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Y.-K. Lee · F. Bekes · R. Gupta
R. Appels · M. K. Morell

The low-molecular-weight glutenin subunit proteins of primitive wheats. I. Variation in A-genome species

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Abstract A Tris-Tricine gel-electrophoresis system (Schaegger and von Jagow 1987), combined with a gradient gel, has been employed to provide an improved resolution of the B and C low-molecular-weight glutenin subunits (LMW-GSs) found in the endosperm of wheat grain. The gel system was used to document the variation in the gluten subunit proteins present in A-genome diploid wheats. The majority of LMW-GSs found in the A-genome diploid wheats were not present in normal bread wheats; the data suggest that they represent a rich source of new variation for the LMW-GSs which are considered to be very important in modulating wheat flour-processing properties. The analysis of variation in the nature of the LMW-GS genes, using PCR, demonstrated that the subclass of C-subunits assayed by primers from a previously published sequence did not show as much variation as the proteins. However, the data collected suggest that sufficient variation may exist in the LMW-GS genes of A-genome diploid wheats to use them as a source of genes for altering the flour-processing properties of hexaploid wheat.

Key words Low-molecular-weight glutenin subunits · A genome wheats · Tris-Tricine PAGE · Variation in genes by PCR

Introduction

Primitive diploid wheats are considered a source of novel genes that encode agronomically valuable traits. The genetic variation of primitive wheats has been extensively studied to evaluate their genetic potential for morphological traits (Sharma et al. 1981), high-molecular-weight glutenin subunits (HMW-GSs), (Kreis et al. 1985; Waines and Payne 1987; Goldsbrough et al. 1989), and gliadins (Ciaffi et al. 1997). In addition, isozymes (Smith-Huerta et al. 1989) and DNA profiling techniques, such as the polymerase chain reaction (PCR) profiles (Vierling and Nguyen 1992) and Restriction Fragment Length Polymorphism (RFLP) patterns (Castagna et al. 1994), have been used to characterize the genetic variation present in the primitive wheats.

Although the LMW-glutenin subunits (LMW-GSs) are known to be essential contributors in determining dough properties, this group of proteins has not been studied in detail (Vensel et al. 1995) and the genetic variation for LMW-GSs in the A-genome wheats has not been reported. In a broad sense the A-genome wheats include four wild species, *Triticum aegilopoides*, *Triticum boeoticum*, *Triticum thaoudar* and *Triticum urartu*, and one cultivated species, *Triticum monococcum*.

The LMW-GSs referred to in this study were defined by the protein extraction method of Gupta and MacRitchie (1991), in order to eliminate gliadin contamination, and a SDS-PAGE system, based on the method of Schaegger and von Jagow (1987), for sharp resolution of both of the B- and the C-subunits. The SDS-PAGE method uses Tris-Tricine gels which provide high levels of resolution of the small C-subunits; in the conventional Tris-Glycine SDS-PAGE system (Laemmli 1970) small proteins migrate together with the bulk of SDS molecules whereas in the Tris-Tricine system they travel faster than the SDS and separate as sharper bands, free from the interferring effects of the SDS. At the genomic DNA level, the LMW-GS genes were characterized in the present study by PCR (Mullis and Faloona 1987; Saiki et al. 1988) using pairs of specific primers based on the sequence data published by Colot et al. (1989).

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Y.-K. Lee · F. Bekes · R. Gupta · R. Appels (✉) · M. K. Morell
CSIRO-Plant Industry, PO Box 1600,
Canberra, ACT 2601, Australia

The studies in the present paper describe the genetic variability of LMW-GS genes in the A-genome wheats at the protein and DNA levels. Comparisons with the respective protein subunits in hexaploid wheats were used to identify novel LMW-GSs of potential interest for introducing new genetic variation into bread wheats.

Materials and methods

Plant materials for SDS-PAGE analysis

A total of 91 accessions of five different A-genome diploid wheat species, originally collected from various geographical locations, were supplied by the Australian Winter Cereal Collection (Tamworth, NSW), Istituto Sperimentale per la Cerealicoltura (Italy), the University of Tuscia (Italy) and ICARDA (Syria). The samples consist of 12 accessions of *T. aegilopoides*, 25 accessions of *T. boeoticum*, 34 accessions of *T. monococcum*, six accessions of *T. thaoudar* and 14 accessions of *T. urartu*. The Australian hexaploid wheat cultivar Suneca was included as a reference sample.

