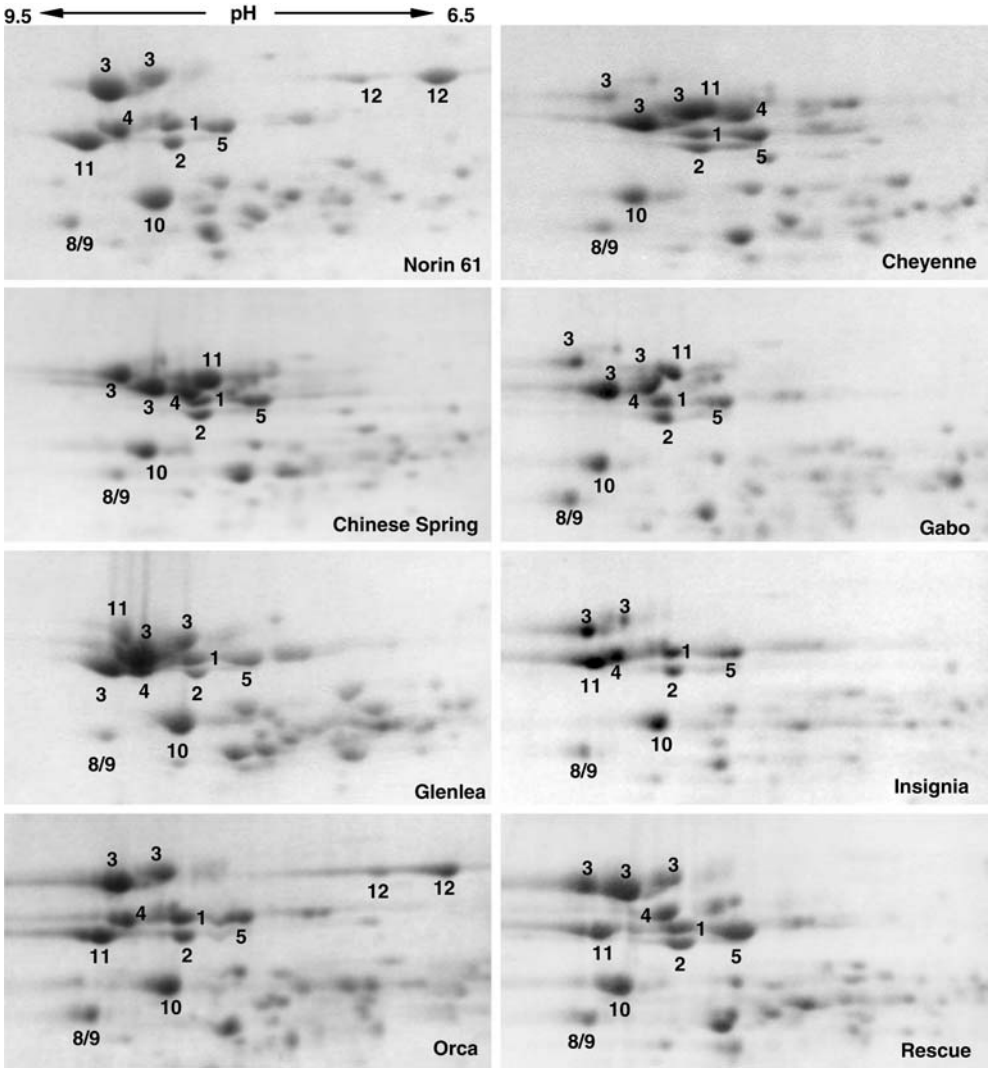

Fig. 5 Comparison of LMW-GS fractions of various wheat cultivars. LMW-GS spots are marked by corresponding groups. The most acidic spot of group 12 (⑤ in Fig. 4) is not present in Norin 61 and Orca. Some alleles were assigned previously as the following alleles: Cheyenne (*Glu-A3c*, *Glu-B3e*), Chinese Spring (*Glu-A3a*, *Glu-B3a*, *Glu-D3a*), Gabo (*Glu-A3b*, *Glu-B3b*, *Glu-D3b*), Glenlea (*Glu-A3 g*, *Glu-B3 g*, *Glu-D3c*), Insignia (*Glu-A3e*, *Glu-B3c*, *Glu-D3c*), Orca (*Glu-A3d*, *Glu-B3d*, *Glu-D3e*), and Rescue (*Glu-A3f*, *Glu-B3 h*, *Glu-D3a*)

