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Biochemical and molecular characterisation of *Glu-1* loci in Argentinean wheat cultivars

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Summary

The high molecular weight glutenin subunit (HMW-GS) composition of a collection of 107 Argentinean bread wheat cultivars was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Allelic variation at the *Glu-1* loci was identified and its frequency calculated. Eleven alleles were detected, three encoded at the *Glu-A1* locus, six at the *Glu-B1* locus and two at the *Glu-D1* locus. A low frequency of the null allele at the *Glu-A1* locus and a high frequency of subunits 5+10 at the *Glu-D1* locus were observed. Reversed phase-high performance liquid chromatography (RP-HPLC) analysis was used to further characterise HMW-GS. Two different types of Bx subunit 8 (named subunits 8 and 8*) were detected, the latter having shorter elution time. Subunit 8* was not identifiable by SDS-PAGE. According to quantification by RP-HPLC analysis, two groups of subunit 7 were observed. One group, with a relatively high proportion of subunit 7 (approximately 39% of the total amount of HMW-GS) appeared in cultivars with allele 7+8* at the *Glu-B1* locus; a second group of subunit 7 (around 24% of the total amount of HMW-GS), was found in alleles 7+8, 7+8* and 7+9. Restriction fragment length polymorphisms (RFLP) analyses of HMW-GS genes were also carried out after digestion of genomic DNA with *Hin*dIII and *Taq*I restriction enzymes. The relationship between DNA fragment size and glutenin subunits, as estimated by electrophoretic mobility in SDS-PAGE, was also examined. The restriction enzyme *Taq*I demonstrated to be a useful tool to detect homozygous plants in selection programs against the *Glu-A1* null allele.

Abbreviations: DMSO – dimethyl sulphoxide; DT – ditelosomic line, DTT – dithiothreitol; HMW-GS – high molecular weight glutenin subunits; LMW-GS – low molecular weight glutenin subunits; RFLP – restriction fragment length polymorphism; RP-HPLC – reversed phase high-performance liquid chromatography; SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA – trifluoroacetic acid

Introduction

Quantitatively, HMW-GS are minor components of the wheat storage proteins but they are key factors in the process of bread-making. These proteins are major determinants of gluten elasticity (Payne et al., 1981a; Branlard & Dadervent, 1985; Dong et al., 1991, MacRitchie, 1992; MacRitchie & Lafiandra, 1997) thus, their genetics, biochemistry and structure have been profusely studied (Payne et al., 1987 and Shewry et al., 1992, for review). They are encoded by the complex *Glu-1* loci situated on the long arm of chromosomes 1A, 1B and 1D (*Glu-A1*, *Glu-B1* and *Glu-D1* locus, respectively), each one displaying extensive allelic variation (Payne & Lawrence, 1983). Each *Glu-1* locus consists of two genes; one encodes a larger x-type subunit, whereas the other encodes a smaller y-type subunit. In hexaploid wheat, six HMW-GS genes

are present, but only those coding for subunits 1Bx, 1Dx and 1Dy are always expressed, 1By and 1Ax subunits are present in some cultivars and 1Ay subunits are not expressed in bread wheat. Nevertheless, a y-type subunit at the *Glu-A1* locus has been reported in Swedish wheat cultivars (Johansson et al., 1993) and in introgressions of diploid wheat into bread wheat (Rogers et al., 1997).

Gene bank collections are assessed based on genetic diversity. A study of these collections is usually based on the identification of distinctive agronomic, physiological and morphological features, but environmental factors may sometimes influence the expression of certain traits. In order to avoid this, biochemical markers such as enzymes, endosperm proteins and DNA have been utilised (Felsenburg et al., 1991; Cooke, 1995; Tahir et al., 1996). For this reason, many collections have been characterised based on HMW-GS. Variation in bread-making quality among cultivars from Great Britain, Spain, Germany, Canada, China, and Japan have been explained by variation in HMW-GS composition (Payne et al., 1987, 1988; Rogers et al., 1989; Lukow et al., 1989, Nakamura, 2000). A quality score assigned to individuals or pairs of HMW-GS was developed in order to systematically assess the bread-making quality of a given cultivar on the basis of its HMW-GS composition (Payne et al., 1987). Other studies have also revealed that variation in the amount of HMW-GS influenced dough properties and bread-making quality (Lawrence et al., 1988; Gupta et al., 1995; Rogers et al., 1997). Furthermore, wheat lines over-expressing specific glutenin subunits showed unusual mixing properties, producing much stronger doughs (Marchylo et al., 1992; Rooke et al., 1999).

Argentina plays an important role in the wheat trade market, with harvest figures fluctuating around 10–12 million tonne and average trade of seven tonne for the last two years (1998–2000). Despite the previous facts, no systematic assessment has been done on its genetic stock using HMW-GS. This study describes the HMW-GS allelic composition of a collection of 107 Argentinean wheat cultivars using SDS-PAGE, RP-HPLC, and RFLPs along with analyses of genetic diversity. Furthermore, quantitative assessments were performed to determine the level of expression of the different subunits using RP-HPLC. The information provided in this study will be valuable for the development of breeding strategies to improve bread-making quality.

Materials and methods

Plant material

Wheat grains used in this study included a total of 107 Argentinean bread cultivars (Tables 1 and 2). These samples were obtained from a germplasm collection maintained at INTA (Instituto Nacional de Tecnologia Agropecuaria) Bordenave and Castelar, Argentina. Chinese Spring ditelosomics 1AS, 1BS and 1DS lines were used to identify the chromosome location of high molecular glutenin genes. Standard wheat cultivars such as Chinese Spring, Gabo, Hartog and Glenlea were also included to identify different alleles at each *Glu-1* locus.

