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PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread-making quality

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Abstract Good or poor wheat bread-making quality is associated with two allelic pairs at the *Glu-D1* complex locus, designated 1Dx5-1Dy10 and 1Dx2-1Dy12, respectively. The polymerase chain reaction (PCR) verified the presence of the HMW-glutenin 1Dx5 gene from genomic DNA extracted from part of the endosperm of a single dry seed, or a small amount of leaf or root tissue, of several bread-wheat cultivars. This easy, quick, and non-destructive PCR-based approach is proposed as a very efficient and safe alternative to standard procedures for selecting bread-wheat genotypes with good bread-making properties.

Key words Wheat · HMW-glutenin genes
Bread-making quality · Polymerase · Chain Reaction (PCR) · Multigene families

Introduction

Gliadins and glutenins are the most abundant storage proteins of wheat endosperm. Glutenins are divided into high (HMW)- and low (LMW)-molecular-weight subunits on the bases of their mobility on SDS-PAGE (Bietz et al. 1975). The HMW-glutenins (for review see: Shewry et al. 1992) are encoded at complex loci, designated *Glu-1*, on the long arm of group-1 homoeologous chromosomes of each genome A, B and D. Molecular analyses have shown that each complex *Glu-1* locus consists of two genes which code for different types of HMW-subunits, one of greater molecular weight, designated the x-type and another of lower molecular weight, designated the y-type (Harberd et al. 1986). Allelic variation for HMW-glutenins has been reported at each of the three complex loci and a specific designation for each of these alleles has been proposed (Payne and Lawrence 1983). Payne and coworkers have also shown that bread-making properties are particularly associated with variation at the *Glu-D1* and *Glu-A1* loci (Payne et al. 1981, Payne et al. 1987). In most bread-wheat cultivars, two allelic pairs at the *Glu-D1* complex locus, designated 1Dx5-1Dy10 and 1Dx2-1Dy12, have been detected and Payne et al. (1987) have demonstrated that good or poor bread-making quality is specially associated with the presence of the HMW-subunits Dx5-Dy10 (5–10) or Dx2-Dx12 (2–12), respectively. The identification of allelic pairs present in a genotype is currently carried out at the protein level by comparing the relative migration of their encoded HMW-subunits on SDS-PAGE. This method has the advantage of rapidly analyzing large numbers of samples. A disadvantage, however, is evident from the nucleotide sequences of some HMW-glutenin genes which show discrepancies between the derived molecular weights of their subunits and the order of migration on SDS-PAGE (Goldsbrough et al. 1989, Shewry et al. 1992). Further uncertainty in utilizing protein migration patterns for identification comes from the report that the Bx7 gene in two different cultivars encodes subunits with at least one difference in the repeat structure (Anderson and Green 1989). For these reasons we have investigated the possibility of developing a more accurate and reliable method for identifying the gene complement of a genotype using the polymerase chain reaction (PCR) (Saiki et al. 1985, 1988).

In the present paper we report the ability to distinguish between bread-wheat cultivars with good or poor bread-making properties by analyzing the PCR product of the 1Dx gene. Since, in fact, the allelic pair 1Dx5-1Dy10 is strictly associated it is possible to select for only one of these two components in order to select for the entire locus.

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Materials and methods

DNA extraction

Genomic DNA was isolated from 5 g of leaves from single plants (see Table 1) as previously reported (D'Ovidio et al. 1992). Rapid DNA extraction was carried out both from 10 mg of roots or the young hypocotyl of a single germinating seed following the procedure reported by Edwards et al. (1991), or else directly, without germination, using part of the endosperm of a single seed following the procedure reported by Benito et al. 1993.

