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PCR analysis of genes encoding allelic variants of high-molecular-weight glutenin subunits at the *Glu-D1* locus

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Abstract Genes encoding high-molecular-weight (HMW) glutenin subunits, present in bread-wheat lines and cultivars, were studied by RFLP (restriction fragment length polymorphism) and PCR (polymerase chain reaction) analyses. In particular, allelic subunits of the x- or y-type, encoded at the *Glu-D1* locus present on the long arm of chromosome 1D, were investigated. The variation in size, observed in different allelic subunits, is mainly due to variation in the length of the central repetitive domain, typical of these proteins. Deletions or duplications, probably caused by unequal crossing-over, have given rise to the size heterogeneity currently observed. The possibility of using the PCR technique for a detailed analysis of HMW glutenin genes in order to obtain a more accurate estimation of the molecular weight of their encoded subunits, and the detection of unexpressed genes, is also described.

Key words HMW-glutenin genes · Electrophoresis · Polymerase chain reaction · Molecular weight determination · Evolution

Introduction

Glutenins are the major determinants of dough strength and elasticity (Kasarda 1989). They are polymeric structures which when treated with reducing agents give rise to two subgroups termed high-molecular-weight (HMW) glutenin subunits and low-molecular-weight (LMW) glutenin subunits. HMW glutenin subunits are encoded at the *Glu-1* loci present on the long arms of chromosomes 1A, 1B and 1D of bread-wheat cultivars (Payne 1987; Shewry et al. 1992). Molecular analyses at the *Glu-1 loci* have indicated that each locus contains two tightly linked, genes, one encoding a subunit of low molecular weight and the other of high molecular weight, designated as y- and x-types, respectively (Harberd et al. 1986). Bread-wheat cultivars usually contain from three to five HMW glutenin subunits when separated by SDS-PAGE; one or none may be encoded by genes at the *Glu-A1* locus, one or two by genes at the *Glu-B1* locus, and two by genes at the *Glu-D1* locus.

Complete amino-acid sequences of these proteins, deduced from DNA sequencing of the corresponding genes, have shown the presence of three distinct domains: a central hydrophobic repetitive domain flanked by non-repetitive N- and C-terminal hydrophilic domains, where most of the cysteine residues are located. The prediction of secondary structure and physical studies have indicated that N- and C-terminal domains form alpha-helical structures, whereas the repetitive domain appears to consist of a spiral based on repetitive beta-turns. The central domain comprises hexa- and nona-peptide motifs in the y-type subunits, whereas in addition to these two the x-type also has a tripeptide motif (Shewry et al. 1989). Differences in subunit size result mainly from variation in the repeat structure and, in particular, from differences in the number of hexapeptide and tripeptides and Shewry et al. (1989) have suggested that the repetitive domains may have evolved by a series of amplification and/or deletion events involving single and multiple blocks of residues with insertions and/or deletions of single residues or part of blocks being rare.

At the *Glu-D1* locus of most bread-wheat cultivars two allelic pairs, indicated as 5 + 10 and 2 + 12, have been detected, and it has been reported that the former pair has the effect of greatly improving flour technological properties compared to the latter (Payne et al. 1981, 1987). Along with pairs 5 + 10 and 2 + 12, the pairs 3 + 12, 4 + 12, 2 + 10, 5 + 12, 2.2 + 12, 2 + 12_1, 2 + 12*, 2.2* + 12 have also been reported to be present at the *Glu-D1* locus and hypotheses on their origin have been postulated (Payne and Lawrence 1983; Margiotta et al. 1993).

Gene cloning and sequencing have made it possible to varify discrepancies between the migration of certain HMW glutenin subunits in SDS-PAGE and their molecular weight. Migration of allelic subunit pairs 1Dx2/1Dx5, on one side, and 1Dy10/Dy12, on the other, is anomalous; subunit 1Dx5 has, in fact, a higher mobility than the smaller allelic subunit 1Dx2, and similarly subunit 1Dy10 has a lower mobility than the larger subunit 1Dy12 (Greene et al. 1988). In addition to exhibiting discrepancies between molecular weight and migration, the molecular weights of HMW glutenin subunits, as determined by SDS-PAGE, appear to be overestimated (Bunce et al. 1985).

