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Effect of allelic variation at the *Glu-3/Gli-1* loci on breadmaking quality parameters in hexaploid wheat (*Triticum aestivum* L.)

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ABSTRACT

Low molecular weight glutenin subunits (LMW-GS) encoded by the *Glu-3* loci are known to contribute to wheat breadmaking quality. However, the specific effect of individual *Glu-3* alleles is not well understood due to their complex protein banding patterns in SDS-PAGE and tight linkage with gliadins at the *Gli-1* locus. Using DNA markers and a backcross program, we developed a set of nine near isogenic lines (NILs) including different *Glu-A3/Gli-A1* or *Glu-B3/Gli-B1* alleles in the genetic background of the Argentine variety ProINTA Imperial. The nine NILs and the control were evaluated in three different field trials in Argentina. Significant genotype-by-environment interactions were detected for most quality parameters indicating that the effects of the *Glu-3/Gli-1* alleles are modulated by environmental differences. None of the NILs showed differences in total flour protein content, but relative changes in the abundance of particular classes of proteins cannot be ruled out. On average, the *Glu-A3f*, *Glu-B3b*, *Glu-B3g* and *Glu-B3i*$_{Man}$ alleles were associated with the highest values in gluten strength-related parameters, while *Glu-A3e*, *Glu-B3a* and *Glu-B3i*$_{Chu}$ were consistently associated with weak gluten and low quality values. The value of different *Glu-3/Gli-1* allele combinations to improve breadmaking quality is discussed.

1. Introduction

Wheat (*Triticum aestivum* L. em Thell) is an important crop in most temperate regions of the world due not only to its yield potential and plasticity, but also to the viscoelastic properties of its grain storage proteins (gluten) that can be processed into a large diversity of food products. Glutenins and gliadins, the polymeric and monomeric fractions of the gluten complex, respectively, are the main determinants of its viscoelastic properties. Glutenins are usually classified into high-molecular-weight (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) based on their different mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). HMW-GS are encoded by genes at the *Glu-1* loci, located on the long arm of homoeologous chromosomes from group 1 (*Glu-A1*, *Glu-B1* and *Glu-D1*). LMW-GS are encoded by multigene families located at three homoeoloci *Glu-A3*, *Glu-B3* and *Glu-D3* located on the distal region of the short arms of chromosomes from homoeologous group 1. These three loci are tightly linked to the *Gli-A1*, *Gli-B1* and *Gli-D1* loci, respectively, which include multigene families encoding γ- and ω-gliadin subunits. As a result of this tight linkage, specific pairs of *Glu-3* and *Gli-1* alleles are frequently associated (Singh and Shepherd, 1988).

Differences among wheat varieties in gluten viscoelastic properties (e.g. strength and extensibility) are mainly associated to

Abbreviations: A-PAGE, acid-polyacrylamide gel electrophoresis; BC, backcross; FPC, flour protein content; GPC, grain protein content; HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits; MDS, midline descending slope; MPA, midline peak area; MPH, midline peak height; MPT, midline peak time; NILs, near isogenic lines; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STS, sequence tagged site; TKW, thousand-kernel weight; ZEL, micro Zeleny sedimentation test.

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different combinations of HMW-GS and LMW-GS (Gianibelli et al., 2001). Most of the *Glu-1* loci include only two functional HMW-GS resulting in simple protein patterns in the upper region of the SDS-PAGE gels. This simplicity has facilitated the identification of specific *Glu-1* alleles and has favored extensive studies of their contribution to breadmaking quality. The focus of wheat breeding programs on the *Glu-1* alleles is reflected in the widely used GLU-1 breadmaking quality score which is based solely on the addition of numerical scores assigned to individual *Glu-1* alleles (Payne, 1987). However, this index explains only a portion of the variation observed in gluten strength, likely because it excludes the contributions of the LMW-GS and gliadins (Gupta et al., 1994; Nieto–Taladriz et al., 1994; Rodriguez–Quijano and Carrillo, 1996; Manifesto et al., 1998). The relative contribution of LMW-GS and gliadins to the total variation in breadmaking quality increases as optimal HMW-GS alleles are fixed in modern wheat breeding programs. For example, in studies of recent Argentine varieties, performed by our group, the GLU-1 index explained less than 20% of the variation in baking quality (Dubcovsky et al., 2000).

LMW-GS are 4–5 times more abundant than HMW-GS and are encoded by a large multigene family (D'Ovidio and Masci, 2004). The high number of LMW-GS results in complex SDS-PAGE patterns that complicate the identification of individual *Glu-3* alleles and of their correlations with breadmaking quality parameters. The tight genetic linkage between *Glu-3* and *Gli-1* loci further complicates this problem because the effects of these linked loci are difficult to separate. These difficulties have hindered the utilization of marker assisted selection for LMW-GS in wheat breeding programs.

To overcome this complexity, we used DNA markers to develop nine near isogenic lines differing in only one *Glu-3/Gli-1* locus at a time in a common genetic background. This strategy allowed us to quantify the effects of individual *Glu-3/Gli-1* loci on gluten strength and other traits associated with breadmaking quality, and to validate the molecular markers required to combine the best alleles by marker assisted selection.

2. Experimental

2.1. Plant material

Argentine spring red wheat variety ProINTA Imperial (BUCK-PUCARA/3/JARAL-F-66//CIANO-67/2*JARAL-F-66) was selected as recurrent parent to develop a set of NILs for allelic variants at *Glu-A3* and *Glu-B3* loci. This variety has a good agronomic performance and an optimal HMW-GS allelic composition (*Glu-A1a*, *Glu-B1al* and *Glu-D1d*) with an index GLU-1 = 10 (Payne, 1987). The *Glu-B1al* allele present in ProINTA Imperial (also known as Bx7 over-expressed or Bx7^{oe}) has a duplication of the Bx7 subunit that results in higher expression levels of this subunit and increased dough strength (Ragupathy et al., 2008). In spite of its optimal HMW-GS composition, ProINTA Imperial has relatively low gluten strength, which suggests the presence of sub-optimal alleles at other loci affecting breadmaking quality (e.g. *Glu-3/Gli-1*).

