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Development of a set of oligonucleotide primers specific for genes at the *Glu-1* complex loci of wheat

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Abstract Specific amplification of the complete coding region of all six high-molecular-weight (HMW) glutenin genes present in hexaploid wheat was obtained by the polymerase chain reaction (PCR). Primers specific for the N-terminal region of the 1Dx gene and for the repetitive domain of the y-type HMW glutenin genes were also developed. Although the primers were constructed on the basis of the nucleotide sequences of HMW glutenin genes present in *T. aestivum* L. cv 'Cheyenne', they were very efficient in amplifying HMW glutenin genes of diploid and tetraploid wheat species. PCR analysis of HMW glutenin genes of *T. urartu* Tuman., *T. longissimum* (Schweinf. & Muschl.) Bowden and *T. speltoides* (Tausch) Gren. ex Richt. showed a high degree of length polymorphism, whereas a low degree of length variation was found in accessions of *T. tauschii* (Coss.) Schmal. Furthermore, using primers specific for the repetitive regions of HMW genes, we could demonstrate that the size variation observed was due to a different length of the central repetitive domain. The usefulness of the PCR-based approach to analyze the genetic polymorphism of HMW glutenin genes, to isolate new allelic variants, to estimate their molecular size and to verify the number of cysteine residues is discussed.

Key words Wheat · HMW glutenin genes · Polymerase chain reaction (PCR) · Multigene families · Evolution

Introduction

High-molecular-weight (HMW) glutenin subunits of the endosperm proteins of wheat are coded by the complex *Glu-1* loci present on the long arm of the group 1 homoeologous chromosomes. Each *Glu-1* locus (*Glu-A1*, *Glu-B1* and *Glu-D1*) consists of two genes, tightly linked, designated as x-type and y-type. The former codes for a subunit of higher molecular weight with respect to the latter. In hexaploid wheat six HMW glutenin genes are present, but only those coding for subunits 1Bx, 1Dx and 1Dy are always expressed, whereas the 1Ax and 1By are not always expressed, and the 1Ay gene is not expressed. Results reported so far have shown that the non-expression of the 1Ay gene can be caused by nucleotide changes in the promoter region (Forde et al. 1985) or by the presence of a transposon-like insertion in the coding region (Harberd et al. 1987).

Both x-type and y-type HMW glutenin subunits consist of three distinct domains: the N-terminal domain, which contains three cysteine residues in the x-type subunits and five cysteine residues in the y-type subunits; the C-terminal domain, containing one cysteine residue in both types; and a central repetitive domain, composed of hexapeptides and nonapeptides in the y-type subunits and, in addition, of tripeptides in the x-type subunits. An additional cysteine residue is present close to the C-terminus in the y-type subunits and, occasionally, close to the end of the N-terminus in the x-type subunits. The central domain forms a β-turn spiral, while the two non-repetitive domains have a globular structure formed by α-helixes (Shewry et al. 1992).

Glutenins are considered to be major determinants of flour quality. Differences in the relative amount of glutenin subunits and in the size of polymers constituted by glutenin subunits have been invoked as being responsible for bread-making (Payne et al. 1981; Payne and Lawrence 1983, Lookhart et al. 1993) or pasta-making characteristics (Branlard et al. 1989). In particular, allelic variation at the *Glu-D1* locus is largely responsible for differences in breadmaking properties of bread wheat flour (Payne et al. 1987).

The genetic variation of HMW glutenin subunits is usually assessed by SDS-PAGE, a system based on differences in the molecular mass of the single protein components (Laemmli 1970). Shewry et al. (1992) found that the high variation in size between the different HMW glutenin sub-

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units results mainly from differences in the number of hexapeptides and tripeptides. Recent results confirmed this by showing that the length variation observed for the HMW glutenin subunits encoded at the *Glu-D1* locus is mainly due to variation in the length of the central repetitive domain (D'Ovidio et al. 1994).

To characterize in more detail the structure and the genetic polymorphism of HMW glutenin genes, the possibility of developing an accurate and rapid approach by which to analyze all the HMW glutenin genes of a genotype was investigated. The polymerase chain reaction (PCR) (Saiki et al. 1985, 1988) was used to amplify specifically the complete coding region of genes encoded at the *Glu-1* loci, the N-terminal region of the 1Dx gene and the repetitive region of the 1Dy genes.