Plant materials for PCR

A total of 17 representative accessions, listed as follows, were used for PCR analysis: AUS 15367 (*T. boeoticum*), AUS 15823 (*T. boeoticum*), AUS 17649 (*T. urartu*), AUS 90350 (*T. thaoudar*), AUS 90381 (*T. aegilopoides*), AUS 90382 (*T. aegilopoides*), AUS 90383 (*T. aegilopoides*), AUS 90384 (*T. aegilopoides*), AUS 90385 (*T. aegilopoides*), AUS 90405 (*T. boeoticum*), AUS 90406 (*T. boeoticum*), AUS 90412 (*T. aegilopoides*), AUS 90443 (*T. boeoticum*), AUS 90458 (*T. boeoticum*), AUS 90459 (*T. urartu*), AUS 90358 (*T. monococcum*) and AUS 90403 (*T. monococcum*). Nullisomic-tetrasomic lines of Chinese Spring (N1AT1D, N1BT1 A, N1DT1 A, N6AT6D, N6BT6 A and N6DT6 A) were used as reference samples.

PCR primers

Primers LMW-GL (5′-CGACAAGTGCAATTGCGCAGATGGA-3′) and LMW-GR (5′-ACCTAGCAAGACGTTGTGGCATTG-3′) were designed using the published DNA sequence of the LMW-GSs (Colot et al. 1989). This pair of primers anneal to gene segments corresponding to middle regions of the signal peptide and the carboxy terminus of the LMW-GSs, thereby flanking the regions coding for the repetitive domains of the proteins. The primers contained fluorescent labels for the detection and analysis of PCR products using an ABI-DNA sequencer.

Protein extraction

Gliadins

In order to verify the homogeneity of the individual accessions, five individual seeds from each accession were randomly selected and one-third of each seed was used for individual gliadin protein extractions in 160 µl of 70% ethanol. The remainder of the seed was kept for the preparation of glutenin proteins and for germination. The extracted gliadins were mixed with 180 µl of SDS buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 30% glycerol and 0.005% bromophenol blue) and incubated for 40 min at 65°C prior to electrophoresis. When more than one gliadin band pattern was detected among the individual samples, eight more seeds from each accession were further screened. Accessions that showed more than two patterns were excluded from the LMW-GS composition analysis, and the seeds showing two different band patterns were separated into two groups and the only major band pattern was selected for the LMW-GS analysis.

LMW-GSs

Glutenins were prepared following the extraction of several seeds from homogeneous accessions. The preparation was performed according to the method described by Gupta and MacRitchie (1991). Final preparations were mixed with 180 µl of SDS buffer (0.1 M Tris-acetate, pH 8.0, 3% SDS, 3 mM EDTA, 30% glycerol and 0.05% Bromophenol Blue) and incubated at 65°C for 30 min.

SDS-PAGE

For electrophoresis, polyacrylamide gels (Schaegger and von Jagow 1987) were cast with a Hoefer electrophoretic apparatus (using a 20-well comb and 1-mm spacers). Gradient gels were electrophoresed at 80 V (1 h) for stacking and at 150 V (18 h) for separating, with water circulation. The anode buffer contained 0.1 M Tricine, 0.1 M Tris (pH 8.2) and 0.1%-SDS. The cathode buffer contained 0.6 M Tris-acetate (pH 8.4) and 0.1% SDS. The gels were stained in Coomassie Brilliant Blue G-250 (2% orthophosphoric acid, 0.6 M ammonium sulphate and 0.1% G-250 dye) overnight with slow shaking, rinsed in 0.03% Brij-35 for 1 h, soaked in 20% ammonium sulphate at 4°C overnight and photographed.

The LMW-GSs of the diploid species were manually scored relative to those of the control hexaploid wheat cultivar (Suneca) in order to generate a profile of band patterns. Scoring of individual bands was conducted using photographs of the gels enlarged to 1.4× the actual size of the gel. For accurate and systematic scoring of the bands from the different gels, the gel pictures were aligned by overlapping identical Suneca bands from each gel. The uppermost B-subunit band from Suneca was defined as the base reference position. A series of horizontal lines were drawn on the gel pictures with a spacing of 1 mm. Bands bisected by a line, or falling below that line, were assigned to one class. This method of scoring was found to be reproducible as the various replicate Suneca bands on each gel were consistently assigned to the same classes. The LMW-GS bands analysed in all of the gels were scored in this way.

