

SELECTION OF CHILEAN WHEAT GENOTYPES CARRIERS OF THE HMW GLUTENIN ALLELE *Glu-D1 x5* THROUGH POLYMERASE CHAIN REACTION

Selección de genotipos chilenos de trigo portadores del alelo glutenina HMW *Glu-D1 x5* mediante reacción en cadena de la ADN polimerasa

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ABSTRACT

Baking quality is a key factor in the development of improved wheat (*Triticum aestivum* L.) cultivars and it is associated with a specific protein fraction known as High Molecular Weight (HMW) glutenins, although there are other proteins involved in baking quality. Current selection of wheat germplasm with improved baking quality is based on gluten and dough analyses, or the identification of HMW glutenin alleles using electrophoretic procedures. This report presents the deployment of an allele-specific Polymerase Chain Reaction (PCR) system to identify the presence of the gene encoding the *Glu-D1* HMWx5 glutenin, associated with good baking quality. Primers targeting part of its promoter and its 5' extreme were used to differentiate it from its allele *Glu-D1* HMWx2. A 450 pairs of bases (pb) band was amplified in genotypes carrying the allele HMWx5, a size consistent with that expected from GenBank sequence data, whereas no amplification was observed in genotypes carrying the HMWx2 allele. The system was validated on a wide range of unrelated Chilean genotypes and allowed the rapid and unequivocal identification of genotypes possessing the HMWx5 allele with high throughput. The development of this and other systems will allow the development of more effective and focused breeding approaches in wheat.

Key words: plant breeding, wheat, *Triticum aestivum* L., biotechnology, PCR.

RESUMEN

La calidad panadera es un importante elemento para el desarrollo de nuevas variedades de trigo (*Triticum aestivum* L.) y se asocia principalmente con un conjunto de proteínas conocidas como gluteninas de elevado peso molecular (HMW), aun cuando existen otras proteínas relacionadas con la calidad panadera. Los procesos actuales de selección de genotipos con calidad panadera se basan en el contenido de gluten de la masa o la identificación de alelos codificadores de gluteninas HMW mediante electroforesis. Se reporta un método basado en la identificación, mediante la reacción en cadena de la ADN polimerasa (PCR), del alelo codificador de la glutenina HMWx5, la cual se asocia con calidad panadera. Mediante el uso de partidores apropiados se amplificó de forma específica un fragmento del promotor de este gen y parte de su extremo 5'. En aquellos genotipos portadores del gen HMWx5 se amplificó un fragmento de aproximadamente 450 pares de bases (pb) el cual es consistente con el tamaño esperado a partir de su secuencia depositada en la base de datos Genbank. Su alelo HMWx2 no amplificó producto PCR alguno. El sistema fue validado en un grupo de genotipos de trigos de diverso origen y permitió la rápida e inequívoca identificación de genotipos portadores del alelo HMWx5. El desarrollo del sistema y otros análogos permitirá el desarrollo de sistemas de mejoramiento genético de trigo más efectivos y focalizados.

Palabras clave: mejoramiento genético, trigo, *Triticum aestivum* L., biotecnología, PCR.

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops of the world, and its baking quality is an increasingly important trait since it defines the uses and marketability of wheat derived products. Although the protein fraction of wheat seed known as gluten is associated with baking quality, High Molecular Weight (HMW) glutenins are the subunits most closely associated with this trait. These proteins have conserved amino and carboxy domains with Cys residues and a repetitive hydrophobic central domain. The homologous loci *Glu-A*, *Glu-B* and *Glu-D* control the synthesis of HMW glutenins and are found on linkage group 1 of their respective homologous genomes in wheat. Each loci encodes two subunits of different molecular weights, x and y . These subunits present a tight genetic linkage and are frequently reported as the $x+y$ pair. Several independent studies report the close association between the allelic pair HMW $x5+y10$ at the locus *Glu-D1* and improved baking quality, whereas the opposite applies to the allelic pair HMW $x2+y12$ (Shewry *et al.*, 1994; Shewry and Tatham, 1997).