We took advantage of microsatellite markers *psp2999* and *psp3000* present within the coding sequences of genes *Glu-A3* and *Gli-B1*, respectively (Devos et al., 1995), to introgress different *Glu-3/Gli-1* alleles into ProINTA Imperial and investigate their effects on gluten strength. We developed Near Isogenic Lines (NILs) through a program of six cycles of backcrossing to ProINTA Imperial (BC_6), self-pollination of heterozygous BC_6 plants and selection of homozygous BC_6F_2 plants. Selection of the introgressed alleles at each step was performed using microsatellite markers *psp2999* and *psp3000*. The resulting homozygous BC_6F_3 seeds were used to generate sufficient seed for the three field trials and to deposit a sample in the National Small Grain Collection (U.S. Department of Agriculture – Agricultural Research Service, Aberdeen, Idaho USA). Germplasm identification number PI 674000 to PI 674009 are described in Table 1.

2.2. DNA isolation and PCR amplification

Genomic DNA was extracted from young leaves following the CTAB procedure (Manifesto et al. 2001). PCR reactions for the microsatellite markers used in the marker assisted backcrossing was performed in a final volume of 18 μl containing 20 ng of genomic DNA, 1X reaction buffer, primers (0.25 μM each), $MgCl_2$ (1.5 mM), 4 dNTPs (125 uM each), and 1 U *Taq* DNA polymerase from Highway Molecular Biology (Argentina). The PCR reaction was carried out in an MJ Research thermal cycler model PT100, with the following cycling conditions: one cycle of 4 min at 94 °C; followed by 40 cycles of 40 s at 94 °C; 45 s touchdown 60 to 55 °C; 50 s at 72 °C, and a final extension at 72 °C for 8 min. The PCR fragments were separated using 6% denaturing polyacrylamide gel electrophoresis and visualized using silver staining. SDS-PAGE was used to select for *Glu-B3* alleles when the *psp3000* PCR amplification product was not easy to separate from other alleles (see next section).

STS markers developed specifically for the *Glu-A3* and *Glu-B3* genes were used in the validation and classification of the introgressed alleles, the PCR reactions were performed as indicated by Zhang et al. (2004) and Wang et al. (2009), respectively. Amplification products were analyzed on a 1.5% (w/v) agarose gels, stained with ethidium bromide and visualized by UV light.

2.3. Protein allelic composition at the Glu-B3/Gli-B1 and Glu-A3/Gli-A1 loci

Gliadins and glutenins were extracted from single seeds. Gliadins were separated by acid-polyacrylamide gel electrophoresis (A-PAGE) (aluminum lactate buffer, pH 3.1) according to Khan et al. (1985). Glutenin subunits were analyzed by SDS-PAGE according to the procedure described by Payne et al. (1981), and using a 10% polyacrylamide gel. When the *Glu-B3* allele could not be identified well using the previous conditions, the polyacrylamide concentration was increased up to 13.5%, and the pH of the TRIS buffer was lowered from the frequently used pH 8.8 to pH 8.5 (Peña et al., 2004). Gels were stained overnight with 12% (w/v) trichloroacetic acid solution containing 5% (v/v) ethanol and 0.05% (w/v)

Table 1
NILs developed for the present study carrying different SSR alleles and their final *Glu-A3*, *Gli-A1*, *Gli-B1* and *Glu-B3* protein composition determined by SDS-PAGE, A-PAGE and PCR markers.

Genotypes[a]	ID	SSR alleles (bp)		Gliadins alleles[b]		LMW-GS alleles[c]	
	NSGC	*Glu-A3*	*Gli-B1*	*Gli-B1*	*Gli A1*	*Glu-A3*	*Glu-B3*
Control	PI 674000	133/142	270	*d*	*a*	*a*	*h*
Man_B3	PI 674008	133/142	252	*p'*	*a*	*a*	*i*
38 MA_B3	PI 674004	133/142	285	*a*	*a*	*a*	*a*
Ret_B3	PI 674006	133/142	213	*b*	*a*	*a*	*b*[b]
Pon_B3	PI 674005	133/142	213	*b*	*a*	*a*	*b*
Chu_B3	PI 674009	133/142	243	*i'*	*a*	*a*	*i*
Tol_B3	PI 674007	133/142	237	*f*	*a*	*a*	*g*
Pon_A3	PI 674002	145/148	270	*d*	*o*	*f*	*h*
Chu_A3	PI 674001	148/157	270	*d*	*m*	*e*	*h*
Tol_A3	PI 674003	148/151	270	*d*	*m*	*g*	*h*

Line's identification refers to the donor parental line and the gene involved. Particularly: Control = ProINTA Imperial, Man = Buck Manantial, 38 MA = 38 MA, Ret = Retacón INTA, Pon = Buck Poncho, Chu = Norkin Churrinche, Tol = Klein Toledo.

[a] NILs with *Glu-A3* and *Glu-B3* introgressed alleles; bp = base pairs.
[b] Allele identified by proteinograms.
[c] Alleles identified by PCR specific-allele.

Coomassie Brilliant Blue R-250. Destaining was carried out with tap water. The characterization of gliadin alleles was performed according to the gliadin blocks described by Metakovsky (1991).

2.4. Field trials

NILs were evaluated in three field trials conducted in 2006 and 2008 in Castelar (34° 40′ South, 58° 39′ West) and La Dulce (38° 20′ South, 59° 12′ West) in the province of Buenos Aires, Argentina. Trials were performed using 1-m long plots of three rows each, organized in a randomized complete block (RCBD) experimental design with ten replications. A ProINTA Imperial sister NIL with the original genotype constitution was used as control. All trials were sown and harvested manually.