PCR analysis

Genomic DNA (25–200 ng) or 3–10 µl of DNA extracted by the rapid procedure was subjected to PCR in a reaction volume of 100 µl with 2.5 units of *Taq* DNA polymerase, 1 *Taq* PCR buffer, 250 ng of each of the two primers and 300 µM of each deoxyribonucleotide. Amplification conditions were for 30 cycles at 94 °C for 1 min, 63 °C for 45 s and 72 °C for 30 s. One-tenth of each total PCR product was analyzed on 1.5% agarose gels. The oligonucleotides used as primers have been synthesized on the basis of the HMW-glutenin 1D5 gene from *Triticum aestivum* cv Cheyenne (Anderson et al. 1989) and have the following sequences: (1) GCCTAGCAACCTTCACAATC, (2) GAAACCTGCTGCGGACAAG.

Digoxigenin labelling by PCR

Plasmid DNA (50–100 ng) was labelled by PCR for 30 amplification cycles in a 100 µl total volume using 2 units of *Taq* DNA polymerase (Boheringer), 1 ×reaction buffer (Boheringer), 50 µM of each dATP, dCTP and dGTP, 45 µM dTTP, 5 µM Dig-11-UTP (Digoxigenin-11-uridine-5′-triphosphate) and 20 ng each of 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 tranferred to nylon membrane (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 which have been digoxigenin-labelled by PCR. The clones used as probes were pHMWF5, which contains the N-terminal partion of the 1Dx5* HMW-glutenin gene (Lafiandra et al. 1993), and p1B9B (Kindly provided by Dr. P. R. Shewry) which contains the 1.8-kb *Hin*dIII fragment of the 1By9 HMW-glutenin gene (Halford et al. 1987). After hybridization, 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 0.1SSC and 0.1% SDS for 30 min each. Immunological detection was performed following the Manufacturer's instructions (Boehringer).

Results

The availability of the nucleotide sequences of the HMW-glutenin genes designated 1Dx2 (Sugiyama et al. 1985) and 1Dx5 (Anderson et al. 1989) allowed us to construct two oligonucleotides specific for the HMW-glutenin 1Dx5 gene. The difficulties of designing two primers specific for 1Dx5 were due to the high degree of homology shown by the 1Dx2 and 1Dx5 genes and by the presence of a large repetitive region which is characteristic of the HMW-glutenin genes. Therefore, the search for appropriate primers was restricted to the terminal non-repetitive coding regions and the immediate flanking non-coding sequences. Nevertheless, it was possible to find two regions that differ between the 1Dx5 and the 1Dx2 genes. Oligonucleotides homologous to these regions in the 1Dx5 gene were constructed and used for PCR experiments. Figure 1 shows the sequences of these primers and their complementary sites which allowed the amplification of a 450-bp fragment from just 3′ of the TATA box to the beginning of the repetitive domain. Electrophoretic analysis of the PCR reactions carried out on genomic DNA of *T. aestivum* cv Cheyenne, which contains the 1Dx5 gene, showed the presence of an intense amplified product of 450-bp which corresponded to the expected fragment size (Fig. 2), along with several fainter, higher-molecular-weight, bands. The same PCR experiment was carried out on genomic DNA of Chinese Spring, a bread-wheat cultivar containing the 1Dx2 gene. No 450-bp fragment amplified from the Chinese Spring DNA. In addition, PCR experiments were performed using varying amounts of leaf DNA of cultivars Chinese Spring and Cheyenne to determine if the primers would amplify the 1Dx2 gene over a range of DNA concentrations. Again, the primer amplified the 1Dx5 fragment but not the 1Dx2 fragment over all DNA amounts tested. As shown in Fig. 2, the 450-bp fragment was obtained only