Electrophoresis

The HMW-GS composition of each cultivar was determined by SDS-PAGE using 10% (w/v) acrylamide gels as described by Lawrence and Shepherd (1980). To establish the presence or absence of subunit 2* in cultivars with subunit 2+12 a further analysis was carried out by SDS-PAGE using 5% (w/v) acrylamide gels (Payne et al., 1981b). The nomenclature system used for the HMW-GS was according to Payne and Lawrence (1983).

Quality score

The quality score, according to the *Glu-1* composition, was calculated. This score is based on the relationship between individual HMW-GS and quality, as determined by the SDS-sedimentation test (Payne et al., 1987).

Biotype identification

To obtain some measurement of the homogeneity of the HMW-GS composition of the analysed wheat cultivars, between 10–15 grains were individually tested in each case. The cultivars studied were subdivided into homogeneous and heterogeneous groups, the latter corresponding to those cultivars with more than one *Glu-1* allele for a given locus. Further analyses of heterogeneous cultivars were performed to determine whether those cultivars had biotypes or were just the result of mixture with other material/s. Individual grains of each type of heterogeneous cultivars were analysed by A-PAGE to determine gliadin composition (Tkachuk & Mellish, 1980) and by one-step

Table 1. HMW-GS composition of Argentinean wheat cultivars

Genotype			Quality score	Frequency (%)	Cultivars		Number of cultivars
Glu-A1	*Glu-B1*	*Glu-D1*					
1	7+8	5+10	10	3.26	Cooperación Maipún	Pro INTA Don Alberto	3
					Klein Granador		
1	7+8*	5+10	10	21.74	Bonaerense Pericón	Klein Orión	20
					Buck Candil	Klein Petiso	
					Buck Ñandú	Klein Sendero	
					Buck Ñapucá	Klein Toledo	
					Buck Ombú	Norkin Caburé	
					Buck Palenque	Pampa INTA	
					Buck Pucará	Pro INTA Redomón	
					Dekalb Quebracho	Retacón INTA	
					Klein Centauro	Thomas Catedral	
					Klein Cobre	Victoria INTA	
1	7+8	2+12	8	2.17	Diamante	38 MA	2
1	7+8*	2+12	8	4.35	Labrador	Sinvalocho	4
					Leones	Klein Atlas	
1	17+18	5+10	10	6.52	Buck Patacón	Norkin Pan 70	6
					Buck Yapeyú	Pro INTA Quintal	
					Marcoz Juárez	Saira INTA	
1	7*+9	5+10	9	7.61	Cooperación Millán	Pro INTA Oasis	7
					Dekalb Tala	Pro INTA Piguüé	
					Klein Atalaya	Pro INTA Querandí	
					Pro INTA Guazú		
1	13+16	5+10	10	1.09	Klein Cartucho		1
1	13+16	2+12	8	1.09	Pro INTA Isla Verde		1
2*	7+8	5+10	10	7.61	Buck Cimarrón	Cooperación Bahía	7
					Buck Manantial	Klein Criollo	
					Buck Namuncurá	Pro INTA Puntal	
					Chasicó INTA		
2*	7+8*	5+10	10	8.70	Buck Catriel	Klein Fortín	8
					Buck Fogón	Norkin Churrinche	
					Buck Napostá	Norkin Lider	
					Buck Poncho	Pro INTA Pincén	
2*	7+8	2+12	8	1.09	Bonaerense Pasuco		1
2*	7+8*	2+12	8	2.17	Buck Charrúa		1
2*	7*+9	5+10	10	16.30	Buck Atlántico	Klein Dorado	15
					Buck Cencerro	Klein Dragón	
					Cooperación Cabildo	La Paz INTA	
					Cooperación Calquín	Olaeta Artillero	
					Cooperación Liquen	Precoz Paraná INTA	
					Cooperación Ninihue	Pro INTA Super	
					Don Ernesto	Thomas Aconcagua	
					Klein Cacique		
2*	17+18	5+10	9	10.87	Buck Guaraní	Klein Salado	10
					Buck Mapuche	Norkin T 82	
					Buck Pangaré	Pionero INTA	
					Chaqueño INTA	Surgentes INTA	
					Ciano 67	Thomas Tronador	
2*	6+8	2+12	8	2.17	Cochicó INTA	Thomas Tupungato	2
2*	7*+9	5+10	7	2.17	Granero INTA	Tucumano Granivo	2
2*	13+16	5+10	10	1.09	Oncativo INTA		1
null	7+8	5+10	8	1.09	Pergamino Gaboto		1

Table 2. HMW-GS composition of biotypes for HMW-GS in Argentinean wheat cultivars

Cultivars	Loci			Biotype	Frequency (%)
	Glu-A	*Glu-B1*	*Glu-D1*		
Bordenave Puán	2*	7+8*	5+10	A	78
	1	7+8*	5+10	B	22
Buck Bagual	2*	7+8	5+10	A	73
	2*	7*+9	5+10	B	27
Buck Pampero	1	7*+9	5+10	A	83
	1	7*+9	2+12	B	17
Cruz Alta	2*	7*+8	5+10	A	51
	1	7*+8	5+10	B	49
Dekalb Chañar	1	7*+9	5+10	A	75
	1	7+8	5+10	B	25
Dekalb Lapacho	1	7+8*	5+10	A	80
	1	7*+9	5+10	B	20
Klein Chamaco	1	7+8	5+10	A	86
	1	17+18	5+10	B	14
Klein Impacto	2*	7+8	5+10	A	67
	2*	7*+9	5+10	B	33
Klein Rendidor	1	7+8*	5+10	A	75
	1	7+8*	2+12	B	25
Klein 32	null	7*+8	2+12	A	80
	null	13+16	2+12	B	20
Las Rosas	1	17+18	5+10	A	67
	2*	17+18	5+10	B	33
Norkin Irupé	1	17+18	5+10	A	88
	1	7*+9	5+10	B	12
Pro INTA Federal	1	7*+9	5+10	A	85
	1	7+8	5+10	B	15
Pro INTA Imperial	1	7*+9	5+10	A	56
	1	7+8	5+10	B	44
San Agustín	1	17+18	5+10	A	53
	1	7+8*	5+10	B	47

one-dimensional SDS-PAGE to assess LMW-GS composition (Gupta & MacRitchie, 1991).