2.5. Quality variables analyzed

The quality analyses in this study included: grain protein content (GPC), flour protein content (FPC), Micro Zeleny sedimentation test (ZEL) and mixograph parameters derived from the midline trace: midline peak height (MPH, also referred in other studies as midline peak value), which is the maximum height of the midline curve expressed as a percentage of full-scale mixograph units; midline peak time (MPT), which is the time in minutes at midline peak height; and midline descending slope (MDS), that is the descending slope at 8 min past peak, and midline peak area (MPA), that is the area under curve from beginning until peak time. Among the different mixograph parameters, MPT shows a good correlation with dough strength (Martinant et al., 1998).

Flour milling was carried out in an experimental automatic mill Buhler MLU-202. Protein content on whole grains was determined by NIR (Near Infrared Reflectance) spectroscopy using an Infratec 1241 Grain Analyzer according to the standard method 39-25 of the American Association of Cereal Chemists (AACC, 2010). Micro Zeleny sedimentation test, that roughly estimates gluten strength, was performed according to method 56-63 of the AACC (AACC, 2010). Dough mixing properties were determined in 10 g of flour, using the computerized National mixograph (Swanson, National MFG. CO) according to method 54-40A of the AACC (AACC, 2010). Thousand-kernel weight (TKW) was determined by manual counting 1000 grains and then weighing them in a precision scale.

2.6. Statistical analyses

Statistical analysis was performed using SAS v.9.3 (SAS Institute, Cary, NC). The analysis of variance (ANOVA) used a MIXED model, where genotypes were included as a fixed effect and both environments and genotype*environment interactions were included as random effects. Blocks were considered nested within environment and were also included as a random effect. As expected from this model, Genotype*Environment was used as error term in comparisons among genotypes. Since this error is usually larger than the residual error of the model, our mean comparisons are more conservative than in a fixed effect model. Experiments were performed in three environments: La Dulce 2006 (LD06), Castelar 2006 (Ca06) and Castelar 2008 (Ca08). For the individual ANOVAs, assumptions of homoscedasticity and normality of errors were tested using Levene's and Shapiro–Wilks tests as implemented in SAS. A logarithmic transformation was used to satisfy these assumptions in MPT, ZEL and MDS data from individual environments.

In order to estimate the effect of protein quality independently of protein quantity, flour protein content was used as a covariate in an ANCOVA analysis for mixograph parameters and for the micro Zeleny sedimentation values, as reported by Branlard et al. (2001). Results between the ANCOVA and ANOVA were compared. Correlations were calculated using Pearson's coefficient (R) and multiple pairwise mean comparisons were conducted using Tukey's test.

3. Results

3.1. Development of isogenic lines

Different *Glu-3/Gli-1* alleles were introgressed into ProINTA Imperial taking advantage of microsatellite markers *psp2999* and *psp3000* present within the coding sequences of genes *Glu-A3* and *Gli-B1*, respectively (Devos et al., 1995). A previous survey of Argentine wheat varieties (Manifesto et al., 2001) identified nine different alleles for *psp2999* and *psp3000* in the varieties 38 MA, Retacón INTA, Buck Poncho, Buck Manantial, Klein Toledo and Norkin Churrinche, which were selected as donor parents of new *Glu-A3/Gli-A1* and *Glu-B3/Gli-B1* alleles in this study (Table 1). NILs were developed through a backcross program with selection assisted by these microsatellite markers. The NILs were analyzed by SDS-PAGE and A-PAGE to determine the resulting changes in LMW-GS and gliadins (Fig. 1). STS markers developed specifically for the *Glu-A3* and *Glu-B3* genes (Zhang et al., 2004; Wang et al., 2009, respectively) were used in the validation and classification of the introgressed alleles (Table 1). Henceforth, the different NILs will be identified with three letters from the donor parent name followed by a letter identifying the genome of the introgressed *Glu-3/Gli-1* locus. For example, Pon_B3 indicates the ProINTA Imperial NIL carrying the *Glu-B3/Gli-B1* allele from Buck Poncho.

3.2. Phenotypic characteristics

After six backcrosses, NILs (BC_6F_3) are expected to be more than 99% identical to the recurrent parent. This was confirmed in the different field trials, where a high phenotypic uniformity in terms of height, phenology and overall agronomic type was observed among the NILs and the ProINTA Imperial control line. However, an interesting difference was observed in NIL Man_B3 which presented glumes with a reddish color similar to the donor parent Buck Manantial (a trait also referred as “bronze glumes”). All other NILs and the recurrent parent have white glumes. The character “red glume color” is determined by the *Rg1* gene located on the 1BS chromosome 2.0 cM from the *Gli-B1* locus (Khlestkina et al., 2009). This genetic linkage indicates that in 98% of the gametes, the *Rg-B1* locus will co-segregate with the *Gli-B1*-252 bp-allele from Buck Manantial, providing a useful morphological marker to trace the Man_B3 allele.

3.3. Protein composition

The analysis of the nine NILs on SDS-PAGE showed uniform HMW-GS composition, as expected from the extensive backcrossing. All NILs presented the same allelic constitution as the recurrent parent ProINTA Imperial: *Glu-A1a* (subunit 1), *Glu-B1al* (subunits 7^{oe}+8*) and *Glu-D1d* (subunits 5 + 10) (Fig. 1A). The identity of the LMW-GS alleles introgressed in the different NILs was further validated using the STS markers for the *Glu-A3* and *Glu-B3* loci (Zhang et al., 2004; Wang et al., 2009).