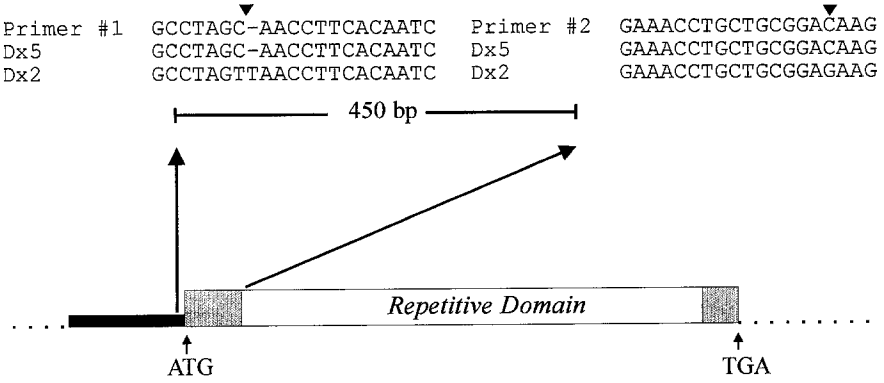


Fig. 1 Design of primers for allele-specific amplification of 1Dx genes. Primers #1 and #2 are identical to the 1Dx5 sequence but each has a single-site difference to the 1Dx2 sequence (*arrowheads* mark positions of difference). The expected amplification product is a 450-bp fragment spanning the sequence from immediately 3′ to the TATA box to the beginning of the repetitive domain. Positions of the start (ATG) and stop (TGA) codons are indicated by *arrows* on the diagram. Non-repetitive terminal regions of the HMW-gene are *hatched*. The *solid black line* is the promoter region, and the *dashed lines* are the flanking DNA sequences.

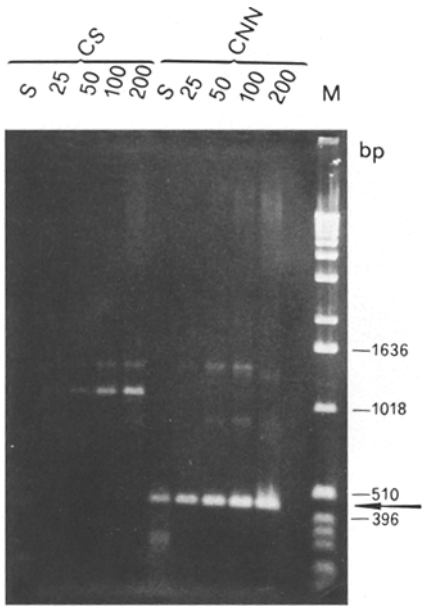
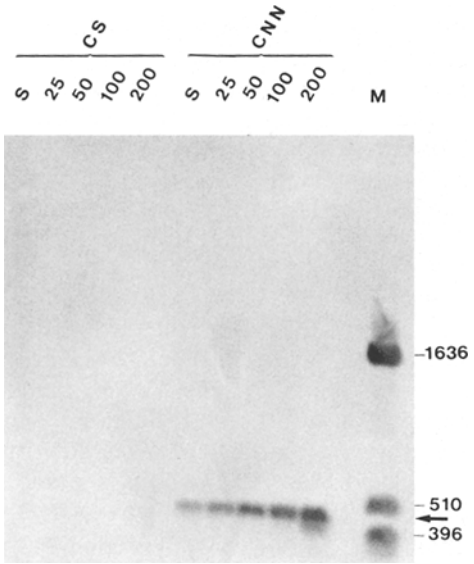


Fig. 2 Primers specific for the 1Dx5 gene were used for PCR reactions with different amounts of genomic DNA (25–200 ng) or 3–10 µl of DNA extracted with rapid preparation (*S*) from part of the endosperm of a dry seed from cultivars Chinese Spring (*CS*) and Cheyenne (*CNN*). *M*, 1-kb DNA ladder; the molecular weight of the fragments are reported in base pairs (bp). The *arrow* indicates the 450-bp specific of the 1Dx5 gene

Table 1 Bread-wheat genotypes analyzed in the present paper and their relative allelic HMW-glutenin subunit composition at the *Glu-D1* locus

Genotypes	*Glu-D1*	
	Dx	Dy
Benito	5	10
Centauro	5	10
Cheyenne	5	10
Chinese Spring	2	12
Columbus	5	10
Conway	5	10
Darius	2	12
Fiocco	5	10
Fiorello	5*	12
Hope	5	10
Liocorno	5	10
Maestra	5	10
Manital	2	12
Marquis	5	10
Melchior	3	12
MG 27078/304	2	12_1
MG 7249	2.2	12
MG 27095/123	2	12*
MG 315	2.2*	12
Newton a	5	10
Newton b	4	12
Pegaso	5	10
Raeder	2	12
Salmone	2	12
San Pastore	2	12
Solar	5	10
Spada	2	12
Talent	4	12
Timstein	2	12