Reversed-phase high performance liquid chromatography

HMW-GS were extracted from a pellet remaining after 25mg of wholemeal were sequentially treated with 70% ethanol (1 mL) and 50% propan-1-ol to solubilise monomeric protein. The residue was then treated with 1 mL of a buffer containing 50% propan-1-ol, 2 M urea, 0.2 M Tris, pH 6.6 to which 1% of DTT was added. The samples were extracted for 1 h at 60 °C in a water bath. This was followed by the addition of 10 μL of 4-vinylpyridine to the samples, which remained in the water bath at 60 °C for an extra 15 minutes. Following centrifugation and filtration through 0.45 μm PVDF filters, samples were ready for injection. Supernatants consisted of reduced and alkylated polymeric protein (mainly HMW-GS and LMW-GS). A Vydac C18 column (Vydac, Hesperia, California) connected to a Waters HPLC system series 600 (Water™717 plus Auto-sampler) was used for analyses. A gradient of aqueous ACN (water and ACN contained 0.07% and 0.05% TFA acid, respectively) at a flow rate of 1 mL/min was used. Integration of chromatograms was performed using Milleniun v. 2.1 software. Subunits 8 and 8* were identified according to their elution times (Marchylo et al., 1992; Margiotta et al., 1993). Quan-

tification of HMW-GS by RP-HPLC was carried out averaging two replicates of each sample.

Genetic diversity

The overall frequencies of alleles at the three *Glu-1* loci were considered in those cultivars with homogeneous composition of HMW-GS. Genetic diversity was calculated using the modified Nei's index (van Hintum & Elings, 1991) using the formula:

$$H = \frac{N}{N-1} \frac{\sum_j [1 - \sum_i (p_{ij}^2)]}{N_j}$$

Where N is the number of cultivars, p_{ij} is the frequency of the allele $_i$ at the locus $_j$ and N_j is the number of loci. The value obtained for the Argentinean collection was also compared with those calculated for other wheat collections.

DNA extraction and southern analyses

Genomic DNA was extracted from leaves of single plants of the cultivars listed in Table 1, following the procedure of Dvorak et al. (1988). Ditelosomic lines for group 1 chromosomes of Chinese Spring were also included as controls to identify the chromosomal location of each fragment. DNA was digested with *Hin*dIII and *Taq*I restriction enzymes, fractionated on 1% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham) following standard procedures. Southern hybridisation was performed as described by Dubcovsky et al. (1994). After hybridisation, the membranes were washed in 2 × sodium citrate buffer (SSC) at 65 °C for 30 min and 1 × SSC at 65 °C for 30 min and a final wash of 0.5 × SSC at 65 °C for 30 min. The clone pDY10A/KS of the high molecular weight glutenin gene described by Anderson et al. (1989) was used in this study.

Results and discussion

Glu-1 *alleles and quality score*

Eleven different alleles were identified among the 107 wheat samples. Three alleles at the *Glu-A1* locus (1, 2* and null phenotype), six alleles at the *Glu-B1* locus (6+8, 7+8, 7+8*, 7+9, 17+18 and 13+16) and two at the *Glu-D1* locus (5+10 and 2+12) were determined. Argentinean wheat cultivars were divided in 18 different groups according to their HMW-GS composition (Table 1). The HMW-GS composition 1, 7+8*, 5+10 was the most frequently found (22%) in the analysed cultivars. At the *Glu-A1* locus, subunits 1 (46.7%) or 2* (52.2%) were present in more than 98% of the Argentinean cultivars. Only two wheat cultivars (Klein 32 and Pergamino Gaboto) had the null form (1.1%), which does not encode for a glutenin subunit. These results are quite different to those observed in Japanese cultivars where allele c is present in 86.8% of the cultivars (Nakamura et al., 1999) or compared to the 36% reported for the world collection analysed by Morgunov et al. (1993).

Larger allelic variation was observed at the *Glu-B1* locus (6+8=2.2%; 7+8=15,2%; 7+8*=35,9%, 7*+9=26,1%, 13+16=3.3% and 17+18=17,4%), although this variability was lower than that previously reported in others countries such as U.K., Spain and Italy (Payne et al., 1987; 1988; Pogna et al., 1989). Two types of subunit 7 were previously identified based on slight differences in electrophoretic mobility (Marchylo et al., 1992). One type, with electrophoretic mobility similar to Chinese Spring, was identified as 7. A second type, with slightly faster mobility than subunit 7, was named 7*. The differences in electrophoretic mobility are due to small differences in their primary structure. Subunit 7* was observed combined with subunit 8 and 9, while subunit 7 was only present in combination with subunit 8. Thus, three different alleles were detected: 7+8, 7*+8 and 7*+9 (Marchylo et al., 1992). In our study, subunit 7* was always present with subunit 9 and it is likely to be the same that previously was considered as 7+9 *Glu-B1* c allele reported in cultivar Bezostaya 1. The combination of subunit 7*+8, designated *Glu-B1 u*, was not detected in the present study. In our collection, we have found that 71 out of 92 cultivars comprised either subunit 7 or 7*. At the *Glu-D1* locus, a high frequency of subunit 5+10 was observed, with this allele representing 88% of the analysed accessions. Three possible factors could affect the distribution of alleles at each *Glu-1* locus: (a) linkage to genes of adaptive value, resulting in the selection of particular alleles for specific areas; (b) the use of a particular parental gene pool; (c) *Glu-1* alleles might be influenced by selection pressure towards specific quality properties such as bread-making quality. It appears that the third option is likely to be the cause of the distribution of the alleles at the *Glu-1* loci in this collection. Since hexaploid wheat in Argentina is basically used in the bread-making industry, those alleles related with good quality have been observed in higher frequency. This particular distribution of alleles has resulted in the high quality score (9.36) detected

