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Identification of new low-molecular-weight glutenin subunit genes in wheat

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Abstract To clarify the composition of low-molecular-weight glutenin subunits (LMW-GSs) in a soft wheat cultivar, we cloned and characterized LMW-GS genes from a cDNA library and genomic DNA in Norin 61. Based on alignment of the conserved N- and C- terminal domains of the deduced amino-acid sequences, these genes are classified into 12 groups. One of these groups (group 5), the corresponding gene of which has not been reported previously, contains two additional hydrophobic amino-acid clusters interrupting the N-terminal repetitive domain. Other groups (groups 11 and 12), which were not identified in other cultivars as a protein product, showed all eight cysteines in the C-terminal conserved domain. With specific primer sets for these groups it was revealed that *Glu-D3* and *Glu-A3* encoded the former and the latter, respectively. Both groups of genes were expressed in immature seeds. The presence of these groups of LMW-GSs may affect the dough strength of soft wheat.

Keywords Soft wheat·Low-molecular-weight glutenin subunits·Low-molecular-weight glutenin subunit genes·*Triticum aestivum*

Introduction

Glutenin consists of high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits. HMW glutenin subunits (HMW-GSs) are encoded by *Glu-A1*, *Glu-B1* and *Glu-D1* on the long arm of chromosomes 1 A, 1B and 1D, respectively (Payne et al. 1980). 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). These glutenin subunits are polymerized by intermolecular disulfide bonds, which are important to the rheological properties of wheat flour doughs. It has been shown that allelic variations of HMW- and LMW- GS influence dough properties in various wheat cultivars (Skeritt 1998 for a review; Nagamine et al. 2000). The role of LMW-GSs is, however, much less well-characterized than that of HMW-GSs, because large numbers of the subunits have similar mobilities in SDS-PAGE analysis. By N-terminal amino-acid sequencing, LMW-GSs were classified into two types based on the first amino acid of mature subunits: in LMW-m and LMW-s types the N-terminal sequence starts with methionine and serine, respectively. The LMW-s type seems to be predominant (Shewry et al. 1983; Kasarda et al. 1988; Lee et al. 1999). Cloned sequences of LMW-GS genes revealed that all of the predicted LMW-GS amino-acid sequences had eight cysteines, which are involved in intra- and inter-molecular disulfide bonds. Although in some LMW-m type LMW-GSs, the first cysteine is at position 5 within the N-terminal conserved domain; in other LMW-m and LMW-s types, the first cysteine residue is found in a repetitive domain (Lew et al. 1992; Masci et al. 1998). The LMW-GS gene family has so far been characterized in only a very limited number of common and durum wheat cultivars. In this study, we attempt to clarify the composition of LMW-GSs comprehensively with a soft wheat cultivar by cloning and characterizing the LMW-GS genes.

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Materials and methods

Plant material

We used a Japanese soft wheat cultivar, Norin 61, for the cloning of LMW-GS genes. This cultivar was maintained in our institute (Nagamine et al. 2000) and is a different biotype from two biotypes of Norin 61 reported by Gupta and Shepherd (1990), because its LMW-GS banding pattern is different from the two. For PCR analysis with specific primer sets, another six common wheat cultivars (Kanto107, Shirasagikomugi, Cheyenne, Falcon, Hope

and Roblin), four durum wheat cultivars (Norba, Produra, Solitario and Langdon) and nullisomic-tetrasomic lines of Chinese Spring lacking group-1 chromosomes (N1AT1B, N1BT1D and N1DT1B) were used.

cDNA library construction and isolation of LMW-GS cDNA clones

Poly (A)+ RNA was prepared from immature seeds harvested 2 weeks after flowering. A cDNA library was constructed with ZAP-cDNA synthesis kit (STRATAGENE). The library was screened with DNA probes specific to LMW-GS genes corresponding to the C-terminal conserved region and the 3′ non-translated region. Oligonucleotides used as primers to amplify the DNA probe were constructed based on LMW-GS genes encoded by *Glu-D3* (LMWG-1D1, Colot et al. 1989). They were as follows:

GluU	CGTCTTGCTAGGTCGCAAATG
GluL	CAGATTGACATCCACACAATGCC.