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The presence of genotypes without the x- or y-type subunit encoded at the *Glu-D1* locus has also been reported (Lafiandra et al. 1988). Results from electrophoretical and chromatographic analyses of allelic subunits at the *Glu-D1* locus and Southern analyses of corresponding genes made it possible to hypothesize that the repetitive region was responsible for the observed variation in size (Margiotta et al. 1993). The possibility of distinguishing all six HMW glutenin genes present at the three *Glu-1* loci and characterizing their N-terminal, C-terminal, and repetitive regions by PCR analyses (D' Ovidio et al. 1993) has allowed the specific analysis of genes encoding the *1Dx* and *1Dy* subunits and the verification of both their sizes and the region responsible for the molecular weight variation observed in the corresponding HMW glutenin subunits.

Materials and methods

Lines and cultivars of bread-wheat (*Triticum aestivum* L.) differing in HMW glutenin subunits at the *Glu-D1* locus were used (Table 1).

One-dimensional electrophoretic SDS-PAGE separations were carried out on 10% gels essentially as described by Payne et al. (1980) but with a bisacrylamide concentration of 0.27%. Samples were run on the same gels with standard reference proteins: myosin (Molecular weight = 205 000), β-galactosidase (116 000), bovine serum albumin (66 000) and phosphorylase B (97 400); the molecular weight of HMW glutenin sub-units was determined by comparison with these proteins.

Genomic DNA was extracted following the procedure reported by D'Ovidio et al. (1992). Southern-blot analyses were carried out on genomic DNA digested with *Hin*dIII and hybridized with the digoxigenin-labelled p1B9B clone* (Halford et al. 1987). Hybridization and washing conditions were those reported in Margiotta et al. (1993). PCR reactions were carried out in a reaction volume of 100 µl with 2.5 units of *Taq* DNA polymerase (Boeheringer), 1 × *Taq* PCR buffer (Boeheringer), 250 ng of each of the two primers and 300 µ*M* of each deoxyribonucleotide. DNA was subjected to an initial denaturation step at 94 °C for 2 min then amplification conditions were for 35 cycles at 94 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min and 30 s followed by a final incubation step at 72 °C for 7 min. The oligonucleotides used as primers for the amplification of the 1D × HMW glutenin genes have been synthesized on the basis of a published sequence of a HMW glutenin gene from *T. aestivum* cv Cheyenne (Anderson et al. 1989) and have the following sequences:

(a) 5′ ATGGCTAAGCGGTTAGTCCT 3′; (b) 5′ CTGGCTGGCCG ACAATGCGT 3′; (e) 5′ CTGGCCGTTGCGGAGAAGCT 3′; (f) 5′ CCACGCTAACATGGTATGAGC 3′. Oligonucleotides used as primers for the amplification of the y-type HMW glutenin genes were synthesized on the basis of a published sequence of a HMW glutenin gene from *T. aestivum* (Halford et al. 1987) and have the following sequences:

(c) 5′ ATGGCTAAGCGGTTGGTCCT 3′; (d) 5′ GGCTAGCCGACAA TGCGTCG 3′; (g) 5′ GGGAACATCTTCACAAAACAGTACAA 3′; (h) 5′ CTGTGTTAACATGGTATGGGTTGTC 3′.

Amplified products were analyzed on 1.5% agarose gels. Lambda DNA, double digested with *Hin*dIII/*Eco*RI, was used as a molecular weight standard to estimate the size of the amplified products.

Table 1 HMW glutenin subunit composition of the genotypes analyzed

Genotypes	1Ax	1Ay	1Bx	1By	1Dx	1Dy
Chinese Spring	–	–	7	8	2	12
MG315	–	–	7	8	2.2*	12
MG7249	2*	–	7	8	2.2	12
Cheyenne	2*		7	9	5	10
Columbus	2*		7	9	5	10
MG27111	–	–	7	8	–	12*
27095/123	–	–	17	18	2	12*
MG27078/304	–	–	7	8	2	12_1