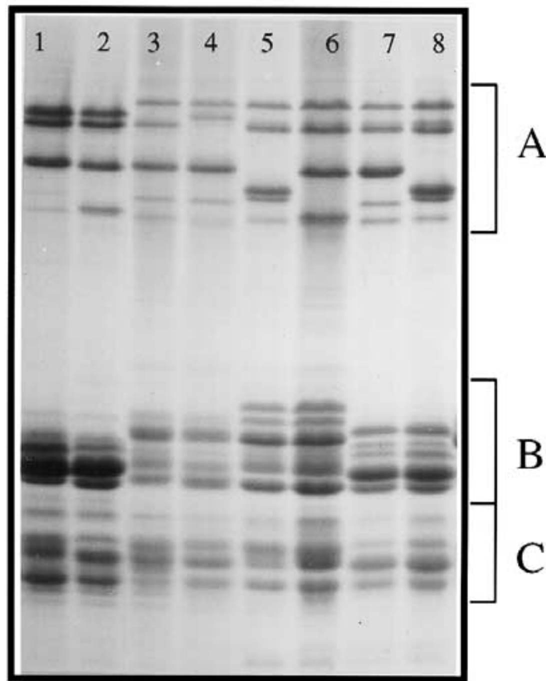

Figure 1. One-step one-dimensional SDS-PAGE of HMW- and LMW-GS of some Argentinean wheat cultivars with different biotypes for HMW-GS. (1–2) Klein Impacto, (3–4) Klein Rendidor, (5–6) Norkin Irupé and (7–8) San Agustín INTA.

in Argentinean cultivars (Table 1). A similar finding is reported by Payne (1987), with Argentina's score (9.5) ranking on top of a list that included collections from other wheat-producing countries. It is likely that breeding selection based on quality related parameters, such as those obtained using the Chopin Alveograph and bread-making test, could be responsible for the higher incidence of HMW-GS alleles which confer good quality. Nevertheless, several studies indicated that HMW-GS were only part of the source of variation in dough-quality correlation. Therefore, an improvement of this correlation could be attained by considering other gluten proteins such as LMW-GS and gliadin, in the quality-score (MacRitchie et al., 1990).

Biotype identification

Out of 107 cultivars screened, 92 were homogeneous for HMW-GS composition (Table 1). Fifteen cultivars were identified to possess two different alleles at a particular *Glu-1* locus (Table 2). A similar level of heterogeneity was also observed in cultivars of other countries, like Australia, Canada and Italy (Lawrence, 1986; Ng et al., 1989; Pogna et al., 1989). Grains from those cultivars considered heterogeneous were further analysed to determine if they could be considered as biotypes of the same cultivar. Analyses of gliadins (results not shown) and LMW-GS (Figure 1) have indicated that the endosperm protein composition was identical. Therefore, we considered them as biotypes for HMW-GS composition. These biotypes could be used for further analyse the effects of particular alleles on bread-making quality. For example, the allele 13+16 has been associated with good quality (Payne et al., 1987; Uhlen, 1990) but due to its narrow distribution in world collections, not much research attention has been given to it, particularly in aspects related with its effect on dough functional properties. Therefore, biotypes found in cv. Klein 32 for the *Glu-B1* (7+8 or 13+16) would be valuable for assessing the effect on quality provided by the 13+16 allele.

According to Cooke (1995), heterogeneity for HMW-GS composition in wheat collections varies from 4% (Zhong-Hu et al., 1992) to 24%. Our results (14%) correspond to a reasonable level of heterogeneity, and match those obtained by Graybosch et al. (1994) for a collection of 100 winter wheat cultivars. An increase in the uniformity of the HMW-GS composition can be expected in the near future due to broader use of SDS-PAGE for screening of HMW-GS, in wheat breeding programs.

RP-HPLC

HMW-GS have been studied in more detail by means of RP-HPLC (Marchylo et al., 1989; Sutton, 1991; Margiotta et al., 1993). This method separates proteins according to their surface hydrophobicities, thus complementing information provided by electrophoresis, which separates proteins according to size and/or charge differences. RP-HPLC has been reported to be a helpful approach to identify alleles of HMW-GS at the *Glu-A1* locus (e.g. to identify subunit 2* when subunits 2 +12 are present) but particularly to differentiate allelic variants at the *Glu-B1* locus. This technique is also appropriate to quantify HMW-GS. Typical profiles of RP-HPLC representing different HMW-GS composition are shown in Figure 2. In a RP-HPLC separation of reduced and alkylated wheat polymeric protein, the first to elute, due to its low surface hydrophobicity, is subunit Dy followed by subunits By, Dx, Bx and Ax. In some cases, subunit By elutes after subunit Dx (e.g. in cv. Chinese Spring). Two types of subunit 8 were previously reported (Marchylo et al.,