For the *Glu-A3* locus, the four microsatellite alleles were linked to the STS alleles *Glu-A3a*, *e*, *f* and *g* as described in Table 1. For the *Glu-B3* locus, the six microsatellite alleles corresponded to only five STS alleles: *Glu-B3a*, *b*, *g*, *h* and *i* (Table 1). Man_B3 and Chu_B3 are polymorphic for the *psp3000* microsatellite marker (252 and 243 bp, respectively) but share the same *Glu-B3i* allele based on the STS marker. This result suggests that these two NILs may have different *Gli-B1* alleles, a hypothesis supported by differences in the A-PAGE

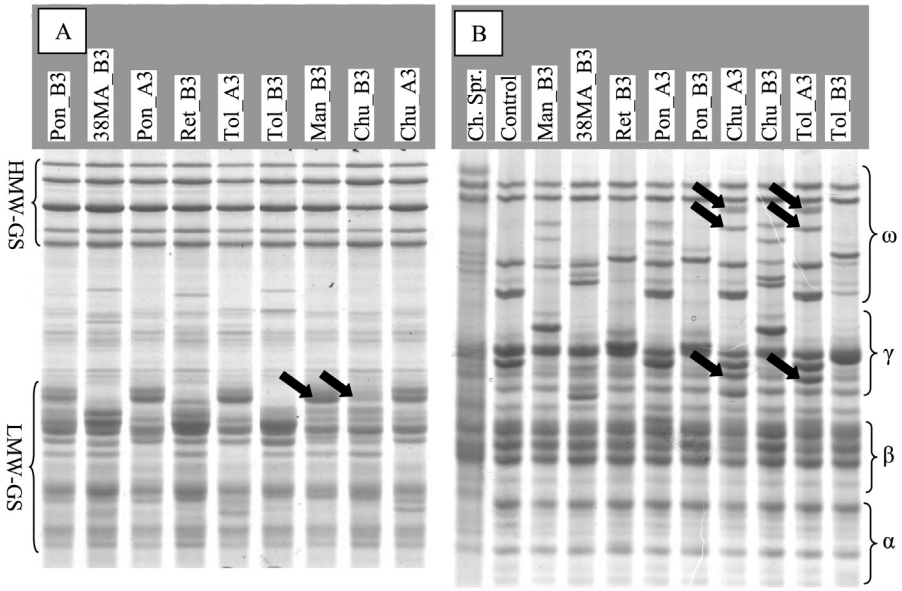

Fig. 1. NILs Protein composition revealed by: A) SDS-PAGE, B) A-PAGE (gliadins). **Note**: Ch. Spr. = Chinese Spring; Control = ProINTA Imperial. Man = Buck Manantial, 38 MA = 38 MA, Ret = Retacón INTA, Pon = Buck Poncho, Chu = Norkin Churrinche, Tol = Klein Toledo. Arrows in A indicate the band with different intensity between Man_B3 and Chu_B3. Arrows in B indicate characteristic bands for the *Gli-A1m* block.

(Fig. 1B). Differences in band intensity were also detected between these two lines in the LMW-GS region of the SDS-PAGE (Fig. 1A). These different storage protein profiles between Man_B3 and Chu_B3 are correlated with significant differences in some of the mixogram parameters as discussed later (see Section 4.2.2 and Table 3). To differentiate the Man_B3 and Chu_B3 alleles, they will be designated hereafter as *Glu-B3i*$_{Man}$ and *Glu-B3i*$_{Chu}$ respectively.

The three NILs carrying LMW-GS alleles *Glu-A3e*, *f* and *g* also showed different gliadin patterns in A-PAGE than ProINTA Imperial, which carries the *Glu-A3a/Gli-A1a* and *Glu-B3h/Gli-B1d* alleles (Fig. 1B). Chu_A3 and Tol_A3 carry the same *Gli-A1* block (*Gli-A1m*) although they differ in the *Glu-A3* allele (Table 1, Fig. 1B). This result indicates either historical recombination between *Glu-A3d* and *Gli-A1m*, or some cryptic heterogeneity within *Gli-A1m*. A similar association between LMW-GS and gliadin profiles was observed in the B genome (Fig. 1B). We observed six different gliadin profiles in A-PAGE that corresponded with the six *psp3000* microsatellite alleles used to develop these lines, and five *Glu-B3 alleles* (Table 1). Four of these gliadins patterns were identified as Metakovsky (1991) gliadin blocks *Gli-B1a*, *b*, *d* and *f*. The other two NILs, Man_B3 and Chu_B3, were similar to Metakovsky (1991) gliadin blocks *Gli-B1p* and *Gli-B1i*, but exhibit a slightly differential mobility in one of the ω-gliadin bands. To reflect these similarities, the Man_B3 and Chu_B3 gliadin blocks were designated as *Gli-B1p′* and *Gli-B1i′*, respectively. The close association between *Gli-B1p′* and the 252-bp *psp3000* allele and between *Gli-B1i′* and the 243-bp *psp3000* allele was confirmed in a survey of a collection of 208 genotypes from South America screened both by A-PAGE and microsatellite *psp3000* (Pflüger, L. and Manifesto, M.M., unpublished).

3.4. Correlation among quality parameters

To determine the relationships among the different quality parameters we calculated correlations between all pairwise combinations of the quality traits included in this study (Table 2). Highly positive and statistically significant correlations were observed between the grain and flour protein content measurement GPC and FPC ($r = 0.94$, $P < 0.001$) and between the mixograph parameters MPT and MPA ($r = 0.92$, $P < 0.001$). Only one of the parameters from these correlated pairs, FPC and MPT were included in further analyses and discussions.

FPC showed positive and highly significant correlations with ZEL and MPH, which were positively correlated with each other (Table 2). These positive correlations are expected given the positive effect of protein content on ZEL and MPH reported in previous studies (Li et al., 2010). FPC also showed a negative correlation with TKW (Table 2), which likely reflects the well-known effect of increased yield in diluting grain protein content. No significant correlation was observed between FPC and MPT.