* Kindly provided by P. R. Shewry

Results

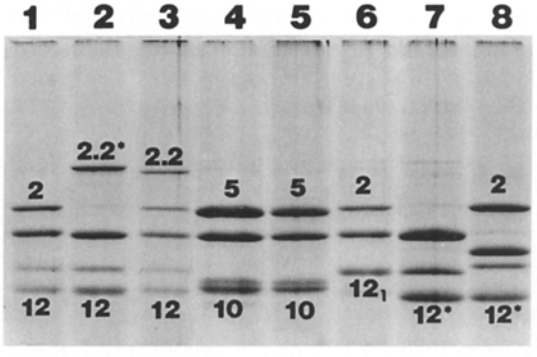
Electrophoretic analysis

One-dimensional SDS-PAGE separation of the materials used, and their specific HMW glutenin composition, are reported in Fig. 1 and Table 1, respectively. The allelic pairs 2 + 12 and 5 + 10 are indicated in the bread-wheat cultivars Chinese Spring (lanes 1), Cheyenne (lane 4) and Columbus (lane 5). Lines possessing HMW glutenin subunits of the x- or y-type, allelic to these pairs but of different molecular sizes, are also reported. In particular, MG 315 and MG 7249 (lanes 2 and 3 respectively) possess a Dx subunit of greater molecular size, compared to subunits 2 or 5; whereas MG 27078/304 (lane 6) possesses a Dy subunit, indicated as 12_1, which is larger than subunits 10 or 12. MG 27111 (lane 7) and 27095/123 (lane 8) have a Dy subunit of faster mobility compared to subunit 12 and hence are presumably of smaller size; the former line is also characterized by the absence of the Dx subunit. The molecular weights of these allelic variants, as determined from their migration in SDS-PAGE in comparison to standard reference proteins, are reported in Table 2.

Southern analyses

Southern analyses, following genomic DNA digestion with *Hin*dIII, indicate that subunits 2.2, 2.2* and 12_1, all possessing higher molecular weight than usually ob-

Fig. 1 One-dimensional SDS-PAGE of total proteins from hexaploid genotypes. *1*, Chinese Spring; *2*, MG315; *3*, MG7249; *4*, Cheyenne; *5*, Columbus; *6*, MG27078/304; *7*, MG27111; *8*, MG27095/123. The allelic designation of subunits at the *Glu-D1* locus is reported close to the polypeptide band



served for the D genome-encoded HMW glutenin subunits, also had fragments of larger sizes (Fig. 2). In particular, a 2.5-kb *Hin*dIII fragment corresponds to the 1Dx gene in Chinese Spring, MG 27078/304 and MG 27095/123 (lanes 1, 6 and 8), whereas 3.15 kb and 2.85 kb are the sizes of allelic fragments present in MG 315 and MG 7249, respectively. The genotype MG 27111, which has been shown by SDS-PAGE not to possess any Dx subunit, has a *Hin*dIII fragment of 2.5 kb similar to that of Chinese Spring.

Genotypes MG 315, MG 7249 and Chinese Spring possess the 1Dy subunit 12 and genomic analysis showed that they have a *Hin*dIII fragment of 1.9 kb (Fig. 2, lanes 1, 2, 3). Cultivars Cheyenne and Columbus, possessing subunit 1Dy 10, have 1Dy *Hin*dIII fragments of about 1.85 kb (Fig. 2, lanes 4, 5), and a similar size for the 1Dy *Hin*dIII fragment has been observed in genotypes MG 27111 and MG 27095/123 (Fig. 2 lanes 7, 8), which both possess subunit 1Dy12*. Finally, MG 27078/304, which possesses a 1Dy subunit of larger size (12_1), showed a 1Dy *Hin*dIII fragment of about 2.05 kb (Fig. 2, lane 6).