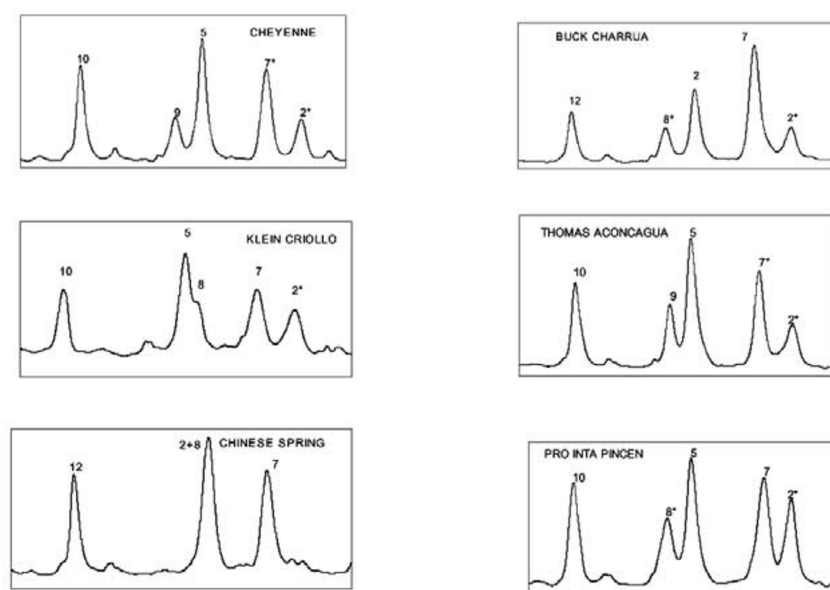

Figure 2. RP-HPLC profile of HMW-GS from Argentinean wheat cultivars with different *Glu-B1* alleles. Controls: Cheyenne and Chinese Spring.

1992; Margiotta et al., 1993). These subunits could only be identified by RP-HPLC. The more hydrophilic is named subunit 8*, whilst the less hydrophilic is named 8 (Figure 2). In this collection 33 cultivars have been identified with subunit 8* as part of the 7+8* allele. The allele *Glu-B1ak* (7*+8*) was not detected in this collection.

Based on quantitative measurements using RP-HPLC, two different groups of subunit 7 have also been reported (Marchylo et al., 1992; Lukow et al., 1992; D'Ovidio et al., 1997). They differed in the proportion of subunit 7 relative to the total amount of HMW-GS, with values of approximately 27% and 41% for the normal and over-expressing, respectively. In this study, one group of cultivars that included all the subunits 7* and some subunits 7, represented around 24% of the total amount of HMW-GS. A second group comprised those cvs. with a higher proportion of subunit 7 representing around 39% of the total amount of HMW-GS (Table 3). In the latter case, the cultivars were identified as over-expressing subunit 7. All cultivars with subunit 7 showing over-expression were present with subunit 8*. The different relative proportion of protein expressed in each case is important, since functional properties such as mixing time and peak resistance (estimated by Mixograph studies) have been related with the total amount of HMW-GS present in a given cultivar. Those cultivars with over-expression of subunit 7 in Canadian cultivars had stronger dough compared with those normally expressing subunit 7 (Marchylo et al., 1992). This increase in dough strength could also be due to an overall increase in the total amount of polymeric protein. A highly significant relationship between total amount of polymeric protein and dough strength has been previously reported (MacRitchie, 1992; MacRitchie & Lafiandra, 1997).

Genetic diversity

The catalogue of the *Glu-1* alleles published by Payne and Lawrence (1983) reports three alleles at the *Glu-A1* locus, eleven at the *Glu-B1* locus and seven at the *Glu-D1* locus. More alleles have been reported later, increasing the number of alleles mostly at the *Glu-B1* locus (Lawrence, 1986; Pogna et al., 1990). In the present study only eleven alleles at the *Glu-1* loci were detected, reflecting a narrow genetic base for this material. It is noteworthy that in this case, six alleles were identified at the *Glu-B1* locus based on RP-HPLC analyses. On the other hand, most of the wheat collections were evaluated only by SDS-PAGE and no differentiation for subunits 8 was considered. Therefore, for the calculation of genetic diversity of this collection, only one allele was considered (7+8).

The high frequency of some alleles indicates that, although variability exists, a few genotypes represent a

Table 3. Quantitative analysis of HMW-GS 7 in Argentinean wheat cultivars

Cultivars	*Glu-1* loci			% subunit7
Bonaerense Pericón	1	7+8*	5+10	38.12
Buck Candil	1	7+8*	5+10	40.20
Buck Catriel	2*	7+8*	5+10	39.24
Buck Charrúa	2*	7+8*	2+12	40.95
Buck Fogón	2*	7+8*	5+10	38.51
Buck Ñandú	1	7+8*	5+10	39.66
Buck Ñapucá	1	7+8*	5+10	39.98
Buck Ombú	1	7+8*	5+10	39.43
Buck Palenque	1	7+8*	5+10	38.25
Buck Poncho	2*	7+8*	5+10	41.37
Buck Pucará	1	7+8*	5+10	37.29
Dekalb Quebracho	1	7+8*	5+10	37.83
Klein Atlas	1	7+8*	2+12	36.00
Klein Centauro	1	7+8*	5+10	43.18
Klein Cobre	1	7+8*	5+10	38.25
Klein Fortín	2*	7+8*	5+10	38.86
Klein Orión	1	7+8*	5+10	37.31
Klein Petiso	1	7+8*	5+10	40.30
Klein Sendero	1	7+8*	5+10	35.83
Klein Toledo	1	7+8*	5+10	37.85
Labrador	1	7+8*	2+12	41.42
Leones	1	7+8*	2+12	40.58
Norkin Caburé	1	7+8*	5+10	36.00
Norkin Lider	2*	7+8*	5+10	35.53
Pampa INTA	1	7+8*	5+10	38.36
Pro INTA Redomón	1	7+8*	5+10	40.09
Retacón INTA	1	7+8*	5+10	41.48
Sinvalocho	1	7+8*	2+12	36.60
Thomas Catedral	1	7+8*	5+10	41.37
Victoria INTA	1	7+8*	5+10	37.72
Average				38.91