Fig. 3A Southern blot of PCR products obtained from Cheyenne (*CNN*) and Chinese Spring (*CS*) hybridized with digoxigenin-labelled HMW-glutenin clones pHMWF5 and p1B9B. *M*, 1-kb DNA ladder; the molecular weight of the fragments are reported in base pairs (bp). The *arrow* indicates the 450-bp specific to the 1Dx5 gene



in PCR experiments of the cultivar Cheyenne containing both a varying amount of genomic DNA (25–200 ng) or 10 µl of DNA extracted from part of the endosperm tissue of a dry seed by the rapid procedure (S). Similar results were obtained using DNA extracted by the rapid procedure from roots or young hypocotyls (data not shown). No 450-bp amplification product was obtained in cv Chinese Spring. To further characterize the origin of the amplified products obtained from Chinese Spring and Cheyenne a Southern-blot analysis was performed using a HMW-glutenin gene as a probe. As shown in Fig. 3 the 450-bp fragment from cv Cheyenne gave a strong hybridization signal, whereas no signal was obtained in this region in the lanes with Chinese Spring DNA. In addition, there was no hybridization with any of the fainter higher-molecular-weight bands, demonstrating that these PCR products do not correspond to HMW-glutenin sequences.

In order to verify the applicability of this PCR-based method for distinguishing between cultivars possessing the HMW-glutenin 1Dx5 gene, or the HMW-glutenin 1Dx2 gene, DNAs of 22 cultivars were analyzed (Table 1). In all cases the amplification product of 450-bp was present only in cultivars known to possess the HMW-glutenin 1Dx5 gene (Fig. 4). Finally, genotypes possessing allelic variants at the *Glu-D1x* locus, encoding subunits 3, 4, 2.2, 2.2* and considered to be similar to subunit 2 (Payne and Law-

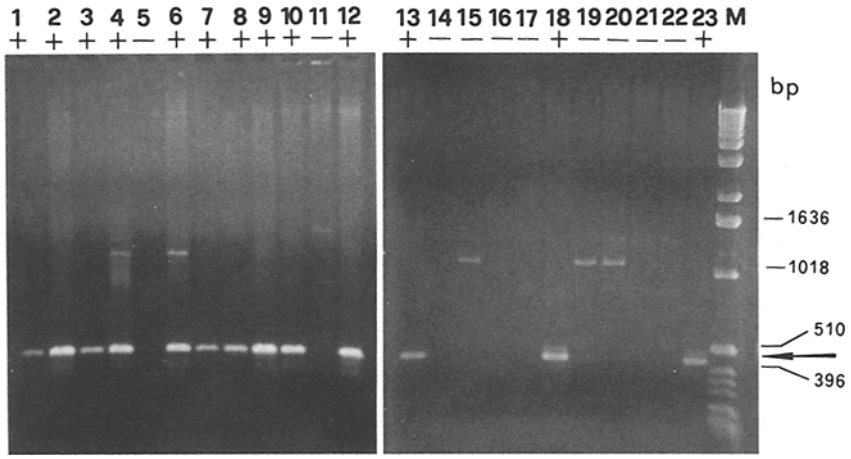


Fig. 4 Primers specific for the 1Dx5 gene were used for PCR reactions on 50 ng of genomic DNA from several bread-wheat cultivars. *1*, Benito; *2*, Conway; *3*, Marquis; *4*, Centauro; *5*, Spada; *6*, Licorno; *7*, Maestra; *8*, Fiocco; *9*, Pegaso; *10*, Solar; *11*, Darius; *12*, Newton a; *13*, Cheyenne; *14*, Chinese Spring; *15*, Salmone; *16*, Melchior; *17*, Newton b; *18*, Hope; *19*, Talent; *20*, MG 7249; *21*, MG 315; *22*, Fiorello; *23*, Columbus. *M*, 1-kb DNA ladder; the molecular weight of the fragments are reported in base pairs (bp). The *arrow* indicates the 450-bp specific to the 1Dx5 gene. +/- indicate the expected presence or absence of the 1Dx5 gene, respectively