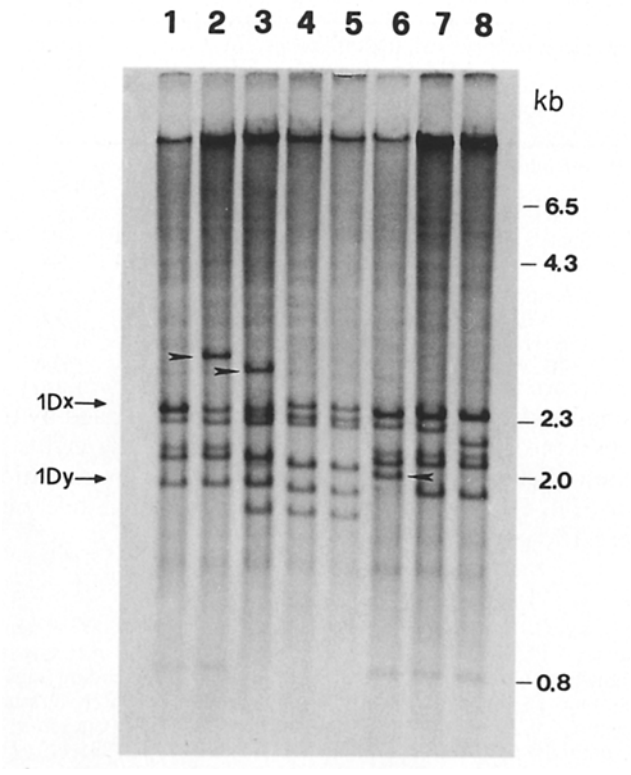


Fig. 2 Southern analysis of genomic DNA digested with *Hin*dIII. *1*, Chinese Spring; *2*, MG315; *3*, MG7249; *4*, Cheyenne; *5*, Columbus; *6*, MG27078/304; *7*, MG27111; *8*, MG27095/123. *Arrows* indicate the *Glu-D1* HMW glutenin fragments of Chinese Spring. *Arrowheads* indicate fragments corresponding to the *Glu-D1x* HMW glutenin gene in MG315 (*lane 2*) and MG7249 (*lane 3*), and to the *Glu-D1y* HMW glutenin gene of MG27078/304 (*lane 6*). The molecular weight marker is shown on the right side of the picture

PCR analysis

PCR analyses were carried out using primers specific for the complete coding region of the 1Dx gene (Fig. 3 primers a and b) and primers specific for the y-type (1Ay, 1By, 1Dy) HMW glutenin genes (Fig. 3 primers c and d). The PCR products obtained have been assigned to specific chromosomes using the genomic DNA of nullitetrasomic lines of *T. aestivum* cv Chinese Spring (D' Ovidio et al. 1993). PCR reactions of the complete coding region of genes at the *Glu-D1* locus of genotypes possessing different 1Dx or 1Dy alleles, showed the presence of amplification products of different sizes (Figs. 4A and 5A, respectively). The amplified product of the Dx gene encoding subunit 1Dx2.2* (Fig. 3, lane 2) was larger than the PCR products of the other 1Dx alleles. This gene was about 100 bp larger than the gene corresponding to subunit 2.2 (Fig. 2, lane 3), which in turn was about 450 bp and 500 bp larger than the genes encoding subunits 1Dx5 of Cheyenne and Columbus (Fig. 3, lanes 4, 5) and 1Dx2 of Chinese Spring, MG 27078/304 and MG 27095/123 (Fig. 3, lanes 1, 6, 8), respectively. The genotype MG27111 showed an amplified product of similar size to that observed in genotypes possessing subunit 1Dx2 (Fig. 3, lane 7). The PCR products of the 1Dx gene of the analyzed genotypes also showed the presence of an additional amplified fragment of about 1.7 kb, which does not represent a partial amplification of HMW sequences because does not hybridizes with HMW probes (data not shown).

Similar results were found by analyzing the PCR products of genotypes possessing different 1Dy alleles. In this case the primers used also amplified HMW glutenin genes corresponding to 1By and 1Ay subunits. In particular, the Ay gene was amplified in MG 7249,

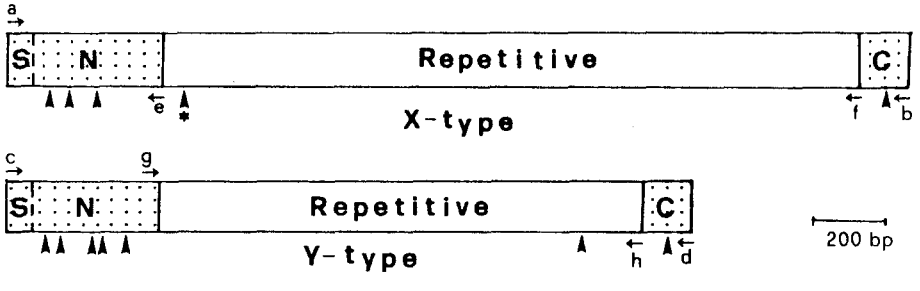


Fig. 3 Diagram of the x- and y-type HMW glutenin genes. *Arrows* indicate the position of the primers used for PCR analysis. *Arrowheads* indicate the position of the codons for cysteine residues. The *asterisk* indicates the position of a cysteine which is present only in the 1Dx5 subunit

Cheyenne and Newton, whereas the other genotypes do not show the amplified product corresponding to the 1Ay gene, most likely because of the presence of a large transposon-like insertion similar to that found in Chinese Spring (Harberd et al. 1987). Even for the y-type genes, the PCR products obtained showed an additional amplified product of about 1.6 kb which does not correspond to an incomplete amplification of HMW glutenin genes. This product, in fact, does not hybridizes with HMW glutenin probes (data not shown).