Table 2
Correlations among quality parameters based on the three field trials combined. The values below the diagonal indicate the Pearson correlation coefficients and the values above the diagonal the corresponding *P* values.

	ZEL	TKW	FPC	GPC	MPT	MPA	MPH	MDS
Zeleny test (ZEL)	1.00	<0.001	<0.001	<0.001	0.075	<0.001	<0.001	0.023
1000 Kernel Weight (TKW)	−0.60	1.00	<0.001	<0.001	<0.001	0.989	<0.001	<0.001
Flour protein content (FPC).	0.90	−0.73	1.00	<0.001	0.160	0.002	<0.001	0.207
Grain protein content (GPC)	0.94	−0.64	0.94	1.00	0.996	<0.001	<0.001	0.435
Midline peak height (MPT)[a]	0.10	−0.20	−0.08	0.00	1.00	<0.001	<0.001	<0.001
Midline peak area (MPA),	0.35	0.00	0.18	0.26	0.92	1.00	0.018	<0.001
Midline peak time (MPH)	0.75	−0.64	0.79	0.77	−0.21	0.14	1.00	0.259
Midline descending slope (MDS)	0.13	0.20	−0.07	0.05	0.46	0.42	−0.07	1.00

[a] MPT and MPA were highly correlated ($r = 0.92$, $P < 0.001$) so MPA was excluded from further analysis.

Table 3
Analysis of variance and covariance for FPC (%), TKW (g), ZEL (ml), MPH (%), MPT (min), MDS (%). Adjusted mean comparison were performed using Tukey at $\alpha = 0.05$. Different letters indicate significant differences. In the ANCOVA, values between brackets indicate significance levels in the corresponding ANOVA without the FPC covariable.

		ANOVA		ANCOVA with FPC as covariable (ANOVA)			
		FPC	TKW	ZEL-L[a]	MPH	MPT-L[a]	MDS-L[a]
Genotype		NS	***	*** (**)	*** (**)	*** (***)	NS (NS)
Environment		***	***	*** (***)	***(***)	*** (*)	** (*)
Interaction GxE		*	NS	** (*)	NS(NS)	*** (***)	***(***)
		$R^2 = 0.90$	$R^2 = 0.84$	$R^2 = 0.95(0.92)$	$R^2 = 0.78(0.77)$	$R^2 = 0.68(0.66)$	$R^2 = 0.69(0.69)$
Glu-3 allele							
Control	*A3a/B3h*	11.37 a	41.78 c	39.30 b	54.37 abc	4.41 abcd	26.21 a
Glu-A3 NILs							
Chu_A3	*e*	11.44 a	38.99 a	32.48 a	54.85 abc	3.84 a	19.81 a
Tol_A3	*g*	11.38 a	40.61 b	37.49 b	55.31 bc	4.09 ab	25.95 a
Pon_A3	*f*	11.33 a	41.82 c	37.20 b	54.23 abc	4.75 cd	23.29 a
Glu-B3 NILs							
38MA_B3	*a*	11.48 a	41.89 c	38.63 b	53.24 ab	4.47 bcd	24.73 a
Tol_B3	*g*	11.59 a	41.99 c	37.90 b	52.64 a	5.01 cd	27.52 a
Ret_B3	*b*	11.22 a	41.86 c	39.76 b	53.46 ab	5.03 d	28.14 a
Pon_B3	*b*	11.54 a	42.89 c	39.69 b	53.78 abc	4.88 cd	30.47 a
Chu_B3	*i*	11.32 a	42.25 c	40.36 b	55.98 c	4.29 abc	23.78 a
Man_B3	*i*	11.35 a	42.33 c	40.89 b	55.76 bc	4.98 cd	28.56 a
Environments							
LD06		13.15 c	38.31 a	45.46 b	57.69 b	4.81 ab	25.65 a
Ca06		11.27 b	43.03 b	40.61 b	53.43 ab	4.76 b	29.42 a
Ca08		9.79 a	43.58 b	29.04 a	51.92 a	4.16 a	22.46 a

Adjusted means (LSmeans) are presented in the original scale. Within each parameter, means followed by the same letter are not significantly different at $p \leq 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.0001$. NS: non-significant.
[a] L indicates a logarithmic transformation for the calculation of the *P* values for the ANCOVA and mean separation.

A negative correlation was detected between mixograph parameters MPT and MPH (Table 2), a result also described in other studies (Li et al., 2010). This negative correlation explains the opposite sign of the correlations of these two parameters with FPC (Table 2). The time to the midline peak (MPT) also showed a positive correlation with the descending slope of the mixogram at 8 min (MDS) ($r = 0.46$, $P < 0.001$). MDS also showed intermediate, but significant positive correlations with TKW and MPA (Table 2).

3.5. Flour protein content

The overall ANOVA model explained 90% of the variation in FPC ($R^2 = 0.90$) in the three experiments, which suggests that the selected model provides an adequate description of the observed variability in this parameter. No significant differences in FPC were detected among NILs but there were significant differences among environments ($P < 0.0001$), with FPC means ranging from 9.79% to 13.15% (Table 3). This result indicates that allelic variation at *Glu-A3* or *Glu-B3* loci have no effect on total flour protein content, regardless of the large variation in protein content observed among environments (Table 3).

3.6. Thousand-kernel-weight

The overall ANOVA model for TKW explained 84% of the observed variation for this trait ($R^2 = 0.84$). Differences in TKW were highly significant ($P < 0.0001$) both among genotypes and among environments (Table 3). Among genotypes, the only significant difference with the control was observed for Chu_A3 (*Glu-A3e*), which showed significantly lower TKW than all other genotypes. Tol_A3 (*Glu-A3g*) allele showed the next lowest values, but was significantly different only from Pon_B3, Chu_B3 and Man_B3, which showed the three highest TKW values (Table 3). By contrast, no significant differences in TKW were detected among the different *Glu-B3* NILs (Table 3). Among the different environment, LD06 had significantly lower TKW than the other two environments, which correlated negatively with the high grain protein content level observed in this environment (Table 3).