Materials and methods

Plants

Bread wheat cultivars (*T. aestivum* L.) 'Cheyenne' and 'Chinese Spring', nullisomic-tetrasomic lines of 'Chinese Spring' (Sears 1966) and accessions of *T. urartu*, T. *longissimum*, *T. speltoides* and *T. tauschii* were used. Accession numbers are specified in the figure legends.

SDS-PAGE analysis

Storage proteins were extracted from single seeds (1:10 w/v) with extraction buffer (0.07 *M* TRIS-HCl, pH 6.8 containing 2% SDS, 10% glycerol, 20% dimethylformamide, 0.02% γ-pironine, 1% dithiotreitol) overnight at room temperature. After centrifugation at 10,000 *g* for 10 min, 3 µl of each sample were loaded in a Mini-Protean II Cell (Bio-Rad, Richmond, Calif.). Stock acrylamide solutions and running buffers were according to the instruction manual. The concentration of acrylamide in the main gel was 8%. Gels were run at 10 mA constant current until the tracking dye had reached the bottom of the gel, stained overnight with 12% trichloroacetic acid solution containing 0.05% Coomassie Brilliant Blue R-250 in absolute ethanol (1% w/v) and destained in tap water.

DNA extraction

Genomic DNA was isolated from 5 g of leaves from single plants as previously reported (D'Ovidio et al. 1992).

PCR amplification

PCR reactions were performed in a final reaction volume of 100 µl using 50–100 ng genomic DNA, 2.5 units *Taq* DNA polymerase (Boehringer), 1×*Taq* PCR buffer (Boehringer), 250 ng each of the two primers and 300 µ*M* of each deoxyribonucleotide. Amplification conditions for the entire coding and repetitive regions of the HMW glutenin genes included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 2 min and 30 s and a final incubation step at 72 °C for 7 min. Amplification conditions of the N-terminal region of the 1Dx gene were for 30 cycles at 94 °C for 1 min, 62 °C for 50 s and 72 °C for 30 s, followed by a final incubation step at 72 °C for 7 min. Primers were prepared on the basis of published sequences of HMW glutenin genes from *Triticum aestivum* cv 'Cheyenne', using a Cyclone Plus synthesizer (Millipore) and have the following sequences: 1Ax gene (Anderson and Greene 1989): a) 5′ AGATGACTAAGCGGTTGGTTC 3′; b) 5′ CTGGCTGGCCAACAATGCGT 3′; 1Bx gene (Anderson and Greene 1989): c) 5′ ATGGCTAAGCGCCTGGTCCT 3′; d) 5′ TGCCTGGTCGACAATGCGTGC 3′; 1Dx gene (Anderson et al. 1989) e) 5′ ATGGCTAAGCGGTTAGTCCT 3′; f) 5′ CTGGCTGGCCGACAATGCGT 3′; i) 5′ CTGGCCGTTGCGGAGAAGCT 3′; y-type genes (Halford et al. 1987): g) 5′ ATGGCTAAGCGGTTGGTCCT 3′; h) 5′ GGCTAGCCGACAATGCGTCG 3′; l) 5′ GGGAACATCTTCACAAAACAGTACAA 3′; m) 5′ CTGTGTTAACATGGTATGGGTTGTC 3′.

Amplified products were analyzed on 1.5% agarose gel.

Digoxigenin labelling by PCR

Plasmid DNA (50–100 ng) was labelled by PCR for 30 amplification cycles in 100 µl total volume using 2 units of *Taq* DNA polymerase (Boehringer), 1×reaction buffer (Boehringer), 50 µ*M* of each dATP, dCTP and dGTP, 45 µ*M* dTTP, 5 µ*M* Dig-11-UTP (Digoxigenin-11-uridine-5′-triphosphate) and 20 ng of each of the T3 and T7 primers. The denaturing step was at 94 °C for 1 min; the annealing step was at 55 °C for 2 min; and the extension step was at 72 °C for 2 min.