The analysis of the amplified product of the 1Dy gene corresponding to subunit 12_1 was shown to be larger than the PCR products of the other Dy alleles analyzed. The amplified product corresponding to this gene was, in fact, found to be about 120 bp, 140 bp and 160 bp larger than the genes corresponding to subunits 1Dy12, 1Dy12* and 1Dy10, respectively.

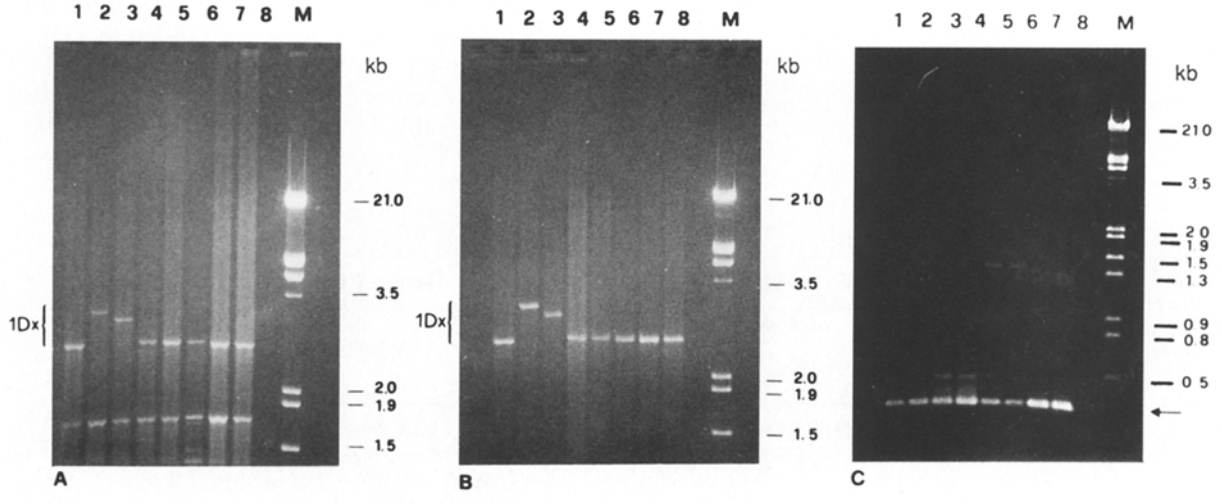
The size of the amplified products corresponding to the 1Dx and 1Dy genes was used to deduce the molecular weight of the corresponding HMW glutenin subunits. A comparison of the molecular weight of mature proteins, as duduced from gene length measurements, the molecular weight of the 1Dx2, 1Dx5, 1Dy10 and 1Dy12 components, calculated from the deduced amino-acid sequences, and the molecular weight calculated from SDS-PAGE, shows that the values of molecular weights obtained by PCR analysis and those deduced from amino-acid sequences are very close (Table 2). The comparison also confirms, the anomalous migration of the allelic components 1Dx2/1Dx5 and 1Dy10/1Dy12 on SDS-PAGE and the over-estimation of the HMW subunits analyzed. In fact, the gene encoding 1Dx5 is larger than that encoding 1Dx2 (Fig. 4A), and the gene encoding 1Dy12 is larger than that encoding 1Dy10 (Fig. 5A). The over-estimation of the analyzed subunits is particularly evident for the 1Dx subunits and ranges from 18 000 daltons for 1Dx5 to 25000 daltons for 1Dx2.2.