3.7. Flour quality parameters

Overall, the analysis of covariance, using FPC as covariable, showed very similar results to the ANOVA without the covariable. This result is not surprising because this particular set of isogenic lines shows no significant difference in FPC, which limits the effects of the correlation between FPC and some of the flour quality parameters (Table 2).

The overall analyses of covariance revealed highly significant differences for genotypes, environments and their interactions for most of the flour quality parameters. The only non-significant comparisons were MDS among genotypes and MPH genotype*environment interaction (Table 3).

All traits showed significant differences among environments, with the lowest values observed in the Ca08 experiment. Since this environment showed the lowest FPC (Table 3) and both ZEL and MPH are positively correlated with FPC (Table 2), their low values in this environment are not surprising. Unexpectedly, MPT values were also significantly lower in Ca08 in spite of the absence of a significant correlation between FPC and MPT (Table 2).

Among the different *Glu-A3/Gli-A1* alleles, Chu_A3 (*Glu-A3e*) showed significantly lower ZEL and MPT values than most of the other NILs, suggesting that this allele has an overall negative impact on breadmaking quality parameters. Tol_A3 showed the second lowest values for MPT but the differences were significant only with the five NILs with highest MPT values (Table 3). These included Pon_A3, Tol_B3, Ret_B3, Pon_B3 and Man_B3. NILs Chu_B3 and Man_B3, which share the same STS marker but differ in microsatellite markers, showing some differences in MPT and MDS values. Man_B3 values for these two parameters were among the three highest, whereas those of Chu_B3 were among the three lowest ones, suggesting that Man_B3 might be a more valuable allele for improving breadmaking quality. Although the differences were not significant in the overall analysis, Man_B3 showed significantly higher MPT values than Chu_B3 in two of the three environments (Table 4).

To understand better the significant genotype by environment interaction observed for most quality parameters, we analyzed

the performance of each NIL for ZEL and MPT in the different environments (Table 4). This analysis revealed that Chu_A3 had the lowest values for both traits in all environments, even though the significance of these differences varied (Table 4). Man_B3 showed a consistent good performance across environments, except for MPT in Ca08 where it showed an intermediate performance. The ranking of other NILs varied across environments, supporting the significant interactions observed in the overall analysis (Table 3). For example, Pon_B3 showed the highest and second highest values of ZEL and MPT, respectively, in LD06, but intermediate values in the other two environments (Table 4).

4. Discussion

Breadmaking quality is a complex trait defined by the individual effects and interactions of different grain components, and the interactions of these components with the environment. Both high- and low-molecular weight glutenins can form inter-molecular disulfide bonds that contribute to the establishment of the extensive protein networks that confer gluten its unique properties. By contrast, gliadins are mainly monomeric proteins (30–78 kDa) with intramolecular disulfide bonds. Some gliadin proteins can form inter-molecular disulfide bonds and act as chain terminators in the gluten networks, whereas others, such as the ω-gliadins lack cysteine residues and do not form disulfide bonds (reviewed by Gianibelli et al., 2001).

Some biochemical and genetic studies suggest that the LMW-GS encoded by the *Glu-3* loci play a more significant role in gluten strength than the linked gliadins (reviewed by D'Ovidio and Masci, 2004). However, more recent studies emphasize the combined effects of these two complex loci. For example, Færgestad et al. (2004) showed significant differences in dough development time between *Glu-3/Gli-1* pairs which differed only in the *Gli-1* alleles (e.g. *Glu-A3f/Gli-A1b* showed better values than *Glu-A3f/Gli-A1f*). Other studies showed that addition of purified gliadin subunits to the dough alters its properties and affects the final breadmaking quality (Khatkar et al., 2002). In our study, the introgressed *Glu-3* and *Gli-1* loci were always linked, so the effects discussed below should be interpreted mostly as the effect of specific *Glu-3/Gli-1* pair. However, we have two particular cases where a similar allele for one locus is combined with a different allele in the other one (*Glu-A3e/Gli-A1m* vs. *Glu-A3g/Gli-A1m* and *Glu-B3i/Gli-B1p′* vs. *Glu-B3i/Gli-B1i′*). These results suggest either historical recombination between *Gli-1* and *Glu-3* loci, or our inability to differentiate variation within the common locus with current marker methods. The tight linkage between *Glu-3* and *Gli-1* is not a major limitation from a practical point of view because *Glu-3/Gli-1* pairs are frequently introgressed together in wheat breeding programs. Moreover, DNA markers can now be used to confirm the simultaneous transmission of both loci.

4.1. Effect of environment

Our study showed significant differences ($P < 0.05$) among the three environments (LD06, Ca06 and Ca08) for all parameters studied (Table 3). This variability was particularly evident for grain and flour protein content, that varied from very low levels in Ca08 (9.8%) to high levels in LD06 (13.2%, Table 3). Since FPC shows a positive and significant correlation with both ZEL and MPH (Table 2), the differences in FPC may explain, at least in part, the significant effect of environment on the last two parameters (Table 3). LD06 also showed the lowest TKW among the three environments, suggesting the possibility that the high FPC observed in this location was associated to a concentration effect of the smaller grains. However, differences in FPC between Ca06 y Ca08 cannot be attributed to differences in grain filling because no significant differences in TKW were detected between these environments (Table 3).

These contrasting environments present a valuable opportunity to evaluate the effect of the *Glu-3/Gli-1* alleles under very different FPC levels. This allowed distinguishing alleles with effects that were consistent from those that were variable across different environments/FPC levels, as discussed in the following section.