Southern blot analysis

Amplification products were fractionated on agarose gel and transferred to nylon membranes (Hybond-N, Amersham) following standard procedures. Prehybridization and hybridization reactions were carried out at 65 °C in a solution containing 5×SSC, 0.02% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) *N*-lauroyl sarcosine and 1% (w/v) blocking reagent (Boehringer). Filters were prehybridized for 4 h and then hybridized for 16–20 h with HMW glutenin clones that had been digoxigenin-labelled by PCR. Clones used as probes were the pHMWF5 clone, which contains the *N*-terminal portion of the 1Dx5* HMW glutenin gene (Lafiandra et al. 1993), and the p1B9B clone, which contains the 1.8 kb *Hin*dIII fragment of the 1By9 HMW glutenin gene (Halford et al. 1987). After hybridization, the filters were washed twice at 65 °C in 2×SSC and 0.1% (w/v) SDS for 15 min, and twice at the same temperature in 0.1×SSC and 0.1% SDS for 30 min each. Immunological detection was performed following the Manufacturer's instructions (Boehringer).

Results

On the basis of the nucleotide sequences reported so far for HMW glutenin genes (Table 1), oligonucleotides specific for genes coding at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci of bread wheat were constructed. As shown in Table 2, the N-terminal and C-terminal nucleotide sequences of the entire HMW glutenin genes are very similar with only a few nucleotide changes. The presence of one or two nucleotide differences in the terminal regions of these genes was enough to enable the specific amplification of the y-type HMW glutenin genes and also the 1Ax, 1Bx and 1Dx genes. The positions and designation of primers used for the specific amplification of the complete coding region of HMW glutenin genes and of their N-terminal and repetitive regions are shown in Fig. 1. In *T. aestivum* cv 'Cheyenne', primers a-b amplify the 1Ax gene; primers c–d, the 1Bx gene; primers e–f, the 1Dx gene; and primers g–h, all three y-type genes (Fig. 2A).

Agarose gel analysis of the PCR products obtained from cv Cheyenne showed the presence of specific amplification products. In order to verify the nature of the amplifi-

cation products, Southern blot experiments using HMW glutenin probes were performed. The results showed that the amplification bands corresponding to the correct molecular size of each HMW gene gave a strong hybridization signal (Fig. 2B), whereas the additional amplification band present in the PCR product of the y-type genes did not show any hybridization signal, demonstrating that they do not correspond to HMW glutenin sequences. Comparison between the size of the six HMW glutenin genes of cv Cheyenne, as determined from nucleotide sequences, and those obtained from electrophoretic analysis of their amplification products showed that the relative differences in length of these genes are maintained (Fig. 2 and Table 1).

Primers used to amplify the HMW glutenin genes of cv 'Cheyenne' were also able to amplify all of the HMW glutenin genes of cv 'Chinese Spring' except, as expected, the 1Ay gene (Fig. 3). In the latter cultivar, in fact, the coding region of the 1Ay gene contains a transposon-like insertion of about 8.0 kb (Harberd et al. 1987), which makes the fragment too long to be amplified by PCR. To verify further the nature of the PCR products, their chromosomal localization was determined using nulli-tetrasomic lines of *T. aestivum* cv 'Chinese Spring', and the results confirmed the correct origin of the amplification products (Fig. 3). The additional bands of about 1.5 kb and 1.7 kb present in the y-type and x-type HMW genes, respectively, did not hybridize with the HMW probe (data not shown).

To analyze in more detail the HMW glutenin genes encoded at the *Glu-D1* locus, oligonucleotides specific for the N-terminal region of the 1Dx gene and for the repetitive domain of the y-type gene were prepared. Even in this case the sequences of the new primers were based on those of the HMW glutenin genes of cv 'Cheyenne'. Amplification reactions using primers l-m, which are specific for the repetitive region of the y-type genes (Fig. 1), gave three bands in cv 'Cheyenne' and two bands in cv 'Chinese Spring' (Fig. 4A), while primers e-i, which are specific for the N-terminal region of the 1Dx gene (Fig. 1), gave only one amplification band of about 400 bp (Fig. 4B). The specificity of the amplification products was verified by chromosomal assignment using nulli-tetrasomic lines of cv Chinese Spring (Fig. 4), by Southern analysis using HMW glutenin probes and by partial nucleotide sequence analysis (data not shown).