GluU and GluL primers correspond to positions 541 to 561 and 1145 to 1167, respectively. Specific PCR products were cloned into the pGEM-T vector (Promega). Two different LMW-GS gene clones, which were highly similar to two LMW-GS sequences (Genbank accs. Y17845 and U86029), were mixed in equal amounts and used as probes. The cDNA library was screened for plaque hybridization with an ECL kit (Amersham Pharmacia). LMW-GS cDNA inserts from individual phage clones were amplified by PCR and sequenced from the 5′-end by automated sequencing with an ABI 373 A (PE Biosystems).

Isolation of LMW-GS genomic clones

Total DNA was prepared from seedlings or embryos by the potassium acetate method described by Dellaporta et al. (1983) followed by phenol/chloroform extraction. LMW-GS gene-specific primers located in the 5′ (Glu4, Glu4.1 and Glu4.2) and 3′ (Glu5, Glu5.1, Glu5.2, Glu5.3 and Glu5.4) non-translated regions, were based on published LMW-GS gene sequence data and the sequence of the cDNA clones, respectively. The locations of these primers correspond to the following positions of LMWG-1D1 (Colot et al. 1989); -174 to –155 (-154 for Glu-4.1), and 992 to 1011, respectively. To clone one group of LMW glutenin genes, we needed to apply a nested-PCR with new sets of primers designed based on clones in the present study. The first reaction was carried out with the Glu18 and Glu1 primer combinations, which were located in the 5′ and 3′ non-translated regions. For the second reaction, 1 µl of the first reaction product diluted 100-fold was used with the primer combinations Glu8 and Glu9, which were located in the 5′ and 3′ end of the translated regions.

Primers that anneal to the 5′ region

Glu4	GCAACTTTGATGATCAATCC
Glu4.1	CTTTGATGATCAATATATCCA
Glu4.2	GCAACTTTGATGATGAATCC
Glu8	CCACCATGAAGACCTTCCTC
Glu18	CATCACAAGCACAAGCATCAA.

Primers that anneal to the 3′ region

Glu1	GACACTTTATTTGTCACCGCT
Glu5	AAACAACGGTGATCCAACTAT
Glu5.1	AGCAACGACAATCCAACTAT
Glu5.2	AACGGTGATCTAACGACTAT
Glu5.3	AACAACGGTGACCCAATTAT
Glu5.4	AAACAAAGGTGATCCAACTAT
Glu9	ACTAGAGATCTTTCCTTATT.

PCR reactions were performed in a total volume of 50 µl containing 1.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 10 pmol of each primer and 1 U of *TaKaRa LA-Taq* DNA polymerase (Takara), ×1 LA PCR buffer II (Takara) and 100 ng of the total DNA. Reactions were performed according to the following protocol: 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. All PCR products were cloned into pGEM-T or pGEM-T Easy vectors (Promega) and sequenced, respectively.

Construction of gene-specific primer sets

Gene-specific primer sets were constructed with primer analysis software, OLIGO ver. 6 (Molecular Biology Insights, Inc), as follows:

Group 5-specific primer set

Glu15	CTGCTGACCCAATTGTTGTAG
Glu26	AAGATCATCACAGGCACAATC.

Groups 11 and 12-specific primer set

Glu13	TTGGGGCTGTTGTTGCTGATA
Glu22	CGTCTTTGCCCTCCTCGCTC.

Sequence analysis:
Alignments of the deduced amino acid sequences of LMW-GS genes were assembled using the CLUSTAL W Program (ver. 1.7, Thompson et al. 1994) and displayed by MacVector, ver 6.5.3 (Oxford Molecular Group, Campbell, Calif.).

Results

Cloning and characterization of the LMW-GS genes

LMW-GS specific probes were used to isolate 47 LMW-GS clones from the cDNA library. All clones contained the 3′ part of the coding regions and the non-translated regions, but none of the clones contained the 5′ part of the coding regions (data not shown). To obtain full-length LMW-GS genes corresponding to these sequences, we carried out PCR reactions using total DNA with LMW-GS gene-specific primers located in the 5′ and 3′ non-translated regions. Although other primer combinations produced no specific amplification of particular LMW-GS genes, the combination of Glu4.1 and Glu5.3, which was constructed based on LMWG-1D1 (Colot et al. 1989), could amplify one LMW-GS gene showing strong sequence similarity to LMWG-1D1. We cloned 106 full-length LMW-GS genes, including pseudogenes showing inframe stop codons, from PCR products with different primer combinations (data not shown). To clone LMW-GS genes corresponding to cDNA clones, which show strong sequence similarity with LMW-s type, we needed to apply a nested PCR method.