Preparation of DNA samples and polymerase chain reaction

Genomic DNA of the wheat plants was prepared using the following procedure described in the DNA Workshop Manual, Plant Science CRC, Canberra. Approximately 1 g of leaf tissue was ground to a fine homogeneous powder in liquid nitrogen and mixed with 3 ml of DNA extraction buffer (4% sarkosyl, 100 mM NaCl, 10 mM EDTA and 100 mM Tris-HCl, pH 8.5). The mixture was ground briefly with 1 ml of phenol, mixed with a further 2 ml of phenol, and inverted on a rotator for 3 h to mix the phases thoroughly. The DNA isolated in this way was then processed using standard procedures.

PCR was carried out in a DNA thermal cycler (Corbett Research, Sydney, Australia) following standard protocols. The DNA was amplified during 35 cycles, each consisting of a DNA denaturation step (92°C, 30 s), a primer annealing step (65°C, 30 s) and an extension step (70°C, 1 min). Following the 35 cycles, the sample was held at 72°C for 5 min. The PCR products were analysed using an ABI DNA sequencer operated in conjunction with GeneScan software.

Results

Verification of homogeneity of the accessions by gliadin screening

Among 91 accessions tested, 23 were found to be internally heterogeneous. These heterogeneous accessions showed two or three different gliadin band patterns among five seeds. When eight more seeds from each accession were screened, up to five different gliadin band patterns were found from some of the accessions. As a result, 68 homogeneous accessions and 17 heterogeneous accessions (showing two major band patterns only) were selected for LMW-GS analysis. The heterogeneity of some of the accessions assayed in this way may have resulted from a variety of sources including spontaneous cross-pollination, mixing during seed handling, or an accumulation of mutations in the protein-coding loci.

LMW-GS composition analysis

Initially, Tris-Glycine gels (10%) were employed to separate the LMW-GSs, and showed excellent resolution for the B-subunits among several different gel concentrations (8, 10, 12 and 14%) tested, but the resolution of the C-subunits was poor. In order to improve the resolution of the C-subunits, Tris-Tricine gels were used. While homogeneous 10% Tris-tricine gels were an improvement on 10% Tris-glycine gels, gradient gels yielded further enhancements in reproducible band sharpness and resolution. Among 5–20%, 7–20% and 10–17% linear gradient gels, the 10–17% Tris-Tricine gels provided the best results, showing extremely clear separation of the B- and C-subunits and convenient for band scoring; the HMW-GSs are not all clearly resolved in this system. The resolution of the 10-% Tris-Glycine gels is compared to that of the 10–17% Tris-Tricine gradient gels in Fig. 1. The results were highly reproducible, although care must be taken to ensure that linear gradients were set-up in the gels. Screening of the LMW-GSs from the 85 A-genome wheat accessions was carried out using the 10–17% Tris-Tricine gradient gels.

From the LMW-GS analysis, two *T. aegilopoides* accessions, AUS 11426 and AUS 15441, showed an unusually high number of HMW-GS bands for diploid wheats. The ploidy levels of these accessions were checked using growing-root-tip analysis. Both accessions were found to be tetraploid containing 28 chromosomes, and they were excluded from the analysis. Therefore, a total of 83 seed samples of the A-genome wheats were screened to analyse the LMW-GS composition. The relative mobility of all of the B- and C-subunits of the diploid wheats was investigated by analysing the different combinations of the samples; and finally all the accessions showing different band patterns were analysed in three gels (Fig. 2). Many of the diploid accessions did not show an obvious division between the LMW-GS B- and C-subunits, unlike the hexaploid wheat which shows a clear gap between these classes. For the purpose of scoring, the LMW-GS B- and C-subunits of the diploid wheats were separately