Table 1 Nucleotide length of the coding region, including the signal peptide, of the HMW glutenin genes

Gene/ subunit	bp	Genotype	Reference
Ax2*	2445	Cheyenne	Anderson and Greene 1989
Ax1	2490	Hope	Halford et al. 1992
Bx7	2373	Cheyenne	Anderson and Greene 1989
Bx17	2270	L86-69	Reddy and Apples 1993
Dx2	2513	Yamill	Sugiyama et al. 1985
Dx5	2543	Cheyenne	Anderson et al. 1989
Ay	1815	Cheyenne	Forde et al. 1985
By9	2115	Cheyenne	Halford et al. 1987
Dy10	1943	Cheyenne	Anderson et al. 1989
Dy12	1978	Chinese Spring	Thompson et al. 1985

Table 2 N-terminal and C-terminal sequences of HMW genes (including signal peptide)

N-terminal	
Ax2*	ATG ACT AAG CGG TTG GTT CTT
Ax1	ATG ACT AAG CGG TTG GTT CTT
Bx7	ATG GCT AAG CGC CTG GTC CTC
Bx17	ATG ACT AAG CGC CTG GTC CTC
Dx2	ATG GCT AAG CGG TTA GTC CTC
Dx5	ATG GCT AAG CGG TTA GTC CTC
Ay	ATG GCT AAG CGG TTG GTC CTC
By9	ATG GCT AAG CGG TTG GTC CTC
Dy10	ATG GCT AAG CGG CTG GTC CTC
Dy12	ATG GCT AAG CGG CTG GTC CTC
C-terminal	
Ax2*	C GAC GCA TTG TTG GCC AGC CAG
Ax1	C GAC GCA TTG TTG GCC AGC CAG
Bx7	C GAC GCA TTG TCG ACC AGG CAG
Bx17	C GAC GCA TTG TCG ACC AGG CAG
Dx2	C GAC GCA TTG TCG GCC AGC CAG
Dx5	C GAC GCA TTG TCG GCC AGC CAG
Ay	C GAC GCA TTG TCG GCT AGC CAG
By9	C GAC GCA TTG TCG GCT AGC CAG
Dy10	C GAC GCA TTG TCG GCT AGC CAG
Dy12	C GAC GCA TTG TCG GCT AGC CAG

In order to evaluate the efficiency of these primers in amplifying HMW glutenin genes from related wheat species, several hexaploid, tetraploid and diploid wheat genotypes were analyzed. In all of the experiments specific amplification products were obtained and their origin ver-

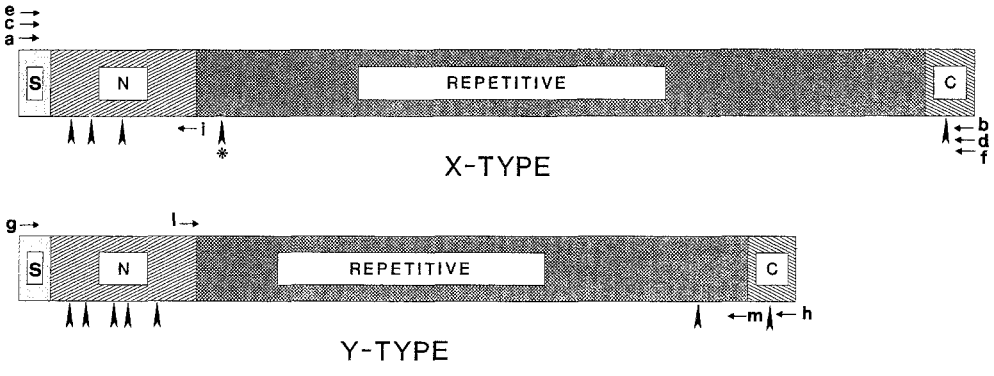

Fig. 1 Diagram of the x- and y-type HMW glutenin genes. *Arrows* indicate the position of the primers used for PCR analyses. *Arrowheads* indicate the position of the codons for cysteine residues. The *asterisk* indicates the position of a cysteine residue that is present only in the Dx5 subunit (*S* Signal peptide, *N* N-terminal domain; *C* C-terminal domain)