Table 3. Continued

Cultivars	*Glu-1* loci			% subunit7
Norkín Churriche	2*	7+8*	5+10	22.52
Pro INTA Pincén	2*	7+8*	5+10	23.00
Buck Napostá	2*	7+8*	5+10	24.49
38MA	1	7+8	2+12	18.53
Bonaerense Pasuco	2*	7+8	2+12	25.64
Buck Atlántico	2*	7*+9	5+10	26.27
Buck Cencerro	2*	7*+9	5+10	28.00
Buck Cimarrón	2*	7+8	5+10	24.25
Buck Manantial	2*	7+8	5+10	23.81
Buck Namuncurá	2*	7+8	5+10	24.22
Chasicó INTA	2*	7+8	5+10	24.22
Cooperación Bahia	2*	7+8	5+10	26.95
Cooperación Cabildo	2*	7*+9	5+10	24.22
Cooperación Calquín	2*	7*+9	5+10	25.00
Cooperación Liquén	2*	7*+9	5+10	27.28
Cooperación Maipún	1	7+8	5+10	26.74
Cooperación Millán	1	7*+9	5+10	25.40
Cooperación Nanihue	2*	7*+9	5+10	19.56
Dekalb Tala	1	7*+9	5+10	23.00
Diamante	1	7+8	2+12	22.00
Don Ernesto	2*	7*+9	5+10	22.87
Granero INTA	2*	7*+9	2+12	27.28
Klein Atalaya	1	7*+9	5+10	22.87
Klein Cacique	2*	7*+9	5+10	25.45
Klein Criollo	2*	7+8	5+10	25.36
Klein Dorado	2*	7*+9	5+10	26.74
Klein Dragón	2*	7*+9	5+10	24.34
Klein Granador	1	7+8	5+10	23.27
Olaeta Artillero	2*	7*+9	5+10	25.76
Precoz Paraná INTA	2*	7*+9	5+10	26.11
Pro INTA Don Alberto	1	7+8	5+10	25.45
Pro INTA Guazú	1	7*+9	5+10	24.78
Pro INTA Oasis	1	7*+9	5+10	25.00
Pro INTA Pigüé	1	7*+9	5+10	25.94
Pro INTA Puntal	2*	7+8	5+10	25.09
Pro INTA Querandí	1	7*+9	5+10	24.24
Pro INTA Súper	2*	7*+9	5+10	22.43
Thomas Aconcagua	2*	7*+9	5+10	22.43
Tucumano Granivo	2*	7*+9	2+12	23.65
Pergamino Gaboto	null	7*+8	5+10	nd
Average				24.46
Controls				
Glenlea (over-expressing)				38.88
Cheyenne (normal expressing)				23.94

high percentage of the population. This is particularly true in this collection, where an unequal distribution of alleles is observed with some of them being rare (null allele at the *Glu-A1*) and some others being widely distributed (5+10 at the *Glu-D1*). The analysis of genetic diversity based on HMW-GS composition of the 92 homogeneous cultivars showed lower value (0.45) for Argentina, than those observed in wheat cultivars of other collections such as Greece (0.68), Spain (0.67), France (0.62), China (0.60), USA (0.59), Australia (0.58) and similar to Russia (0.46) (Cook et al., 1995).

An extensive genetic diversity study based on microsatellite and AFLP markers has recently shown that the Argentinean bread wheat germplasm has main-

tained a relatively high and constant level of genetic diversity during the last half century (Manifesto et al., 2001). This result indicates that the observed reduction in diversity associated with the HMW-GS composition is probably the result of strong selection pressure for good bread-making quality at these loci, and that this is not a representative estimate of the diversity of the Argentinean wheat germplasm. Increasing diversity of the Argentinean HMW-GS composition would be worthwhile, in order to produce new cultivars for alternative end-use products (e.g. noodles, cookies).

Restriction fragment length polymorphism

Restriction fragment length polymorphisms (RFLP) were used to identify genetic variation in HMW-GS among wheat cultivars as used previously by other authors (Harberd et al., 1986; Reddy & Appels, 1993). The coding regions of the HMW-GS genes contain a variable central repetitive region flanked by shorter non-repetitive regions corresponding to the N- and C-terminal regions of the HMW-GS (Thompson et al., 1985). Restriction enzymes *Hin*d III and *Taq* I generally cut at both sizes of the repetitive region generating useful allelic variation.

The determination of the relationship between restriction fragments and alleles is now facilitated by the availability of numerous HMW-GS genomic sequences including alleles Ax1 (X61009), Ax2* (X13928), AxNull (AF145590), Ay2* (X03042), Bx7 (X13927), Bx17 (Reddy & Appels, 1993), By9 (X61026), Dx2 (X03346), Dx5 (X12928), Dy10 (X12929), and Dy12 (X03041). In many cases, these sequences were long enough to predict the expected sizes of the RFLP fragments. The assignment of alleles to RFLP bands was also confirmed by the use of ditelosomic lines (Figure 3), and the association between hybridisation patterns of HMW-GS genes and SDS-PAGE HMW-GS protein patterns in the Argentinean germplasm. A diagrammatic representation of fragments corresponding to each *Glu-1* locus is shown in Figure 4. The sizes of the RFLP fragments attributed to each gene are shown in Table 4.

All HMW-GS sequences mentioned above show a conserved *Hin*d III site at the 5' boundary between the end of the N-terminal region and the beginning of the central repetitive domain (359–383 bp downstream from the start codon in the x-type subunits and 428 bp in the y-type subunits). These eleven sequences have a second *Hin*d III site in the 3' region just beyond the end of the C-terminal region, which determines *Hin*d III fragments ranging from 2020 to 2246 bp for the x-type subunits and from 2000 to 800 bp for the y-type subunits. (Figure 3a and Table 4).

A relationship between sizes of *Hin*d III RFLP bands and the size of the protein subunit was observed between some subunits from the *Glu-A1* and *Glu-B1* locus confirming previous results in bread and durum wheats (Harberd et al., 1986; Margiotta et al., 1993), and *Triticum tauschii* (Mackie et al., 1996). For example, relative sizes of RFLP fragments estimated from sequence data and SDS-PAGE bands were consistent for subunits Ax1 (2246 bp) and Ax2* subunit (2201 bp), Bx7 (2139 bp) and Bx17 (2020 bp), and By8 (1900 bp) and By9 (1817 bp). However, Dx5 (2264 bp) and Dx2 (2261 bp) have similar *Hin*d III fragment lengths but different SDS-PAGE mobility. In addition, Dy10 *Hin*d III fragment (1647 bp) is smaller than the Dy12 fragment (1683 bp), but has a protein with a slower relative mobility in SDS-PAGE, indicating that the size of the repetitive domain is not the only factor affecting relative migration in SDS-PAGE gels.