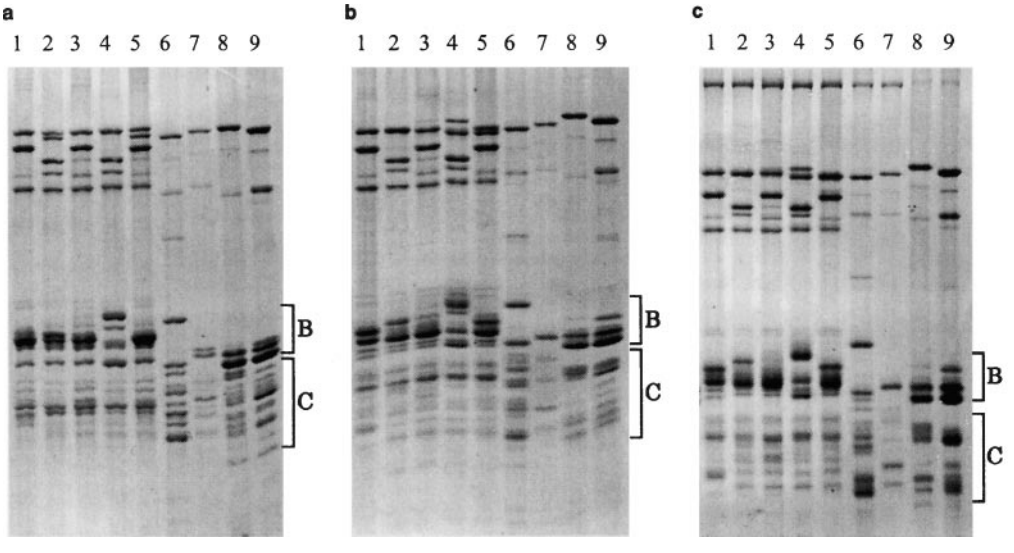

Fig. 1 A comparison of the resolution of LMW-GS separated in: **(a)** a 10–17% Tris-Tricine gradient gel, **(b)** a 10–17% Tris-Glycine gradient gel, **(c)** a 10% Tris-Glycine gel. The overall data need to be considered in making an assessment of the comparisons. The Tris-tricine gradient gel gives the most consistent and even spread of sharp bands across the LMW-GS distribution. This allows for a simplified and more reliable scoring of differences. Gel-to-gel variation, encountered in any analytical system, is adequately compensated for by using appropriate controls. *Lanes 1–5* hexaploid wheat cultivars, Chinese Spring, Gabo, Kite, Suneca and Sunstar. *Lanes 6–9* AUS15367 (*T. boeoticum*), AUS 90383 (*T. aegilopoides*), AUS90384 (*T. aegilopoides*) and CPI136589 (*T. boeoticum*), respectively. The *B* and *C* designations refer to the groups of proteins that most likely belong to the B- and C-subunits of the low-molecular-weight glutenin proteins