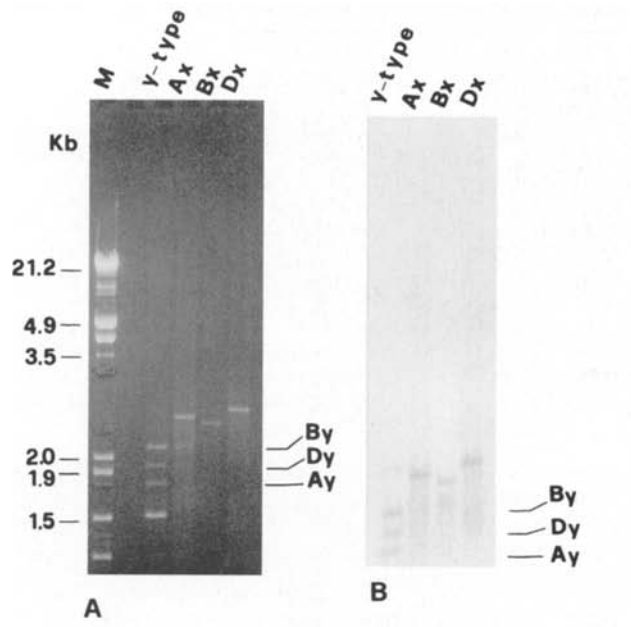

Fig. 2 **A** 1.5% agarose gel of PCR products of the complete coding region of all six HMW glutenin genes of bread wheat cv 'Cheyenne'. **B** Southern blot analysis of the amplification products of all six HMW glutenin genes of cv 'Cheyenne' using HMW glutenin probes. The amplification product of the specific HMW gene is indicated at the *top* of the picture

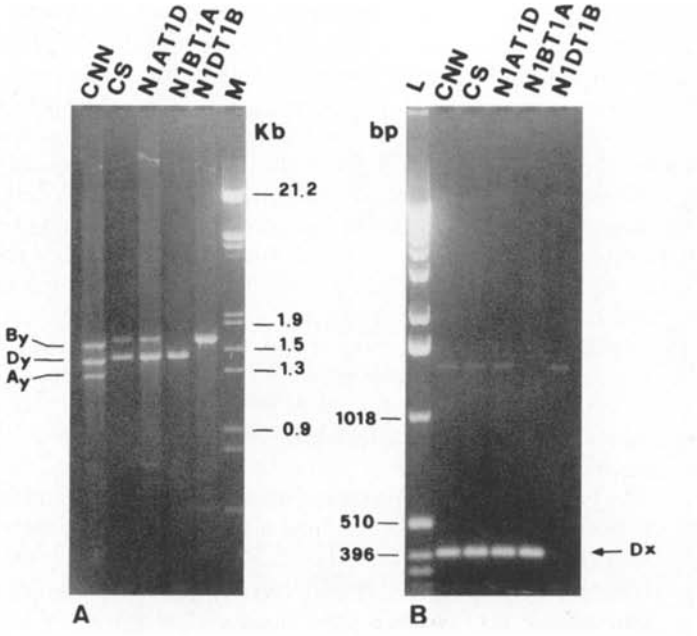

Fig. 4A, B 1.5% agarose gel of PCR products of HMW glutenin genes of the bread wheat cultivars 'Cheyenne' (*CNN*) and 'Chinese Spring' (*CS*), and of the nulli-tetrasomic lines of homeologous chromosome group 1 of 'Chinese Spring' (*NT*). **A** PCR products of the repetitive region of the y-type HMW glutenin genes; **B** PCR products of the N-terminal region of the 1Dx gene. The lack of some amplification products in nulli-tetrasomic lines indicates the chromosomal localization of that specific PCR product