Although no sequences that were cloned were the same as previously published LMW-GSs, the deduced amino-acid sequences of the cloned LMW-GS genes show strong sequence identity with previously reported LMW-GS sequences. These sequences were classified into 12 groups based on the alignment of the N- and C-terminal conserved domains of the deduced amino-acid sequences (Table 1, Fig. 1).

The amino-acid sequence identity between the groups was less than 90%. Some of these groups contain different-sized polypeptides due to the presence of various deletions/insertions within repetitive and glutamine-rich domains (data not shown). The cDNA clones corre-

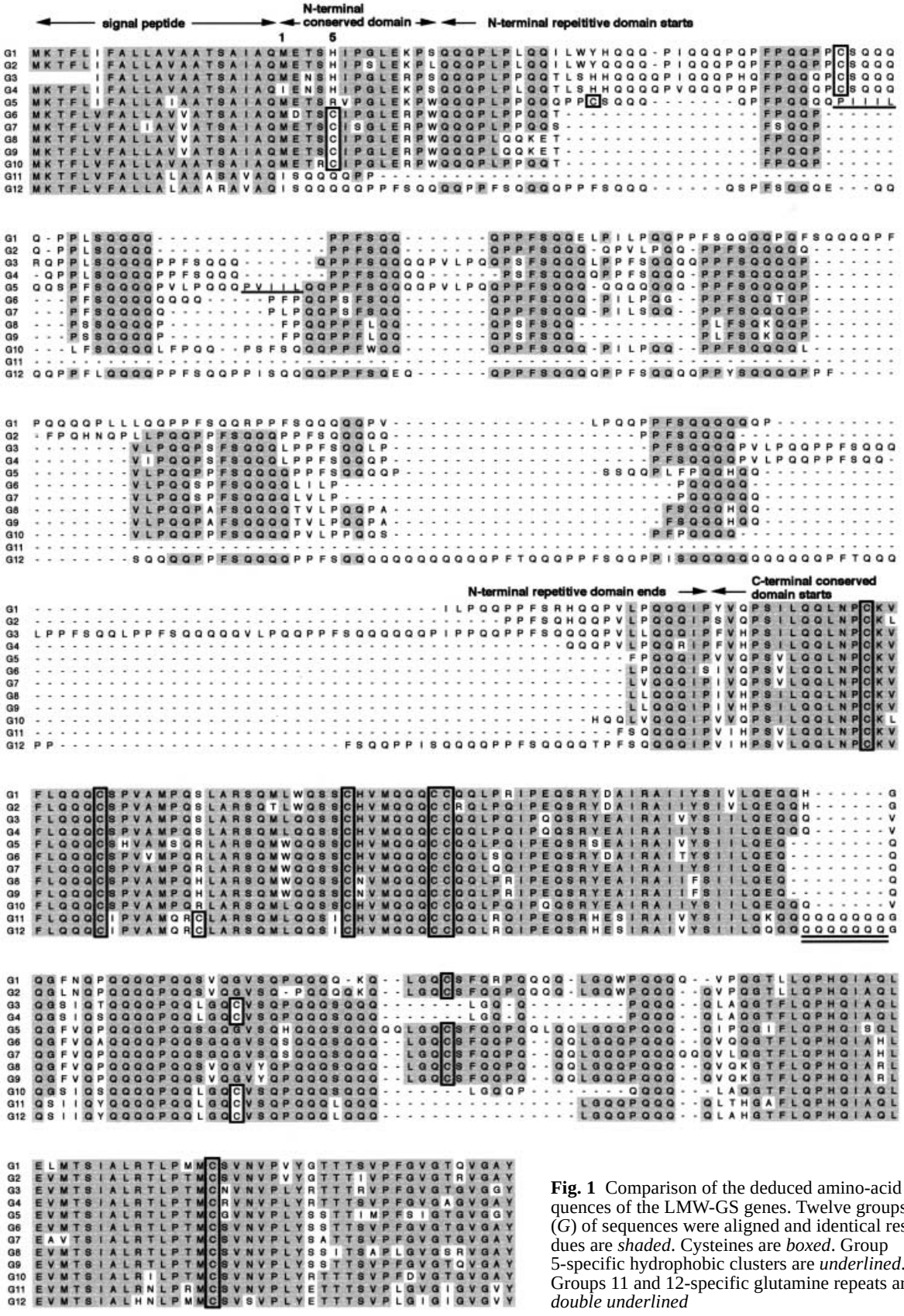

Fig. 1 Comparison of the deduced amino-acid sequences of the LMW-GS genes. Twelve groups (*G*) of sequences were aligned and identical residues are *shaded*. Cysteines are *boxed*. Group 5-specific hydrophobic clusters are *underlined*. Groups 11 and 12-specific glutamine repeats are *double underlined*

Table 1 Classification of the deduced amino-acid sequences of the LMW-GS genes

Type	Group	Primer pair	N-terminal sequence	C-terminal sequence	Amino acid number	No. of genomic clone	No. of cDNA clone	Similar published sequence (locus, sequence identity)
I	1	4/5	METSHIPG	VGTQVGAY	365	1	1	CAA59340 (*Glu-D3*, 99%)
	2	4.2/5.1	METSHIPS	VGTRVGAY	343	1	7	CAA59339 (*Glu-B3*, 97%) CAA74550 (*Glu-B3*, 97%)
II	3	18/1→8/9 18/1→8/9.2	MENSHIPG	VGTGVGGY	393 378 377 376 348 334 319 304 267 243	1 1 1 1 1 1 1 1 1 1	17	CAB40553 (97%) CAA76890 (*Glu-B3*, 98%) CAB41921 (96%)
	4	4/5.4 4.2/5.4	IENSHIPG	VGAGVGAY	340 297	2 1	2	n.a.[c]