rence, 1983; Margiotta et al. 1993), were analyzed. As expected, they did not show the characteristic amplification band of 450-bp (Fig. 4). This analysis also included the cultivar Fiorello which was reported to possess subunit 5 (Pogna et al. 1987) but as with the other allelic variants it did not show any amplification product (Fig. 4, lane 22).

Discussion

The possibility of distinguishing between cultivars possessing the HMW-glutenin subunits 2 or 5 has already been reported at the biochemical level by comparing the relative migration on SDS-PAGE. This method is currently used in breeding programs to select genotypes possessing the 1Dx5 glutenin subunit gene. As alternative to this method we have developed a PCR-based approach for selecting the 1Dx5 gene. The presence of some differences in the nucleotide sequences of the 1Dx2 and 1Dx5 glutenin genes allowed us to develop oligonucleotides which are able to identify the presence of the 1Dx5 glutenin gene in a genotype. One of the nucleotide differences between 1Dx2 and 1Dx5 used to select the oligonucleotides resides in the codon for the cysteine residue at the beginning of the repetitive region. This cysteine is characteristic of subunit 5 and has been implied to play an important role in influencing the gluten properties of flour (Greene et al. 1988). This procedure has thus far accurately confirmed the presence or absence of the 1Dx5 gene in all cultivars tested. It also was not sensitive to a range of DNA concentrations which is known to create problems for PCR in some systems.

The use of PCR analysis with primers specific not only for specific genes, but also for nearly identical alleles of a given gene, represents a useful new tool for genotype analysis. The results of the current report show that PCR analysis can help avoid the misleading interpretation of the results obtained on SDS-PAGE analysis of HMW-glutenin subunits. An example of misinterpretation of results obtained on SDS-PAGE and a demonstration of the effectiveness of the PCR-based approach is represented by the cv Fiorello whose amplification product, obtained using primers specific for the 1Dx5 gene, do not show the 450-bp fragment. This cultivar was, in fact, reported to be a recombinant at the *Gli-D1* locus giving the allelic pair 1Dx5/1Dy12 (Pogna et al. 1987). By analyzing the progeny obtained from crosses between this recombinant and cultivars possessing either the allelic pair 1Dx5/1Dy10 or 1Dx2/1Dy12 it was suggested that the y-type subunits 10 or 12 were the main components responsible for differences in the technological characteristics of flour, while the x-type subunits 2 or 5 would have only minor effects. The recent demonstration that Fiorello possesses a Dx subunit which does not correspond to the typical Dx5 (Lafiandra et al. 1993) confirms the result and the efficiency of the proposed PCR approach.

The use of primers may also help establish relationships among alleles. As more sequence information and PCR results become available new additional alleles can be more accurately characterized. For example, we have shown that 1DX3, 1Dx4, 1Dx2.2, 1Dx2.2*, and 1Dx5* are not likely to be 1Dx5 variants since the 1Dx5 primers do not amplify the expected 450-bp DNA fragment. Further experiments could determine if these additional alleles are more-closely related to the 1Dx2 gene. The accuracy, rapidity and simplicity, as well as the avoidance of toxic substances such as acrylamide, make the proposed PCR-based approach a valid alternative to standard techniques for selecting genotypes possessing the 1Dx5 gene. Moreover, the possibility of using small amounts (about 10 mg) of leaves, roots or endosperm tissue for PCR analysis could allow the screening of hundreds of plants in 1 day and could be useful in breeding programs for a quick and early evaluation of progeny. The selected plants or the remaining embryo-parts of the selected grains can then be grown

and evaluated for other characters in subsequent generations.

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