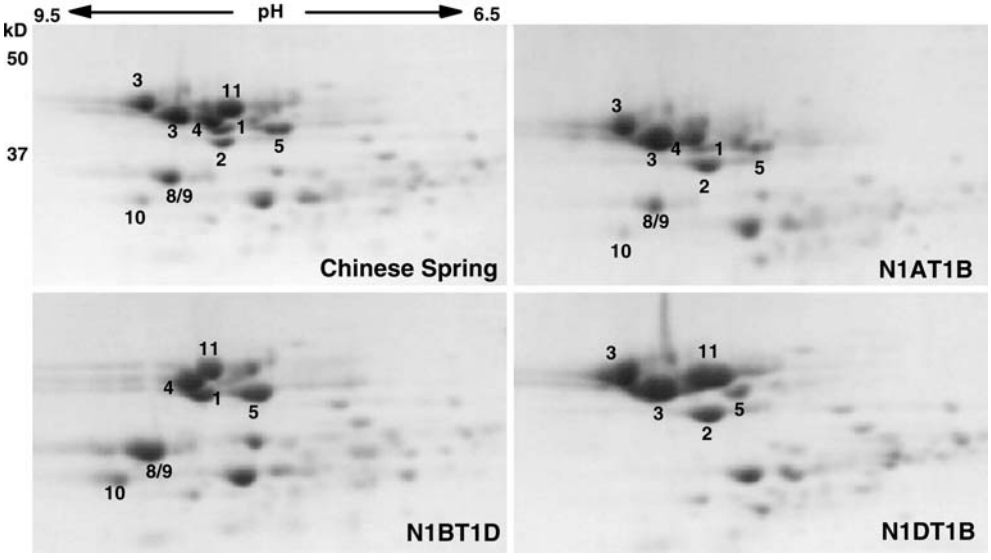

Fig. 6 Nullisomic–tetrasomic analysis of LMW-GSs separated by 2DE. Chinese Spring and its nullisomic–tetrasomic lines (N1AT1B, N1BT1D, and N1DT1B) were used. Group numbers are shown beside each spot

Glu-D3e. Gupta and Shepherd (1990) identified two biotypes of Norin 61; one was classified as *Glu-A3e*, *Glu-B3i*, and *Glu-D3d*, and the other as *Glu-A3d*, *Glu-B3i*, and *Glu-D3e*. We classified Norin 61 in our stock as *Glu-A3d*, *Glu-B3i*, and *Glu-D3d*. For the *Glu-A3* allele, the other alleles showed only one spot instead of three in the

case of *Glu-A3d* that might have three genes. One encodes group 11 and the other three encode group 12. Different modifications might also produce the three spots of group 12. We also found the one spot of group 11 in Insignia at the same position as that of Rescue (*Glu-A3f*). Although Insignia was used as one of the standard cultivars for *Glu-A3e* as a null allele, it should be classified as *Glu-A3 f*. Therefore, it is necessary to reassign standard cultivars for LMW-GS allele identification. Cheyenne, the *Glu-D3* of which was not assigned, previously, had a spot of group 4 different from others, which should be assigned as a new allele, *Glu-D3f*.

It should be noted that there was clear variation in spot intensities among cultivars and among spots. Spots of group 3 of *Glu-B3* and group 10 of *Glu-D3* were the most abundant, followed by spots of group 11 of *Glu-A3* among cultivars. However, spots of group 3 encoded by *Glu-B3c* in Insignia were less abundant than those of group 11. The small amount of group 3 of *Glu-B3c* might be associated with the weak gluten property (Gupta et al. 1994).

Genomic analysis of chromosome 1AS of diploid wheat, *Triticum monococcum*, showed that at least three copies of *Glu-A3* genes were present (Yahiaoui et al. 2004). It is expected that the copy number of *Glu-3* genes might be different among *Glu-3* loci and affect the abundance of each subunit in hexaploid wheats. Further analysis is necessary to clarify whether the differences of individual LMW-GS amounts were caused by the differences of the copy number of the corresponding genes, or the strength of their promoters.

Although with 2DE it is difficult to detect small variation in molecular weight, the characterization of individual LMW-GSs by a combination of 2DE analysis, amino acid sequencing, and grouping based on N-terminal amino acid sequence identity will be useful to study their allelic variations, and to identify LMW-GSs affecting gluten quality.

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