III	5	4/5.1, 4/5.2 4/5.2	METSRVPG	IGTGVGGY	350 348 321	3 1 1	2	n.a.[c]
IV	6	4.2/5, 4/5.4 4/5	MDTSCIPG	VGTGVGAY	303 302 290 279	7 1 1 1	0	CAA44473 (97%) LG-AQ1 (*Glu-A3*)[a]
	7	4/5, 4/5.4 4.2/5	METSCISG	VGTGVGAY	303	8	1	AAA34285 (99.6%) AAB48477 (99.3%) LMW-14/1 (*Glu-D3*)[a]
	8	4/5, 4/5.4, 4.2/5	METSCIPG	VGSRVGAY	298	9	0	AAB48475 (99.6%)
	9	4.2/5	METSCIPG	VGTQVGAY	298	1	0	AAB48475 (98%)
V	10	4.1/5.3	METRCIPG	VGTGVGAY	304	11	1	AAB48476 (98%) AAB48478 (98%) CAA31685 (*Glu-D3*, 99%) LMW-16/10 (*Glu-D3*)[a]
VI	11	4/5	ISQQQQQP	VGIGVGVY	212	1	11	BAA23162 (91%)[b]
	12	4.2/5, 4.2/5.4	ISQQQQQP	IGIGVGVY	390 389	2 1	5	CAA30570 (92%)[b] AAB48479 (93%)[b] AAC26220 (91%)[b]

[a] Sequences are not listed in Genbank
[b] Sequence identity with group 12
[c] n.a.: not available
These new genomic sequences were submitted to DDBJ (AB062851 to AB062878)

sponding to groups 3 and 11 were the most abundant and there were no cDNA clones corresponding to groups 6, 8 and 9 (Table 1). The deduced N- and C- terminal sequences of mature proteins of these groups are also listed in Table 1. The N-terminal end includes sequences previously published. Groups 1, 2, 5, 6, 7, 8, 9 and 10 share the same N-terminal sequence as the LMW-m type (Lew et al. 1992). Group 1 shares strong sequence similarity with a sequence encoded by *Glu-D3* (Van Campenhout et al. 1995). Group 2 shows strong sequence similarity with sequences encoded by *Glu-B3* (Van Campenhout et al. 1995; D'Ovidio et al. 1997). Group 3 has the N-terminal sequence of the LMW-s type with asparagine at position 3 (Masci et al. 1998; D'Ovidio et al. 1999;