The genetic analysis of wheat is hampered by its large genome size (3.500 Mb, being 1 Mb 1.000.000 pairs of bases) and hexaploid nature (there are three genomes: A, B and D in cultivated wheat). Traditional methods to select wheat segregant lines carrying "good" baking quality alleles are based upon sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) systems or the rheological analysis of advanced breeding lines. However, according to Goldsborough *et al.* (1989) there are known discrepancies between derived molecular weights and their order of migration on SDS-PAGE for some HMW glutenins. Since even medium size wheat breeding programs handle over 10.000 genotypes per year, steady and cost-effective systems based on the identification of genes rather than gene products are required. Once such systems are developed and verified they can be easily upscaled and expanded, taking advantage of Polymerase Chain Reaction (PCR) procedures.

Here we report the deployment of a diagnostic system for the allele *Glu-D1* HMW $x5$, hereafter referred to as HMW $x5$, based upon PCR procedures. Results are discussed from a wheat breeding standpoint.

MATERIALS AND METHODS

Experiments were conducted in the Biotechnology Unit of the Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación Carillanca, Temuco, IX Region, Chile. Cultivars carrying the alleles HMW $x2$ and HMW $x5$, namely Chinese Spring and Dalcahue INIA, were used as control genotypes. Total endosperm protein from wheat grains was extracted and separated using SDS-PAGE according to Hewstone and Hinrichsen (1994). Genomic deoxyribonucleic acid (DNA) was extracted from young, clean leaves according to Campos and Ortega (2001). DNA quantification was conducted with a spectrophotometer (MBC 2000, Perkin Elmer, USA) and its integrity assessed on agarose gels.

The following primers (5'-3') D'Ovidio and Anderson (1994) were custom synthesized at BIOS Chile:

Primer 1: GCCTAGCAACCTTCACAATC

Primer 2: GAAACCTGCTGCGGACAAG

Primer 3: GTTGGCCGCTCGGCTGCCATG

Primer 4: TGGAGAAGTTGGATAGTACC

Optimized PCR conditions were 50 ng of DNA; 300 μ M each dNTP (Promega, USA), 3 mM MgCl₂ (Sigma, USA); 10 pmol each primer and 0,6 U *Taq* DNA polymerase (Promega, USA), in a 25 μ l volume. A 9700 thermocycler (Perkin Elmer, USA) was used and the PCR program included 30 cycles at 94°C for 1 min; 63°C for 45 s and 72°C for 30 s and final extension at 72°C for 5 min. PCR products were soaked at 4°C and separated through electrophoresis. Agarose (Promega, USA) gels (1,5%) were prepared in 1X TAE buffer and electrophoresis was conducted at 100 V. Gels were then stained with Ethidium Bromide (Sigma), digitized and stored. Sequence comparisons were conducted with the ALIGN tool available in PC-GENE.

RESULTS AND DISCUSSION

According to Shewry *et al.* (1992), there is a clear genetic association between baking quality and HMW glutenins allelic composition in wheat. Although there are also HMW loci in genomes A and B, their allelic richness precludes the development of gene-specific detection systems. Since there are only two main reported alleles in the HMW-D locus, detecting the presence or absence of just one allele allows deducing the allelic composition of any given wheat sample.

The alignment of HMW_{x5} (GenBank accession X12928) and HMW_{x2} (GenBank accession X03346) sequences showed extensive conservation at the DNA level (similarity at the nucleotide level reached 98.5%). There were however some differences which allowed the specific amplification of the *Glu-D1* HMW_{x5} over the *Glu-D1* HMW_{x2} allele (Figure 1). The amplification of the *Glu-D1* HMW_{x2} allele was attempted with primers 3 and 4, however a reduced reproducibility was observed (data not shown).

Primers 1 and 2 amplify the HMW_{x5} allele residing on the D genome. Their length (19-20 nucleotides) provides the reliability required and their targets are shown in Figure 1. The optimized PCR mixture

described, i.e., the use of a 25 µl reaction volume and 0.6 units of *Taq* polymerase, is much more cost effective than the method described by D’Ovidio and Anderson (1994), that used a reaction volume of 100 µl and 2.5 units of *Taq* DNA polymerase. This would further simplify its wide application in wheat breeding programmes since *Taq* DNA polymerase is the single most expensive reagent of PCR reactions.