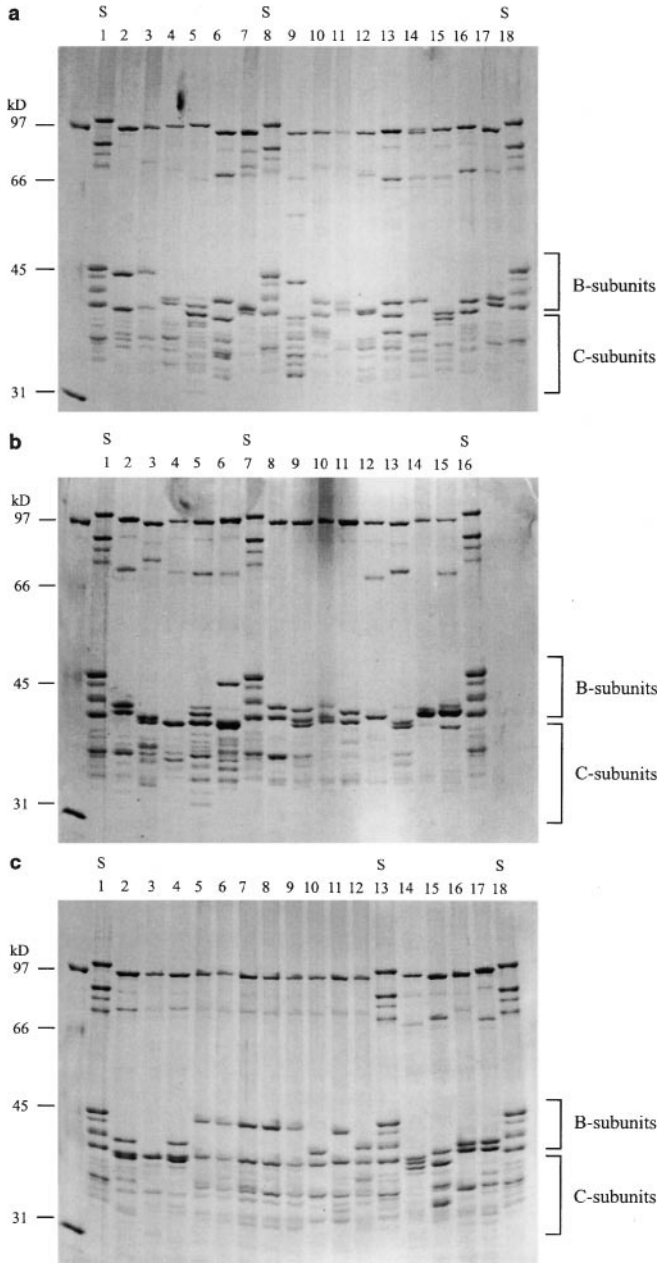

Fig. 2a–c SDS-PAGE analysis of the LMW-GSs from A-genome diploid wheats. Glutenin proteins of representative accessions of each species are shown with those of the control sample (Suneca) in the gels. Loading volumes of the protein samples were adjusted for these gels in order to obtain optimal band intensity for band scoring. **(a)** *Lanes 1, 8 and 18* Suneca; *lanes 2–7 T. aegilopoides*; *lanes 9–17 T. boeoticum*. **(b)** *Lanes 1, 7 and 16* Suneca; *lanes 2–6 T. boeoticum*; *lanes 8–15 T. urartu*. **(c)** *Lanes 1, 13 and 18* Suneca; *lanes 2–12 T. monococcum*; *lanes 14–17 T. thaoudar*

scored on the basis of the mobilities of the respective subunits in the hexaploid wheat cv Suneca. A total number of 36 LMW-GSs were scored from the 83 accessions. The number of B-subunit bands observed from individual accessions was between one and five, and the total number of different B-subunit bands scored was 16. The number of C-subunit bands per accession was between two and 11, and the total number of different bands scored was 20.

There were band-intensity variations observed between samples, relative to the intensity of bands in the wheat cv Suneca protein sample. All of the bands from the accessions of *T. thaoudar* were relatively faint; the loading volumes of the protein samples of *T. thaoudar* had to be approximately three times that of the protein samples of the other species, in order to obtain clear bands. This observation was reproducible between different gels as well as by a comparison of the relative band intensity of the diploid wheat samples.

It was found that accessions containing the same band patterns of B-subunits showed very similar, or identical, band patterns of C-subunits. This is not surprising as some of the B- and C-subunits of glutenins are controlled by the same loci. There were 43 different LMW-GS band patterns; *T. aegilopoides* (7 band patterns), *T. boeoticum* (15 band patterns), *T. monococcum* (11 band patterns), *T. thaoudar* (4 band patterns) and *T. urartu* (8 band patterns). The numbers of B- and C-subunit bands from the hexaploid control line, Suneca, were five and nine, respectively. The numbers of bands and band patterns found from each species are summarised in Table 1. Many of the band patterns were unique to a single accession, except for the accessions of *T. monococcum*. Similar band patterns for *T. monococcum* (see Fig. 2 c, lanes 2, 5, 7, 8 and 9) were frequently found among 34 accessions of this species. Other exceptions include band pattern 1 of *T. aegilopoides* (Fig. 2, shared with an accession of *T. boeoticum*), and band pattern 4 of *T. thaoudar* (Fig. 2, shared with an accession of *T. aegilopoides*).

PCR analysis

The specificity of the primers for amplifying the LMW-GS protein genes from the group-1 chromosomes was checked by analyzing the products amplified from nullisomic-tetrasomic lines of Chinese Spring lacking either group-1 or group-6 chromosomes. Lines lacking the group-6 chromosomes showed a uniform banding pattern while nullisomic-tetrasomic lines for group 1 indicated that specific amplification products were missing in particular lines (Table 2); this provided evidence that the primers assayed only genes on the group-1 chromosomes. The PCR profiles of the lines lacking group-1 chromosomes indicated that band "f" was amplified from the 1A chromosome, as this band is missing from the N1AT1D line; similarly bands "c" and "h" were derived from the 1D chromosome. The primers did not produce 1B-specific bands.