RFLP fragments obtained when DNAs from the same cultivars were digested with the restriction enzyme *Taq*I varied between 0.9 and 3.4 Kb. The x-type subunits at the *Glu-A1* locus Ax1 and Ax-null alleles showed no size variation with *Taq* I (3.4 kb), but the Ax2* fragment was slightly smaller (3.3 kb). The two smallest *Taq* I fragments (1.3 and 9 kb) were assigned to the *Glu-A1* locus using ditelosomic lines (Figure 3b). These two y-type subunits correspond to the silent *Glu-A1*-null allele, associated with poor bread-making quality. The 1.3 and 0.9-kb *Taq* I fragments were also present in Argentinean cultivars Klein 32 (biotype 7+8) and Pergamino Gaboto, the only two cultivars in this collection with the null allele at the *Glu-A1* locus (Figure 3b). These two bands are allelic to a 2-kb band present in all cultivars with the Ay1 and Ay2* (expected *Taq* I fragment = 2059bp) alleles. The polymorphic 1.3–0.9-kb and 2.0 kb bands can be used as co-dominant markers in breeding programs aimed to replace the Ay-null allele by the Ay1 or Ay2* in segregating populations. Plants homozygous for the 1 or 2* alleles can only be differentiated from heterozygous plants carrying the null allele, on SDS-PAGE by band intensity. This differentiation is easier with the co-dominant RFLP marker. A third Ay allele was observed in cultivars 38MA, Buck Atlántico, Cooperación Maipún, Klein Petiso, Klein Rendidior, Klein Toledo and Sinvalocho after digestion with *Taq* I. These cultivars did not show the 1.3–0.9-kb nor the 2.0 kb bands, but showed double intensity in the By8

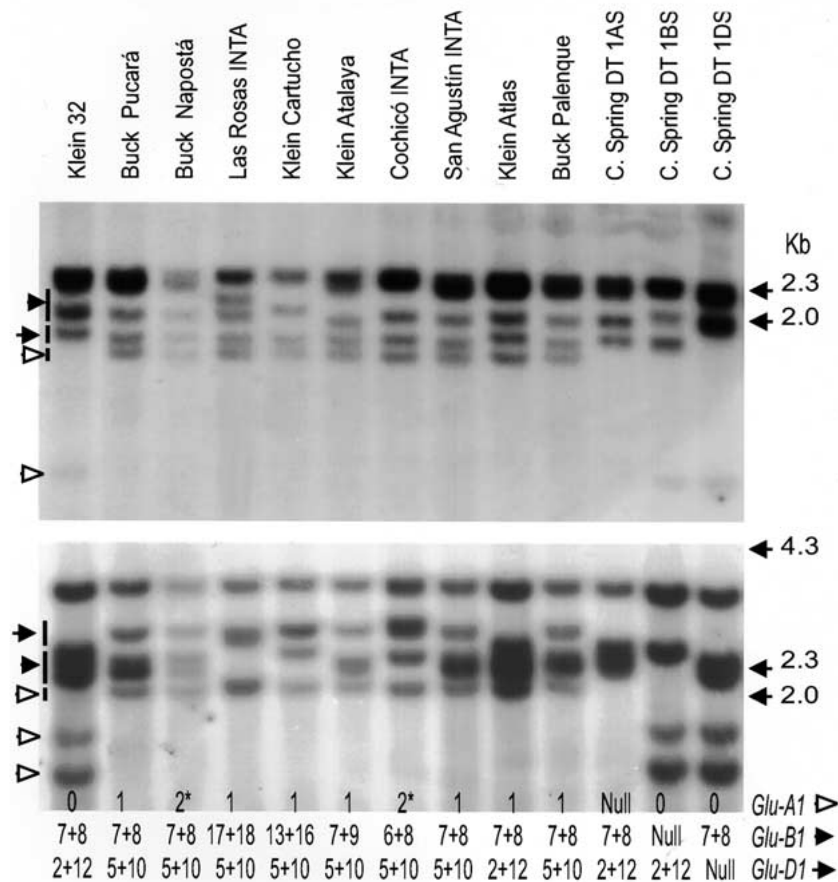

Figure 3. Hybridisation of *Hin*dIII (a) and *Taq*I (b) digested DNAs of Argentinean wheat cultivars (From left to right: cultivars Klein 32, Buck Pucará, Buck Napostá, Las Rosas INTA, Klein Cartucho, Klein Atalaya, Cochicó INTA, San Agustín INTA (biotype 7+8), Klein Atlas, and Buck Palenque) and Chinese Spring ditelosomic lines (DT-1AS, DT-1BS and DT-1DS).

(1900 bp) fragment. The old cultivar 38MA is present in the pedigree of the other six cultivars carrying this new Ay allele and is the most likely source in this Argentinean germplasm.

At the *Glu-B1* locus, two fragments with small size differences were attributed to subunits B7x and B8y (2.2 and 2.3 kb). Variation between fragments corresponding to glutenin subunits genes of the *Glu-B1* locus were observed (Figure 3b). At the *Glu-D1* locus, two large *Taq* I fragments of similar size (3.5kb) were associated with the x-type subunits Dx5 and Dx2. These two fragments cannot be distinguished from the Ax subunits (3.4kb) in Figure 3b. HMW-GS Dy10 (2.8 kb) and Dy12 (2.6 kb) were easier to differentiate in the *Taq* I digestions than in the *Hin*d III digestions.