A perfect match was observed between the results of the PCR system and the actual allelic composition of wheat samples deduced from SDS-PAGE (Figure 2A). As shown in Figure 2A, the identification of *Glu-D1* HMW_{x5} carrier genotypes is more straightforward at the gene rather than at the gene product level. Furthermore, in all blind experiments including a wide array of wheat genotypes the PCR system correctly detected the presence of the *Glu-D1* HMW_{x5+y10} pair. *Glu-D1* HMW_{x5+y10} and *Glu-D1* HMW_{x2+y12} alleles are expected to occur in different wheat breeding programmes although at different frequencies. Such a feature would allow the application of the diagnostic system reported here to other wheat breeding programmes and to select carriers of the *Glu-D1* HMW_{x5} allele onto which other desired traits could be introgressed.

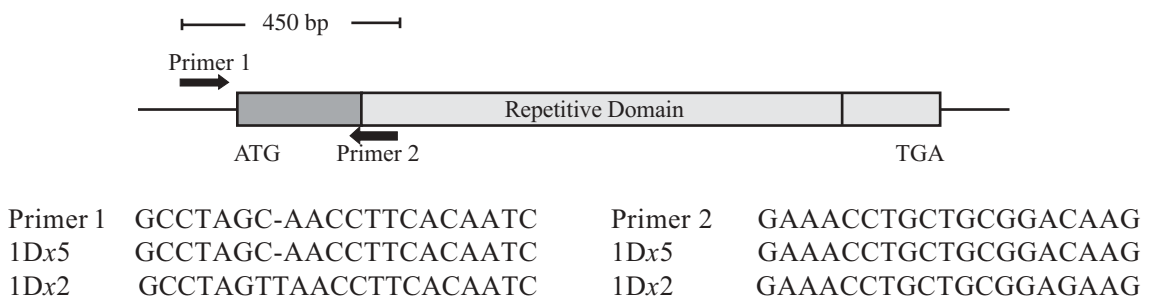


Figure 1. HMW_{x5} allele showing position and primer sequences. A, C, T and G correspond to Adenine, Cytosine, Thymine and Guanine, respectively.

Figura 1. Alelo HMW_{x5}, secuencia y posición de partidores. A, C, T y G corresponden a Adenina, Citosina, Tiamina y Guanina, respectivamente.

The tight genetic linkage existing between subunits x and y has hampered the development of genetic stocks to identify the actual subunit responsible for the baking quality properties related to them. Nevertheless, this is a significant advantage from a diagnostic standpoint, since the detection of either of them suffices to ascertain allelic composition.

Figure 2B presents the results of the routine analysis of wheat genotypes through PCR. Discriminating between DNA samples carrying "good" or "bad" baking quality alleles is straightforward and simple and can be achieved in less than 3 hours with agarose gels. It also eliminates the use of hazardous reagents such as acrylamide.

This diagnostic system shown eliminates inaccuracies due to the visual scoring of SDS-PAGE gels, since the molecular weights of the proteins encoded by alleles $x5$ and $x2$ are very similar (their molecular weight in Daltons deduced from DNA sequence are 89.059 and 90.486 respectively). Also, the mobility of HMW glutenins subunits is not always correlated to their actual molecular weights. Selection systems such as those reported here allow the detection of mutant HMW $x5$ alleles lacking a specific Cys residue, which are associated with reduced baking quality (D'Ovidio and Anderson, 1994). Such mutation cannot be detected with SDS-PAGE systems and would therefore go unnoticed, resulting in mistaken selection decisions. Selection errors can be very expensive for breeding programs in terms of time and money. Although it takes between 12 and 14 years to release a new winter wheat cultivar, they generally last in the market for no more than 5 years on average, mainly due to the loss of genetic tolerance to biotic stresses or the appearance of newer, higher yielding cultivars. Therefore it is necessary for breeding programmes to avoid any selection errors in order to remain efficient and competitive.

A further advantage of this system is the use of any plant tissue (leaves, roots, seeds, stems), whereas conventional SDS-PAGE systems rely upon the availability of seed tissue. Being PCR based it only requires minute amounts of DNA, whereas alternative molecular methods for allele

detection such as Southern blots would require micrograms of DNA. Therefore, it provides more flexibility and hastens the selection process, while reducing the volume of segregant lines reaching more advanced steps of the breeding programme. This system is currently used on a regular basis in the wheat breeding programme based at the Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación Carillanca.