Masci et al. 2000). The N-terminal sequence of this group is expected to start from serine at position 4 (Table 1). Group 4 has an isoleucine residue at position 1 and asparagine at position 3 instead of methionine and threonine, respectively. This sequence was not reported previously. Group 5 shows two additional repeats consisting of five hydrophobic amino acids (PIIIL and PVIIL) interrupting the N-terminal repetitive domain (underlined in Fig. 1). Although the same N-terminal sequence (METSRVPG) was found by protein-based sequencing (Lew et al. 1992), its corresponding DNA sequence was not reported previously. Group 6 shows a strong sequence similarity (97%) to one of the LMW-GSs in the durum wheat cultivar (D'Ovidio et al. 1992). This group also shares the same N-terminal sequence encoded by a gene isolated from an A-genome species (Lee et al. 1999). Group 7 has almost the same amino-acid sequence as two LMW-GSs isolated from common wheat except for one glutamine insertion (Okita et al. 1985, Genbank acc. AAA34285) and two amino-acid substitutions (Cassidy et al. 1998, Genbank acc. AAB48477) in the C-terminal conserved domain. This group also show a strong sequence similarity to one of the LMW-GSs (LMW-14/1) isolated from D-genome species (Ciaffi et al. 1999). Group 8 has the same amino-acid sequence as a LMW-GS isolated from common wheat (Cassidy et al. 1998, Genbank acc. AAB48475) except for one amino-acid substitution in the C-terminal conserved domain. Group 9 shares its amino-acid sequence with group 8, except at the C-terminal end (Fig. 1). Group 10 shows strong sequence similarity (99%) to LMW-GSs located on *Glu-D3* (LMWG-1D1, Genbank acc. CAA31685) and LMW-16/10 (Ciaffi et al. 1999). Only one primer set (Glu4.1 and Glu5.3), constructed based on the LMWG-1D1 sequence, specifically amplified this group. Groups 11 and 12 lacking the N-terminal conserved domain share the N-terminal sequence reported in common wheats (Cassidy et al. 1998, Maruyama et al. 1998; Pitts et al. 1988; Cloutier et al. 2001), but this sequence was not found by protein-based sequencing of LMW-GSs (Lew et al. 1992; Sissons et al. 1998). These groups show an additional six glutamine repeats in the C-terminal domain (double-underlined in Fig. 1) and share a relatively weak sequence identity (91 to 93%) with published sequences (Genbank accs. AAB48479, BAA23162, S01992) and a partial sequence isolated from an A-genome species, *Triticum monococcum* (Genbank acc. AAC26220). These previously published sequences also show additional glutamine repeats (six to eight repeats) at the same position (data not shown).

All LMW-GS groups contain eight cysteine residues, which are conserved among all of the previously published LMW-GS sequences. The relative positions of cysteines are also conserved, except those of the first and seventh.