A total of seven bands (Fig. 3a–g) in the size range of 392–445 bp were amplified from the 17 accessions of the A-genome wheats. The annealing sites of the PCR primers are illustrated in Fig. 4. The resolution limit in

Table 1 Summary of the LMW-GS analysis by SDS-PAGE. The numbers of B- and C-subunit bands and band patterns found from each A-genome diploid wheat species are listed with the number of accessions studied

Species	No. of accessions	No. of different band patterns (B and C together)	B-subunits		C-subunits	
			Total no. of bands	Different patterns	Total no. of bands	Different patterns
T. aegilopoides	10	7[a]	7	6	17	7[a]
T. boeoticum	20	15[a]	11	14	21	15[a]
T. monococcum	34	11	9	7	13	11
T. thaoudar	6	4	4	2	17	4
T. urartu	13	8	9	8	14	8
Total	83	43		37		43

[a] These two species share one of the band patterns with another species (see text for details)

Table 2 PCR profiles of the 17 A-genome wheat accessions and nullisomic-tetrasomic lines of Chinese Spring (CS). A cross indicates the presence of a band. The size ranges of the bands are shown at the top of the table

DNA sample	Bands size (bp)							
	a (395–400)	b (401–408)	c (409–416)	d (417–421)	e (422–426)	f (427–432)	g (441–448)	h (464–467)
CS			+			+		+
N1AT1D			+					+
N1BT1A			+			+		+
N1DT1A						+		
N6AT6D			+			+		+
N6BT6A			+			+		+
N6DT6A			+			+		+
AUS15367		+	+	+				
AUS15823	+				+		+	
AUS17649		+	+					
AUS90350	+						+	
AUS90381		+	+					
AUS90382						+		
AUS90383	+		+					
AUS90384	+		+					
AUS90385	+	+			+			
AUS90405	+		+					
AUS90406	+	+	+					
AUS90412	+				+			
AUS90443	+	+	+					
AUS90458	+		+					
AUS90459		+	+					
AUS90358	+		+					
AUS90403	+		+					

this experiment was approximately 7 bp. The number of bands obtained from individual accessions was between one and three (Table 2). The sizes of the PCR products were similar to those predicted from the DNA sequence of the gene used in designing the primers. The size differences between these PCR fragments from the diploid wheats were between 6 bp and 50 bp (see Fig. 3). Bands "a" and "c" were found most frequently and appeared together from accessions of *T. aegilopoides*, *T. boeoticum* and *T. monococcum*. Bands "a" and "b" were not obtained from the hexaploid lines used as controls but were frequently observed from the diploid accessions (Table 2). Bands "f" and "h" were obtained only from the hexaploid wheat control lines (see Table 2).

Discussion

It was possible to accurately predict the homogeneity of LMW-GS compositions within accessions by screening gliadin proteins of individual seeds because the genes coding for the gliadin and LMW-GS proteins are closely linked. The *Glu-B3* locus is only 1.8–2.0 cM away from the *Gli-B1* locus (Singh and Shepherd 1988;

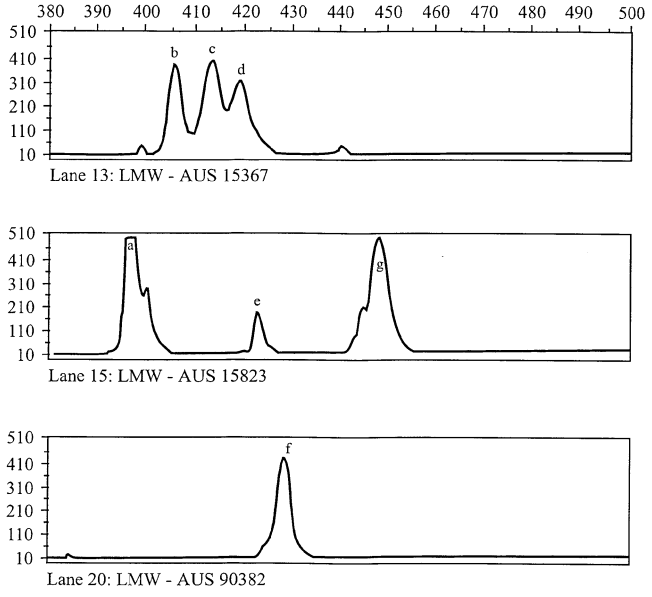

Fig. 3 Electrophoretic patterns of the PCR products analysed in a polyacrylamide gel using ABI DNA sequencer-GeneScan software. Among the 17 samples, three showing all of the seven bands are presented in an electropherogram. The X-axis indicates the size of bands in base pairs. The bands were named as *a–g*. AUS 15367 and AUS 15823 are accessions of *T. boeoticum*, and AUS 90382 is *T. aegilopoides*