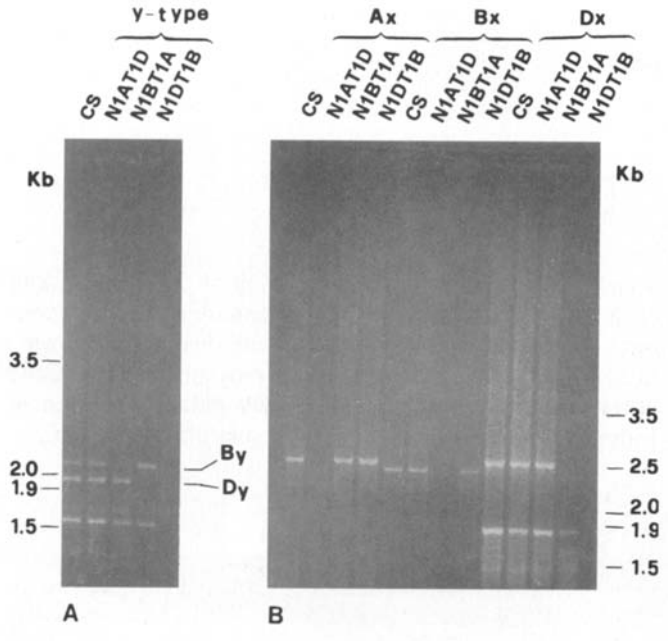

Fig. 3 1.5% agarose gel of PCR products of the complete coding region of HMW glutenin genes of bread wheat cv 'Chinese Spring' (*CS*) and nulli-tetrasomic lines of homeologous chromosome group 1 of the same cultivar (*NT*). The amplification product of the specific HMW gene is indicated at the *top* of the picture. The lack of amplification products in nulli-tetrasomic lines indicates the chromosomal localization of that specific PCR product. The amplification bands of about 1.5 kb and 1.7 kb do not correspond to HMW glutenin sequences

ified by Southern analysis (data not shown). In particular, the analysis was extended to several accessions of wild diploid wheat species and a high degree of length polymorphism was found by analyzing their y-type HMW glutenin genes (Fig. 5B). The PCR data were compared to those obtained by SDS-PAGE analysis, and analogous results were obtained (Fig. 5A). Nevertheless, in some cases the correspondence between gene length, as determined by PCR, and the molecular size of the corresponding subunit, as determined by SDS-PAGE, is missing. An example is represented by the Dy subunit of *T. tauschii* accession 2100; in this case the size of the encoding gene is larger than that expected from the subunit size (Fig. 5A, B).

Finally, in order to establish the origin of the size variation observed in the y-type HMW genes, the primers specific for the repetitive domains of these genes were used. Results from amplification reactions clearly demonstrated that the central repetitive domain was in all cases responsible for the observed size variation (Fig. 5B, C).

Discussion

PCR analysis is an extremely effective method for detecting genetic polymorphism in that it can distinguish between allelic variants that may differ only by a single nucleotide. In previous papers the PCR approach was proposed as a valid alternative to standard techniques for de-

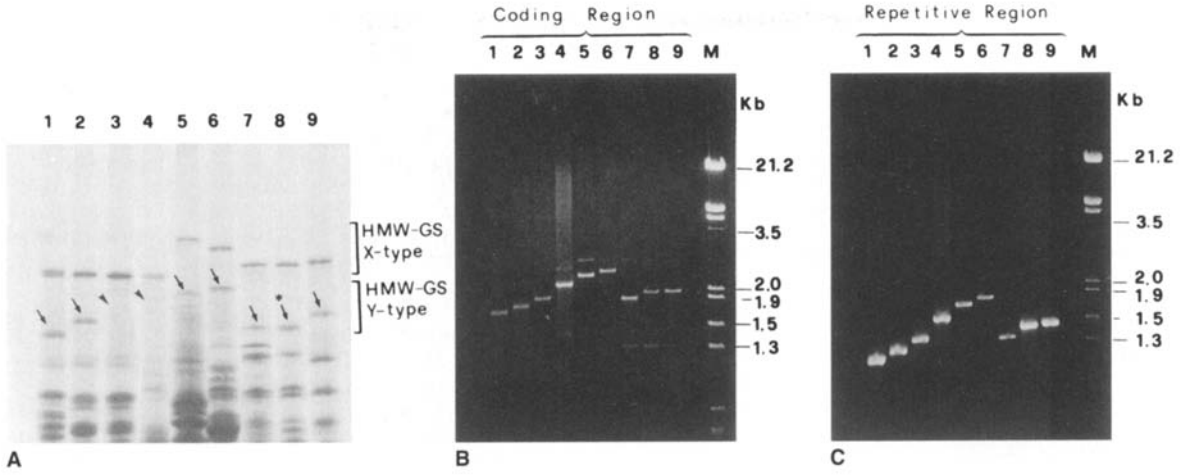

Fig. 5 **A** One-dimensional SDS-PAGE of total seed proteins. **B** PCR products of the complete coding region of the y-type HMW glutenin genes. **C** PCR products of the repetitive region of the y-type HMW glutenin genes. *Lane* 1 *T. urartu* ATRI 11496/82 SKL, *2 T. urartu* MG 29945, *3 T. urartu* PI 428308, *4 T. speltoides* 2033, *5 T. longissimum* 4-1, *6 T. longissimum* 4-5, *7 T. tauschii* 20-9, *8 T. tauschii* 2100, *9 T. tauschii* G1276. *Arrows* indicate y-type HMW glutenin subunits. The *arrow* with the *asterisk* indicates the Dy subunit with anomalous migration. *Arrowheads* indicate the lack of y-type HMW subunits

tecting and isolating new allelic variants of wheat storage protein genes (D'Ovidio et al. 1990, 1991).