The system reported here relies upon the Mendelian segregation of already existing or introgressed HMW alleles associated with baking quality and their monitoring through PCR. There is an alternative approach to introduce baking quality alleles into wheat, such as transgenesis (Alvarez *et al.*, 2000). However, it may raise unexpected silencing and transgene expression issues derived from the high homology existing between HMW $x2$ and HMW $x5$ genes and the sheer complexity of the wheat genome. Furthermore, such transgenic genotypes may require regulatory approval before their commercialization that may increase their development expenses.

The development of automatic systems for calling PCR amplicons will eliminate the use of agarose gels and further ease the application of this and other diagnostic systems used on a routine basis by wheat breeding programmes. Such research is ongoing and manuscripts are being prepared. Furthermore, alternative systems based upon HMW y alleles are also available which can also be used in selecting wheat genotypes with high baking quality (Ahmad, 2000).

The increasing amount of plant derived Expressed Sequence Tags (ESTs) (Campos, 2002) deposited in databases represents a unique source of protein encoding DNA fragments that could be used to develop assays such as that reported here once their allelic relationships are fully understood. The coordinated effort of molecular geneticists and plant breeders should allow the use of such information to develop new methods of genetic selection directly based on DNA data. Through multiplex PCR it becomes possible to amplify several different alleles per reaction, further reducing selection expenses.