In order to establish the region responsible for the size variation of the analyzed subunits, primers which allowed the amplification of the repetitive region (Fig. 3 primers g and h), the N- terminal region (Fig. 3 primers a

Table 2 Size of the PCR products of some 1Dx allelic variants and the deduced M_r of the encoded subunit compared with the M_r deduced from amino acids and SDS-PAGE. The gene length was determined using lambda DNA degested with *Hin*dIII/*Eco*RI as a molecular weight marker and represents the complete coding region including the signal peptide which is 63 bp. The deduced M_r of the encoded subunit refers to the mature protein without the signal peptide. The M_r was calculated from the amplification product by dividing the length of the PCR product (gene length) by 3 and multiplying by 107. 107 represents the mean molecular weight of the individual amino acids in a HMW glutenin subunit and was found by dividing the molecular weight of the published sequences of 1 Dx2, 1Dx5, 1Dy10 and 1Dy12 by the number of residues in the sequence. To demonstrate the accuracy of such a calculation the length of the nucleotide sequence of the same genes as deduced by **a**, Sugiyama et al. (1985); **b** and **c**, Anderson et al. (1989); **d**, Thompson et al. (1985) are shown in brackets next to the PCR-deduced value for gene length. In brackets with an asterisk is reported the M_r mean as estimated for the actual gene size using the above formula

Gene length (bp)	M_r (dalton) mean from gene length	M_r from deduced amino-acid sequence	M_r from SDS-PAGE
1Dx subunits			
2 2560 (2514, a)	89.059 (87.419)*	87.000	109.500
5 2600 (2544, b)	90.486 (88.489)*	88.137	106.500
2.2 3050	106.536	–	131.400
2.2* 3180	111.173	–	133.500
1Dy subunits			
12 2000 (1980, c)	69.086 (68.373)	68.696	73.000
10 1960 (1944, d)	67.659 (67.089)	67.495	74.200
12_1 2120	72.653	–	81.600
12* 1980	67.303	–	71.900

Fig. 4A–C Agarose gels of PCR products of 1Dx HMW glutenin genes. **A** PCR products of the complete coding region including the signal peptide. **B** PCR products of the complete coding region without the C-terminal region. **C** PCR products of the N-terminal region. *1*, Chinese Spring; *2*, MG315; *3*, MG7249; *4*, Cheyenne; *5*, Columbus; *6*, MG27078/304; *7*, MG27111; *8*, MG27095/123. The *arrow* indicates the PCR products corresponding to the N-terminal portion of the 1Dx HMW glutenin genes



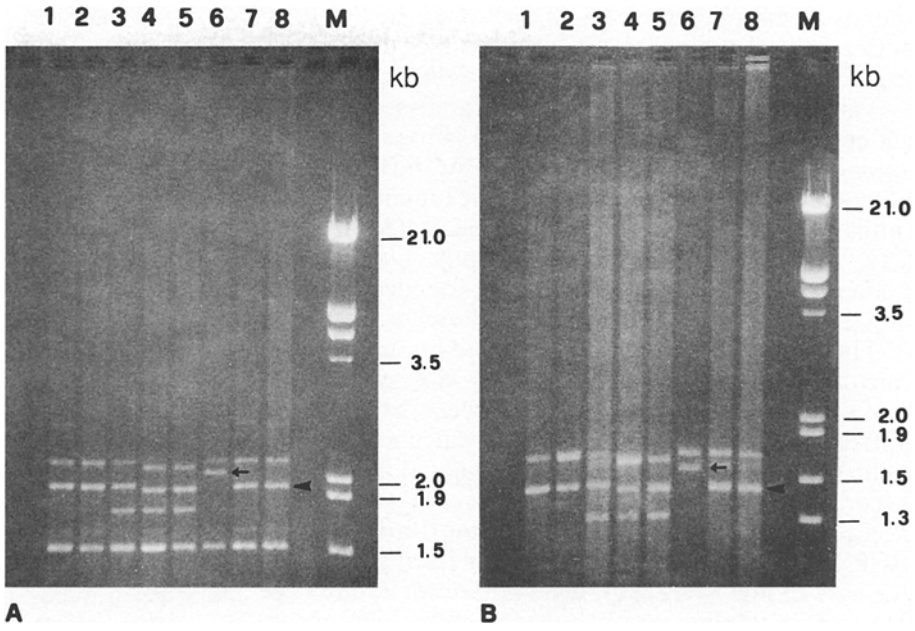


Fig. 5A, B Agarose gels of PCR products of y-type HMW glutenin genes. **A** PCR products of the complete coding region including the signal peptide. **B** PCR products of the repetitive region. *1*, Chinese Spring; *2*, MG315; *3*, MG7249; *4*, Cheyenne; *5*, Columbus; *6*, MG27078/304; *7*, MG27111; *8*, MG27095/123. *Arrows* indicate the PCR product of the HMW glutenin gene encoding subunit 12_1. *Arrowheads* indicate the location of the PCR products of the HMW glutenin genes encoding subunits 12, 10 and 12*