4.2. Effect of individual locus

The presence of significant interactions between genotypes and environments for all traits except TKW and MPH indicate that results varied across environments. Therefore, for the variable parameters we discuss the general effect as well as the effects on the different environments.

4.2.1. Effect of Glu-A3/Gli-A1 alleles on quality traits

Interestingly, significant differences in TKW were detected for two of the three A genome introgressions but for none of the six B genome introgressions. We currently do not know if the

Table 4
Mean comparisons analysis of ZEL and MPT parameters by individual environment using ANCOVA (FPC as covariable).

Location		ZEL-L[a] (mL)			MPT-L[a] (min)		
		LD06	Ca06	Ca08	LD06	Ca06	Ca08
ANCOVA Genotype		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
R^2		$R^2 = 0.72$	$R^2 = 0.52$	$R^2 = 0.60$	$R^2 = 0.68$	$R^2 = 0.69$	$R^2 = 0.61$
Genotype	***Glu-3* alleles**						
Control	*A3a/B3h*	53.36 bc	39.40 ab	25.12 b	4.02 abc	4.85 cdef	4.37 abc
Glu-A3 NILs							
Chu_A3	*e*	41.27 a	37.51 a	18.88 a	3.69 a	4.00 a	3.82 a
Tol_A3	*g*	51.15 bc	38.40 a	22.94 ab	3.89 ab	4.31 ab	4.07 ab
Pon_A3	*f*	48.74 b	38.59 ab	24.19 b	4.47 cd	5.07 def	4.72 c
Glu-B3 NILs							
38MA_B3	*a*	51.92 bc	40.29 ab	23.63 b	4.28 bc	4.51 bc d	4.63 bc
Tol_B3	*g*	52.33 bc	39.31 ab	22.22 ab	4.90 de	5.40 f	4.75 c
Ret_B3	*b*	53.74 c	41.24 abc	24.28 b	4.93 de	5.36 ef	4.78 c
Pon_B3	*b*	55.73 c	40.92 abc	22.34 ab	5.12 e	4.77 bcde	4.75 c
Chu_B3	*i*	53.16 bc	42.02 bc	25.88 b	4.24 bc	4.36 abc	4.25 abc
Man_B3	*i*	53.28 bc	44.00 c	25.20 b	5.20 e	5.25 ef	4.50 bc

Adjusted means are presented in the original scale. Values with the same letter did not differ significantly at $p \leq 0.05$ (Tukey test).
[a] L indicates a logarithmic transformation for the calculation of the *P* values for the ANCOVA and mean separations.

significantly lower TKW observed in the Chu_A3 and Tol_A3 NILs is the result of pleiotropic effects of these particular alleles or the result of a linked QTL for TKW. The *Glu-A3e* allele present in Chu_A3 is a null allele associated with the absence of proteins (Gupta et al., 1989), which may explain the significantly negative effect of the *Glu-A3e* allele on almost all the quality parameters (ZEL, MPT and MDS). The negative impact of this allele on breadmaking quality parameters was consistent across the three evaluated environments (Table 3) and is also consistent with results from other studies (Branlard et al., 2001; Flæte and Uhlen, 2003; Zhang et al., 2004, 2012). This negative effect of *Glu-A3e* is most likely caused by the *Glu-A3* deletion, since both Chu_A3 and Tol_A3 carry the same *Gli-A1* allele (*Gli-A1m*) and still showed differences in quality parameters. However, we cannot rule out the possibility that the negative effect of the null *Glu-A3e* allele can be due to the reduced amount of LMW relative to other proteins.

By contrast, the *Glu-A3f* present in Pon_A3 was associated with high MPT values both in the overall ANOVA and in the three individual environments (Table 4). The MPT values in this line were 23.7% higher than the lowest values observed in Chu_A3 (Table 5). The good performance of *Glu-A3f* allele has been observed also by Tsenov et al. (2010), who reported a strong positive effect of this allele on end-use quality.

In this study, we observed a significantly negative correlation between MPT and MPH (Table 2), that was reported also by Jin et al. (2013). In the last study, the *Glu-A3e* and *Glu-A3f* alleles showed an opposite ranking for these parameters, an observation that is consistent with our results. In the overall analysis, the *Glu-A3e* allele has the lowest value for MPT and the highest value for MPH (Table 3), although the differences in MPH were not significant in this study.

4.2.2. Effect of Glu-B3/Gli-B1 alleles

Among the NILs with different *Glu-B3* alleles, Ret_B3 and Pon_B3 carry similar *Glu-B3b/Gli-B1b* alleles. As expected, none of the quality traits showed significant differences between these two NILs and the actual values were very similar for all traits (Tables 3 and 4). This provides a positive control of the reproducibility of the quality analyses.

Among the different quality parameters, MPT is a good predictor of gluten strength and showed good discrimination in the ANCOVA, so we focused our initial analyses on this parameter. NILs Tol_B3, Ret_B3, Pon_B3 and Man_B3 showed the highest MPT values, and were significantly higher than Chu_A3, Tol_A3 in the overall ANCOVA (Table 3) and also higher than 38 MA_B3, Chu_B3 and the control in some of the individual locations (Table 4). These results suggest that alleles *Glu-B3g*, *Glu-B3b*, and *Glu-B3i*$_{Man}$ confer longer mixing time (~17% longer) than alleles *Glu-B3a*, *Glu-B3i*$_{Chu}$ and *Glu-B3h* (Table 5) and might be a better target for breeding programs interested in improving gluten strength. A similar ranking for these alleles was proposed by Zhang et al. (2012) analyzing Aroona NILs, where quality parameters for lines carrying *Glu-B3i*, *Glu-B3g* and *Glu-B3b* were better than those in lines carrying *Glu-B3a* and *Glu-B3h* alleles.