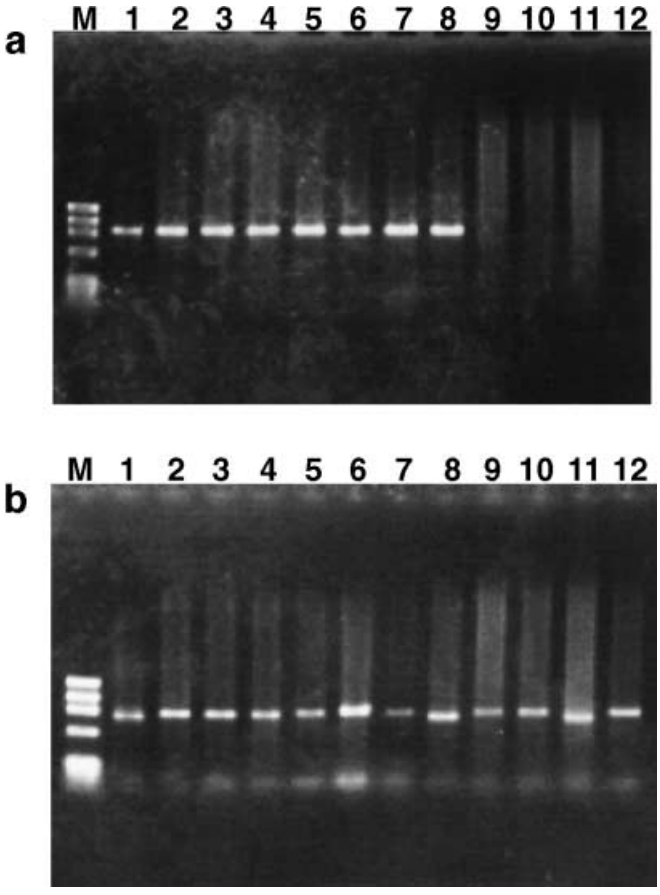

Fig. 2 PCR using group 5 (**a**), and groups 11 and 12 (**b**) – specific primer sets with various common (*1* to *7*) and durum (*8* to *11*) wheats: *1* Norin 61; *2* Kanto 107; *3* Shirasagikomugi; *4* Haruno-akebono; *5* Cheyenne; *6* Falcon; *7* Hope; *8* Roblin; *9* Norba; *10* Produra; *11* Solitario; *12* Langdon. M, ∅×174 digested with *Hae*III

Construction of LMW-GS group-specific primers

To amplify specific LMW-GS genes by PCR, we have constructed primer sets specific for group 5, and groups 11 and 12. The group 5-specific primer set amplified a single product, whereas the primer sets for groups 11 and 12 amplified diffused PCR products in Norin 61 (Fig. 2).

With the group 5-specific set, only one group-5 sequence was detected (data not shown). With the groups 11- and 12-specific primer set, various sized sequences were detected (data not shown). In other wheat cultivars using the group-5 primer set, the same-size single PCR product was amplified among common wheat cultivars, but we could not amplify a PCR product from durum wheats (Fig. 2). With the primer set for groups 11 and 12, other cultivars showed a single PCR product with size polymorphism. To identify the chromosomal locations of these groups, we carried out PCR with nullisomic-tetrasomic lines of Chinese Spring lacking group-1 chromosomes (Fig. 3).

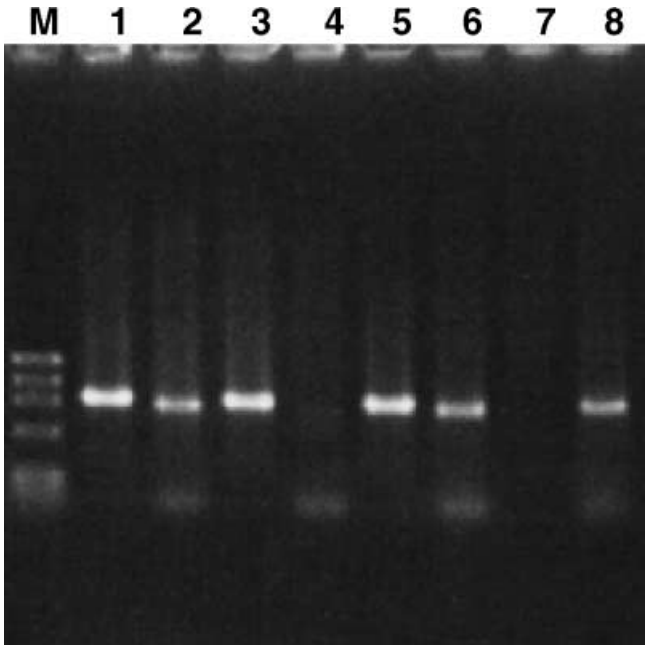

Fig. 3 PCR using group 5 (*1*, *3*, *5* and *7*), and groups 11 and 12 (*2*, *4*, *6* and *8*) -specific primer sets with nulli-tetrasomic lines of Chinese Spring lacking group-1 chromosomes. *1* and *2* Chinese Spring; *3* and *4*, N1AT1B; *5* and *6*, N1BT1D; *7* and *8* N1DT1B. *M* ∅×174 digested with *Hae*III

With the group 5-specific primer set, we failed to amplify PCR products with N1DT1B. With the groups 11- and 12-specific primer set, we failed to amplify PCR products with N1AT1B. These results suggest that group 5, and groups 11 and 12 are encoded by *Glu-D3* and *Glu-A3*, respectively.