and e), and the complete coding region without the C-terminal region (Fig. 3 primers a and f) of the Dx and Dy genes, have been constructed. Using these primers it was possible to demonstrate that the internal repetitive domain was responsible for the size variation of the anlayzed HMW glutenin genes. In fact, when primers specific for the N-terminal region of the 1Dx gene were used, the amplified products obtained showed a uniform size for the different 1Dx components, indicating that the N-terminal region was similar in the HMW glutenin genes analyzed (Fig. 4C). In contrast, when primers specific for the complete coding region without the C-terminal portion were used, the PCR products showed the same differences already observed in the amplified products of the complete coding regions, demonstrating that the repetitive region was responsible for the size variation (Fig. 4B). The PCR analysis of the 1Dy genes was carried out using primers which allowed the amplification of the repetitive region of the 1Dy gene. As for the case of the 1Dx genes, the PCR products of the 1Dy genes showed the same differences already observed in the amplified products of the complete coding region (Fig. 5B).

Discussion

By analysing bread-wheat lines and cultivars possessing different allelic variants at the *Glu-D1* locus, Margiotta et al. (1993) observed that size variation did not affect the isoelectric points and surface hydrophobicities of the corresponding HMW glutenin subunits. Southern analysis of HMW glutenin genes of the above mentioned allelic variants showed the presence of *Hin*dIII fragments of different sizes. Because it has been reported that the *Hin*dIII sites are located upstream of and downstream from the repetitive region of HMW glutenin genes (Harberd et al. 1986), and that these sites are conserved in bread-wheat cultivars (Anderson et al. 1988), it was hypothesized that the size variation displayed at the DNA and protein level could be due to differences in the number of repeats in the central repetitive domain (Margiotta et al. 1993). Nevertheless, the fact that the positions of the *Hin*dIII sites are not conserved in some durum wheat genotypes (Margiotta et al. 1993) could make some results questionable. The development of PCR conditions which allow each gene at the *Glu-1* loci to be specifically analyzed (D'Ovidio et al. 1993) provide the opportunity to verify this hypothesis.

The results obtained by PCR analysis using primers specific for the N-terminal, C-terminal, and repetitive regions of HMW glutenin genes indicated that the different size of the allelic subunits detected at the *Glu-D1* locus is due to variation in the central repetitive domain.

According to Shewry et al. (1989) the N- and C-terminal domains evolved by the combination of single amino-acid substitutions and small insertion and/or deletions, whereas the repetitive block structure of the central domain provided the basis for a more rapid evolution and divergence by duplication and/or deletion of whole blocks, or several blocks, of residues, mostly as a result of unequal crossing-over.

The specific amplification of the complete coding region of HMW glutenin genes from genomic DNA of wheat genotypes allowed the length of these genes, and consequently the molecular weights of the encoded proteins, to be determined. Therefore, this PCR approach could be very useful to verify whether the deduced protein size of a specific HMW glutenin subunit corre-

sponds to its migration on SDS-PAGE, so avoiding misleading results due to anomalous migration as already reported by Goldsbrough et al. (1989).

This is of particular interest for the analysis of HMW glutenin subunits, whose designation is based on their apparent molecular weight on SDS-PAGE (Payne and Lawrence 1983). Thus, different HMW glutenins with a similar electrophoretic migration on SDS-PAGE could lead to misinterpretation, as recently reported by Lafiandra et al. (1993) for the *Glu-D1*-encoded subunits present in the bread-wheat cultivar Fiorello.

The molecular weight of the encoded mature protein, calculated as reported above, is very close to the real molecular weight of the protein and, because the relative differences with other components calculated in the same way are conserved, this method could be useful in experiments where equimolar amounts of proteins are needed. The reported results also demonstrate the use of PCR to detect the presence of unexpressed genes as in the case of line MG27111, which does not contain the 1Dx subunit. Increasing knowledge of the relationship between gluten structure and the technological properties of flour makes the PCR approach one of the most suitable methods of analysis for the rapid and specific identification of regions apparently responsible for qualitative gluten differences.

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