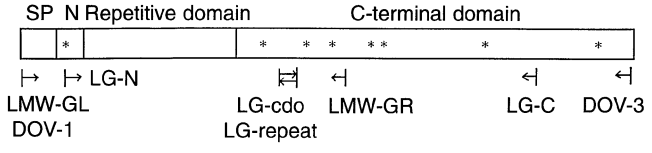

Fig. 4 Diagram of the LMW-GS genes (Colot et al. 1989). The length of the gene is 926 bp. *Arrows* indicate annealing sites of the PCR primers, the LMW-GLs and LMW-GRs, used for the PCR analysis. *SP* indicates the signal peptide and *N* is the N-terminal domain. The positions of the conserved cysteine residues are marked with *asterisks* (*)

Redaelli et al. 1995). Heterogeneity of some of the accessions assayed in this way may have resulted from a variety of sources including spontaneous cross-pollination, mixing during seed handling, or an accumulation of mutations in the protein-coding loci.

The SDS-PAGE system involving Tricine used to analyze the LMW-GSs improved the separation of both the B- and C-subunits, compared to the systems used previously. The C-subunits separated in this study using the Tris-Tricine system (Schaegger and von Jagow 1987) could be easily scored and allowed comparisons between accessions; the system offered an improved resolution relative to the Tris-Glycine SDS-PAGE system (Laemmli 1970). Tricine, a substitute for glycine, migrates much faster than glycine in a stacking gel and allows the small proteins to be separated from the SDS; as a result the stacking limit is shifted toward the low-molecular-mass range, producing sharper bands for the small proteins (Schaegger and von Jagow 1987).

The diploid wheats studied in this paper possessed highly variable band compositions not only between species but also within species. The accessions of cultivated diploid wheat (*T. monococcum*) showed the least variation in band pattern, possibly because they may represent a relatively small number of genotypes fixed during cultivation. The accessions of *T. thaoudar* consistently gave relatively faint LMW-GS bands in comparison to those of the other accessions.

The diploid wheats showed 16 different B-subunit and 20 C-subunit bands in this study, whereas there have been only five B-subunit and three C-subunit bands identified from the A-genomes of hexaploid wheat (Gupta and Shepherd 1990). The levels of polymorphism of hexaploid and diploid wheats are therefore remarkably different, and this suggests that almost all of the bands from the diploid wheats are novel subunits. The extensive variation of the protein band patterns in these diploid wheats has resulted from point mutations and deletions (Mecham et al. 1978; Lee et al. 1998). It has also been speculated that there may be a post-translational modification of polypeptides produced from the same genes (Mecham et al. 1978). The narrow range of diversity among LMW-GSs in the A-genome of hexaploid wheats is probably due to the limited number of accessions involved in the derivation of modern hexaploid wheat. The narrow range of diversity could also be due to preferential selection and the fixation of certain genotypes by breeding or the suppression of certain forms of gene expression in the A-genomes of hexaploid wheat.

The PCR primers for the LMW-GS genes were targeted to reveal length variation from the relatively unconserved repetitive domains. The small length variations of the PCR bands are due to deletions, or additions, in short repetitive domains and the non-conserved repeat motifs (Lee et al. 1998). In contrast to the LMW-GS protein-band patterns, the PCR band profiles did not reveal high variability in these genes. This might have resulted from several factors. Firstly, post-translational modification of the proteins produced from the same genes might produce polymorphic band patterns (Mecham et al. 1978). Secondly, the annealing sites of the primers used for this study may target the PCR analysis to particular subclasses of the LMW-GS genes. For example, these primers may anneal to the genes of only the C-subunits, and not the B-subunits, because the length of the gene sequence used for a primer design is 926 bp, which would express an approximately 31-kDa polypeptide. This is too short for a gene coding B-subunits, which are expected to have polypeptides between 38 kDa and 45 kDa in these diploid wheats. Thirdly, these genes may contain another variable region in the C-terminus, which was outside of the amplified region using the LMW-GL and LMW-GR primers; this is unlikely to be a major factor

as judged from the complete structure of several genes (Lee et al. 1998).

Analysis of LMW-GS variation at both the protein and DNA levels shows that there is considerable variation within the A-genome diploid wheats. The data suggest that the genes from A-genome wheats should provide a valuable resource of new genes that can be used to modify wheat flour-processing properties.

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