The development of primers specific for the complete coding region of all six HMW glutenin genes of bread wheat and for their internal domains enables the isolation of specific allelic variants, the analysis of the polymorphism at each specific *Glu-1* locus, and the determination of the possible domains responsible for length variation of these genes. In the present paper we demonstrate that the length variation of HMW glutenin genes from diploid wild wheat is due to variation in the central repetitive domain. The same result was also obtained for HMW glutenin genes from some bread wheat genotypes (D'Ovidio et al. 1994). These findings confirm the hypothesis of Shewry et al. (1992) that the central repetitive domain is responsible for the length variation of HMW glutenin subunits.

SDS-PAGE is one of the most widespread techniques used in wheat breeding laboratories for detecting allelic forms related to good or poor quality. Nevertheless, it is well known that the mobility of HMW glutenin subunits in SDS-PAGE is not always correlated to their actual molecular weights (Shewry et al. 1992). Anomalous migrations on SDS-PAGE might be a problem for breeders when it is necessary to select parental lines for breeding programs. This problem might be overcome by using RP-HPLC, as recently reported by Margiotta et al. (1993), or by using PCR. The greater reliability of the PCR approach proposed here in amplifying the complete coding regions of HMW glutenin genes allows, in fact, the determination of the actual molecular weight of the HMW glutenin genes, thereby avoiding the misinterpretation of results from SDS-PAGE. In this respect, the determination by PCR analysis of the molecular size of the 1Dx and 1Dy HMW glutenin genes has been reported (D'Ovidio et al. 1994). Moreover, the possibility to amplify specific domains of these genes, such as the N-terminal domain, allows a rapid determination, by nucleotide sequence analysis, of the number and position of the codons for cysteine residues (Lafiandra et al. 1993). Since, in fact, the quality of flour seems to be strictly correlated with the size and type of glutenin polymers (Huebner and Wall 1976; Dachkevitch and Autran 1989; Singh et al. 1990), whose subunits are held together by disulphide bonds, the number and/or position of cysteines is likely to influence directly quality characteristics. In particular, it has been hypothesized that the additional cysteine residues located in the N-terminal region of the 1Dx5 gene play an important role in determining a better quality of bread wheat flour as opposed to the 1Dx2 HMW glutenin subunit, which does not possess this cysteine residue (Greene et al. 1988). Recent results in this direction have been reported for bread wheat cultivar Fiorello, which is supposed to possess the 1Dx5 HMW glutenin subunit, as deduced from SDS-PAGE analysis (Pogna et al. 1987). Using the primers here reported, which are specific for the N-terminal region of the 1Dx gene, we were able to amplify the N-terminal domain of the 1Dx gene of cv Fiorello. The nucleotide sequence of this region demonstrated the absence of the codon for the cysteine residue peculiar to the 1Dx5 subunit, thereby showing that the N-terminal region of the 1Dx HMW glutenin subunit of Fiorello is more similar to that of subunit 1Dx2 (Lafiandra et al. 1993).

Besides enabling the characterization of HMW glutenin genes, the possibility to amplify, clone and express specific regions of HMW glutenin genes in heterologous systems allows many different studies to be performed in a relatively easy way. The possibility to produce single domains and to modify them by site-directed mutagenesis could be useful in structural studies. The patterns of folding of repetitive and non repetitive domains, which have different structures, might be studied individually, thereby bypassing the difficulty of preparing them from purified proteins because of the lack of suitable sites for proteolytic or chemical cleavage (Maddgwick et al. 1992). It should be pointed out, however, that PCR amplification of the re-

petitive region could introduce some nucleotide changes as a result of *Taq* DNA polymerase activity in such highly repetitive region.

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