Discussion

From the cultivar Norin 61, a Japanese soft wheat variety characteristic for weak gluten, we cloned a wide variety of LMW-GS genes with LMW-GS specific primers. This is the first comprehensive study of LMW-GS genes in common wheats. The deduced amino-acid sequences of the LMW-GS genes were classified into 12 groups based on the conserved N- and C-terminal domains. Since some of the cloned LMW-GS genes may contain deletions of the repetitive domain that occurred during the cloning process (Masci et al. 1998), a grouping based on sequence similarities of the conserved domains rather than size should help to simplify the characterization of LMW-GS sequences. Among these LMW-GSs, we identified two unique LMW-GS groups (groups 4 and 5) which were not previously reported as nucleotide-base sequences. Group 4 contains IENSHIPG in the N-terminal domain with isoleucine at position 1 and asparagine at position 3. If the isoleucine at position 1 does not affect a cleavage site, this group should show the same N-terminal amino-acid sequence of the mature protein as the LMW-s type (MENSHIPG) which also has asparagine at position 3 (Masci et al. 1998). Group 5 contains additional hydrophobic repeats within the N-terminal repetitive domain. These repeats make the N-terminal repetitive domain more hydrophobic than other groups. In contrast to other LMW-GSs, particularly the 42 k LMW-GS which shows relatively regular repeat sequences of a flexible nature in the N-terminal repetitive domain (Masci et al. 1998), group 5 seems to have less flexiblity due to the presence of hydrophobic repeats within this domain, which may affect the elasticity of dough (Belton 1999). The position of the first cysteine of group 5 is located in between the first cysteine positions of groups 1–4 and groups 6–10 (Fig. 1). A deletion before the first position of cysteine in groups 1–4 might have occurred in group 5. Group 3, for which we need to apply a nested PCR to amplify the corresponding genes, includes ten different sized LMW-GSs. One of these clones encodes 388 amino acids (370 amino acids as a mature protein) and shares strong sequence similarity to the 42 k LMW-GS gene encoding 369 amino acids as a mature protein (98% amino-acid identity), which is a major component of the glutenin fraction in some good quality common and durum wheat cultivars (Masci et al. 1998; D'Ovidio et al. 1999; Masci et al. 2000). Although we need to study the amount of this 42 k-like LMW-GS in Norin 61 seeds, it suggests that subunits of this group do not necessarily improve good baking quality. We need further analysis of the possible role of these subunits in a proper background.

In the case of groups 11 and 12, the cysteine in the N-terminal region was relocated to the C-terminal conserved domain. Since the repetitive domain is predicted to have a positive influence on gluten quality (Masci et al. 1998), polymerization of this group of LMW-GSs, lacking the repetitive domains as part of the network frame formed by disulfide bonds, should affect the glutenin polymer structure. Another possibility is that the cysteine relocated from the N-terminal domain does not precipitate in the intermolecular disulfide bond. In such a case, these groups of LMW-GSs might act as a chain-terminator instead of a chain-extender in glutenin polymerization; this would reduce the size of glutenin polymers and result in a weak gluten property. Further analysis is necessary to clarify the involvement of this cysteine in disulfide bond formation.

The number of cDNA clones corresponding to each group may not represent the relative amounts of transcripts in immature seeds, since the DNA probes used for screening the cDNA library showed strong similarity with groups 3, 11 and 12. Although we could not confirm whether the individual LMW-GS genes were expressed, it is clear that the data supports that at least nine groups of LMW-GS genes were expressed in immature seeds of Norin 61. As for groups 11 and 12, these LMW-GS genes have been found in other foreign cultivars, but not as protein products. This is the first direct evidence that this group of LMW-GS genes were expressed as mRNA in immature seeds. In our preliminary amino-acid sequencing of LMW-GSs (data not shown), we found the same