NILs Man_B3 and Chu_B3, which have the same *Glu-B3i* allele for the STS showed differences for MPT in two of the three individual environments (Table 4). Man_B3 (*Glu-B3i*$_{Man}$) showed the highest values for ZEL, the second highest for MPH and MDS and the third highest MPT. By contrast, NIL Chu_B3 had the lowest value among the B-genome NILs in MPT in the three environments (Table 4). In the LD06 environment, for example, Man_B3 MPT values were 22.6% higher than those of Chu_B3 (Table 5). These large differences in quality parameters can be caused by differences between protein encoded by genes located in the *Glu-B3*, *Gli-B1* or both loci. The differences in the protein profiles between the *Gli-B1p′* and *Gli-B1i′* alleles are obvious in the A-PAGE (Fig. 1B) and are also associated with differences in the *Gli-B1* microsatellite marker. However, we cannot rule out the possibility that proteins at the *Glu-B3* locus also contribute to the differences in MPT and MDS. The identical STS marker only indicates identity in one or a few of the genes present in this locus and other linked LMW-GS can still exhibit differences in their coding sequences. Alternatively, as these two NILs showed the same migration pattern in SDS-PAGE, but also show a small difference in the intensity in some bands (Fig. 1A), we can consider the possibility of differences in gene expression, or copy number variation.

A previous study in our group showed a significant positive effect linked to the 252 bp *psp3000* allele. In a cross between Argentine cultivar Klein 32, which has the 252 bp *psp3000* allele, and Chinese Spring, which has identical HMW-GS as Klein 32 but a 285 bp *psp3000* allele, the 252 bp allele was associated with significant increases in sedimentation volume and mixograph parameters (Manifesto et al., 2001). From this result, and its A-PAGE profile, we infer that Klein 32 has the same *Glu-B3/Gli-B1* combination as Buck Manantial (the donor of Man_B3). The 252-bp allele is also present in the Argentine varieties Buck Manantial, Buck Cencerro and Buck Pangaré, which are well-known for their excellent breadmaking quality, confirming the positive value of this allele.

The *psp3000* microsatellite marker, located within a γ-gliadin in the *Gli-B1* locus, provides a simple tool to trace the introgression of the 252 bp in wheat breeding programs. When Buck Manantial or NIL Man_B3 developed in this study are used in crosses with white glume varieties, breeders can use the linked "bronze glume" trait to enrich these populations in the favorable 252 bp allele. Since recombination events can still occur between these two loci located 2 cM apart, breeders should use the *psp3000* or A-PAGE protein gels to confirm the introgression of the desired allele in the selected lines. Still, the use of a morphological marker in the early cycles of selection can help breeders to screen large segregating populations.

Table 5
Difference between alleles (values to the left), within each locus, contributing to the highest and lowest value (genotypes in parentheses) of MPT and ZEL. To the right the differences expressed in percentage. Pon = Buck Poncho, Man = Buck Manantial, Tol = Klein Toledo, Ret = Retacón INTA, Chu = Norkin Churrinche.

Param.	Env.	*Glu-A3*	*Glu-B3*
MPT	LD06	0.78 (Pon-Chu) 21.1% $f > e$	0.96 (Man-Chu) 22.6% $i_{Man} > i_{Chu}$
	Ca06	1.07 (Pon-Chu) 26.7% $f > e$	1.04 (Tol-Chu) 23.8% $g > i_{Chu}$
	Ca08	0.90 (Pon-Chu) 23.6% $f > e$	0.53 (Ret-Chu) 12.5% $b > i_{Chu}$
	Pooled	0.91 (Pon-Chu) 23.7% $f > e$	0.74 (Ret-Chu) 17.3% $b > i_{Chu}$
ZEL	LD06	9.88 (Tol-Chu) 23.9% $g > e$	3.81 (Pon-38 MA) 7.3% $b > a$
	Ca06	1.38 (Pon-Chu) 3.7% $f > e$	4.69 (Man-Tol) 11.93% $i_{Man} > g$
	Ca08	5.31 (Pon-Chu) 28.1% $f > e$	3.66 (Chu-Tol) 16.5% $i_{Chu} > g$
	Pooled	0.29 (Tol-Chu) 0.8% $g > e$	2.99 (Man-Tol) 7.9% $i_{Man} > g$

5. Conclusions

Our study was able to detect significant differences among *Glu-3/Gli-1* alleles in breadmaking quality parameters by using near isogenic lines and a large number of replications in field trials. The use of NILs greatly reduced the variability caused by different genetic backgrounds, whereas the large number of replications in the field reduced the environmental variability. This experimental design increased our power to detect small differences among *Glu-3/Gli-1* loci and accounted for most of the variation in the tested quality traits (see large R^2 values in Table 3).

The use of multiple locations with contrasting grain protein levels, also allowed us to determine which of these effects were consistent across locations. Based on these results, we established the following recommendations for our breeding programs. First, we decided to select against the null-*Glu-A3e* and the *Glu-A3g* alleles based on their negative impact on gluten strength in all environments (Table 4). Second, we recommended selecting in favor of the *Glu-B3i*$_{Man}$/*Gli-B1p*′ based on its positive effect across quality parameters and environments (Tables 3 and 4). This allele can be enriched by selecting for the linked "bronze glume" or directly by using the *psp3000* microsatellite marker. The *Glu-B3g/Gli-B1f* and the *Glu-B3b/Gli-B1b* are equally positive combinations that should be favored in marker assisted selection strategies to improve breadmaking quality. The *Glu-B3b/Gli-B1b* was also suggested as a positive combination by Flæte and Uhlen (2003).

In summary, our study provides information on the effects of different *Glu-3/Gli-1* alleles on breadmaking quality traits that can be used to complement current selection strategies based only on the selection of optimum HMW-GS. This additional information has the potential to increase the proportion of variation in breadmaking quality controlled by breeders, and therefore to accelerate the rate of improvement in breadmaking quality.

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