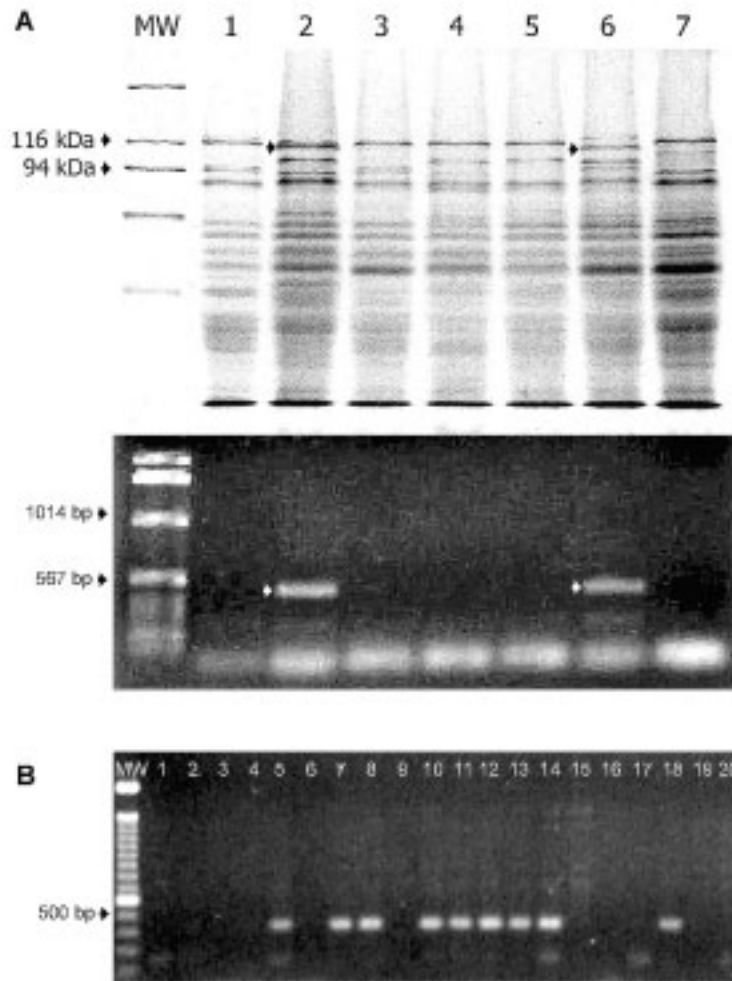


Figure 2. A: Comparison between allelic composition assessed through PAGE-SDS (top panel) and PCR (bottom panel). Genotypes were as follows: 1) Renaico INIA; 2) Kona INIA; 3) Pukem INIA; 4) Pankul INIA; 5) Tukan INIA; 6) Dalcahue INIA and 7) Metrenco INIA. Kona INIA and Dalcahue INIA carry the HMWx5 allele. Black (top panel) and white (bottom panel) arrows correspond to the HMWx5 allele. **B:** Analysis of 19 experimental wheat genotypes. MW corresponds to a 100 bp ladder. Genotypes were as follows: 1) Car 3945; 2) Car 3946; 3) Car 3947; 4) Car 3948; 5) Car 3949; 6) Car 3951; 7) Temu 1032; 8) Temu 1042; 9) Temu 1048; 10) Temu 1049; 11) Temu 1052; 12) Temu 1056; 13) Temu 1067; 14) Temu 1072; 15) Temu 1075; 16) Temu 1076; 17) Temu 1092; 18) Temu 2019; 19) Temu 2025 and 20) Check, Chinese Spring ($x2+y12$).

Figura 2. A: Comparación entre composiciones alélicas definidas mediante PAGE-SDS (sección superior) y PCR (sección inferior). Los genotipos fueron: 1) Renaico INIA; 2) Kona INIA; 3) Pukem INIA; 4) Pankul INIA; 5) Tukan INIA; 6) Dalcahue INIA y 7) Metrenco INIA. Kona INIA y Dalcahue INIA portan el alelo HMWx5. Flechas negras y blancas indican el alelo HMWx5. MW corresponde al marcador de peso molecular 100 pb.

B: Análisis de 19 genotipos experimentales de trigo. Los genotipos fueron: 1) Car 3945; 2) Car 3946; 3) Car 3947; 4) Car 3948; 5) Car 3949; 6) Car 3951; 7) Temu 1032; 8) Temu 1042; 9) Temu 1048; 10) Temu 1049; 11) Temu 1052; 12) Temu 1056; 13) Temu 1067; 14) Temu 1072; 15) Temu 1075; 16) Temu 1076; 17) Temu 1092; 18) Temu 2019; 19) Temu 2025 y 20) Control, Chinese Spring ($x2+y12$). MW corresponde al marcador de peso molecular 100 pb.

CONCLUSIONS

The direct use of DNA information is a valuable tool to identify segregating breeding lines useful for plant breeders.

The system reported here allows the steady identification of wheat breeding lines carrying HMWx5 alleles.

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REFERENCES

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- Ahmad, M. 2000. Molecular marker-assisted selection of HMW glutenin alleles related to wheat bread quality by PCR-generated DNA markers. *Theor. Appl. Genet.* 101:892-896.
- Alvarez, M.L., S. Guelman, N. Halford, S. Lustig, M. Reggiardo, N. Ryabushkina, P. Schewry, J. Stein, and R. Vallejos. 2000. Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theor. Appl. Genet.* 100:319-327.
- Campos, H. 2002. Plant genomics: an overview. *Biol. Res.* 35:385-399.
- Campos, H., and F. Ortega. 2001. Genetic variability of red clover breeding populations as revealed by RAPD molecular markers. *Euphytica* 122:61-67.
- D'Ovidio, R., and O. Anderson. 1994. PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread-making quality. *Theor. Appl. Genet.* 88:759-763.
- Goldsborough, A., N. Bulleid, R. Freedman, and R. Flavell. 1989. Conformational differences between two wheat (*Triticum aestivum* L.) "high molecular weight" glutenin subunits are due to a short region containing six amino-acid differences. *Biochem. J.* 263:837-842.
- Hewstone, N., and P. Hinrichsen. 1994. Composición de subunidades de gluteninas de alto peso molecular de trigos chilenos de pan (*Triticum aestivum* L.). *Agric. Téc. (Chile)* 54:211-218.
- Shewry, P., N. Halford, and A. Tatham. 1992. High molecular weight subunits of wheat glutenin. *J. Cereal Sci.* 15:105-120.
- Shewry, P., M. Miles, and A. Tatham. 1994. The prolamin storage proteins of wheat and related cereals. *Prog. Biophys. Molec. Biol.* 61:37-59.
- Shewry, P., A. Tatham, and P. Lazzeri. 1997. Biotechnology of wheat quality. *J. Sci. Food. Agric.* 73:397-406.