The diagrammatic representation of the *Taq* I fragments presented in Figure 4b shows differences to that suggested by Reddy & Appels (1993). Prediction of fragment sizes based on current sequence information suggest that the bands indicated as 10 and 12 (Reddy & Appels, 1993) correspond to alleles Ay1 and Ay2* (expected *Taq* I fragment = 2059bp). In addition, bands 17 and 18 (Reddy & Appels, 1993) are inverted (expected *Taq* I fragment Bx17= 2146bp), and bands indicated as 2 and 5 are actually Dy10 (expected *Taq* I fragment >2417bp) and Dy12 (expected *Taq* I fragment = 2568 bp).

Conclusions

The biochemical and molecular characterisation accomplished in this study provided detailed information about the composition of the *Glu-1* loci in Argentinean wheat cultivars. RP-HPLC and RFLP represents a powerful tool to characterise and identify allelic variants at the *Glu-1* loci. As a main feature, this collection

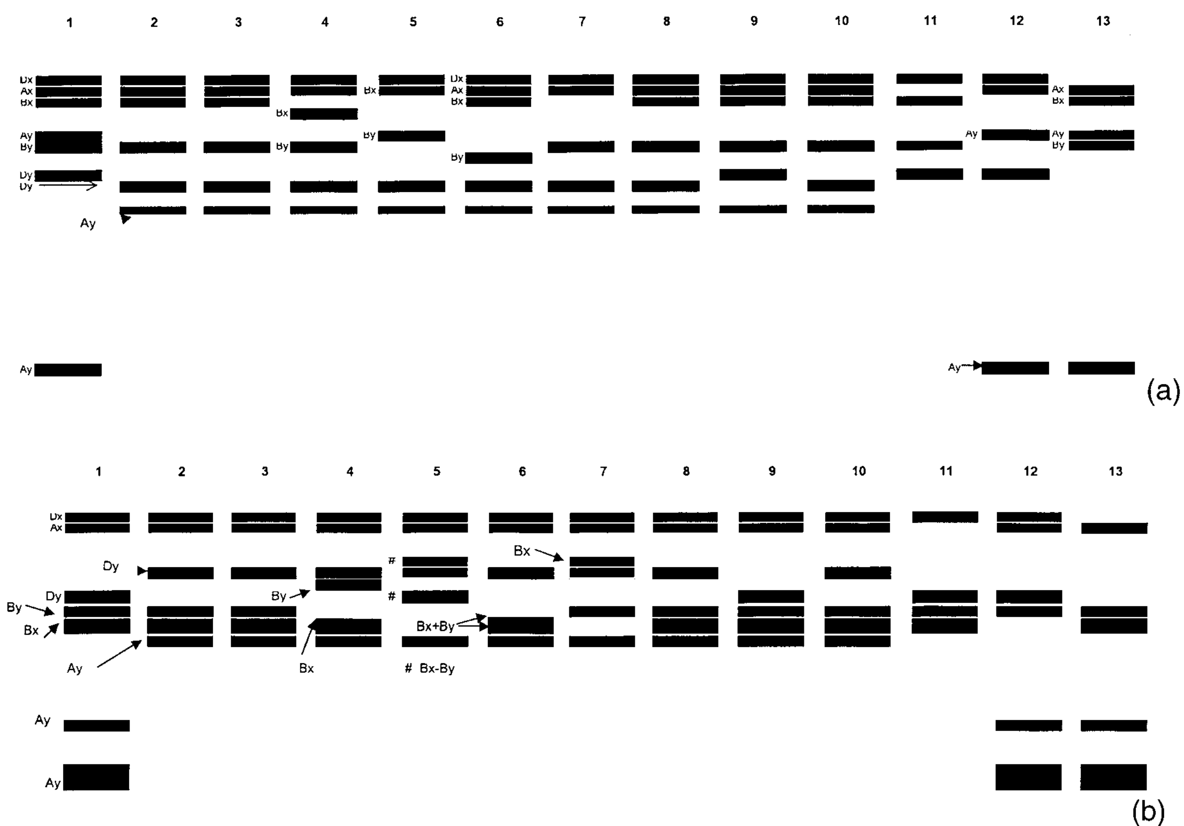

Figure 4. Diagrammatic representation of restriction enzymes fragments (*Hin*dIII: a and *Taq*I: b) hybridising to the HMW-glutenin cDNA (From left to right: cultivars Klein 32, Buck Pucará, Buck Napostá, Las Rosas INTA, Klein Cartucho, Klein Atalaya, Cochicó INTA, San Agustín INTA (biotype 7+8), Klein Atlas, and Buck Palenque) and Chinese Spring ditelosomic lines (DT-1AS, DT-1BS and DT-1DS).

Table 4. Molecular sizes of restriction fragment corresponding to the HMW-GS genes present in Argentinean wheat cultivars. Sizes in Bold were confirmed from sequence information

Restriction enzymes	*Hind*III		*Taq* I	
Glutenin subunits	x-type	y-type	x-type	y-type
Glu-A1				
1	**2.2**	1.5	3.4	2.0
2*	**2.2**	**1.5**	**3.3**	**2.0**
null	2.2	1.9+0.80	3.4	1.3+0.9
Glu-B1				
6+8	2.4	1.9	3.0	2.4
7+8	**2.1**	1.9	**2.3**	2.4
7+9	**2.1**	**1.8**	2.3	2.3
13+16	1.9	1.9	2.9*	2.5*
17+18	**2.0**	1.9	**2.1**	2.7
Glu-D1				
2+12	**2.3**	**1.7**	3.5	**2.6**
5+10	**2.3**	**1.6**	3.5	2.9

* x-y correspondence has not been confirmed.

showed high quality scores based on HMW-GS. A particular distribution of alleles was also noted, with high frequency of subunit 5+10 at the *Glu-D1* locus and low frequency of null alleles at the *Glu-A1* locus. Overexpressed subunit 7 was also found in high frequency. This information could be used by wheat breeders to select appropriate sources of specific genes related with end-product quality.

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