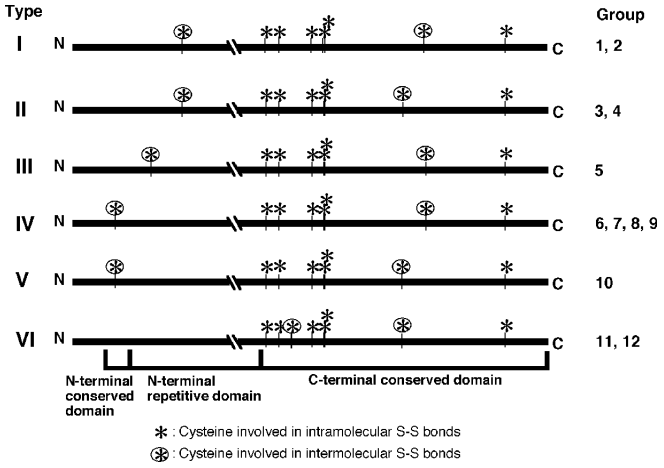

Fig. 4 Classification of the deduced amino-acid sequences of the LMW-GS genes based on the distribution of cysteine. The positions of cysteines are shown as *asterisks* (*)

amino-acid sequences as those of groups 3 (or 4), 5, 10 and 11 (or 12). Therefore, these groups should be translated into protein products in Norin 61 immature seeds.

Group 5-specific PCR shows uniformly sized products among common wheat cultivars. On the other hand, groups 11 and 12-specific PCR shows size heterogeneity among common wheat and durum wheat cultivars. The amino-acid sequence diversity of groups 11 and 12 from other cultivars is much higher than that of other groups (Table 1). These results suggest that the degree of sequence variability of LMW-GSs is different among groups. The high variability in groups 11 and 12 may in part be responsible for the difference in gluten quality among wheat cultivars.

As for the chromosomal location of these LMW-GS genes, group 5, and groups 11 and 12, seem to be encoded by *Glu-D3* and *Glu-A3*, respectively. The lack of PCR products obtained with the group 5-specific primer set using durum wheats also supports that group 5 is encoded by a D-genome chromosome. Van Campenhout et al. (1995) reported that a LMW-GS sequence showing strong similarity with groups 11 and 12, was encoded by *Glu-A3*. We can predict the loci of other groups based on amino-acid sequence similarity to previously reported sequences of loci which have been identified (Table 1): Group 6 (*Glu-A3*), groups 2 and 3 (*Glu-B3*), and groups 1, 7 and 10 (*Glu-D3*).

To classify these LMW-GS groups based on the functional aspects of dough, we further divided the 12 LMW-GS groups into six types based on the position of the first and seventh cysteines (Fig. 4).

Type I includes groups 1 and 2, which have the first cysteine in the N-terminal repetitive domain instead of position 5 in the N-terminal conserved domain. Type II includes groups 3 and 4, which show the seventh cysteine located at a different position from type I (Fig. 1). Type III corresponds to group 5 and has the first cysteine residue in the repetitive domain much closer to the N-terminal end than types I and II (Fig. 1). Type IV includes groups 6, 7, 8 and 9. Type V corresponds to group 10. Both types have the first cysteine at position 5 in the N-terminal conserved sequence domain, as for most of the nucleotide-based sequences of LMW-GS. These two types are distinguished from each other by the position of the seventh cysteine in the C-terminal domain. The position of the seventh cysteine of type IV and type V corresponds to that of types I and II, respectively (Fig. 1). Type VI includes groups 11 and 12. In contrast to other types, it lacks the first cysteine residue in the N-terminal region, but shows a new cysteine within the C-terminal conserved domain (Fig. 4). This classification simplifies the difference of LMW-GSs related to glutenin polymer formation by disulfide bonds, and should be useful for further analysis of the involvement of specific LMW-GSs in relation to disulfide bond formation.

In this study, we characterized various LMW-GS sequences from a soft wheat cultivar, but there is a need to carry out quantitative analyses of these LMW-GSs as protein products in seeds for the identification of functionally important LMW-GSs. The construction of group- and/or type-specific primer sets should help us to select specific LMW-GSs by PCR in breeding programs for improvement of wheat flour quality.

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