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Contributions of individual and combined Glu-B1x and Glu-B1y high-molecular-weight glutenin subunits to semolina functionality and pasta quality

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ABSTRACT

Durum wheat is an important food crop used primarily for pasta production. High-molecular-weight glutenin subunits (HMW-GS) encoded by the closely linked genes *Glu-B1x* and *Glu-B1y* are known for their combined effects on pasta quality, but their individual contributions and interactions remain poorly understood. In this study, we show that individual loss-of-function mutants of *Glu-B1x* (ΔBx6) and *Glu-B1y* (ΔBy8) were associated with significant reductions in gluten strength compared to the wildtype, with stronger effects in the ΔBxy double mutant. Reductions in gluten strength were reflected in reduced mixograph and alveograph parameters, gluten index, faster extrusion flow rates and increased cooking loss. Interestingly, the *Glu-B1x* mutation was also associated with significant increases in grain and semolina protein content, increased pasta firmness, reduced starch viscosity and increased amylose in ΔBx6 and ΔBxy. In general, the ΔBx6 mutation had stronger effects than the ΔBy8 mutation, and significant interactions between the two genes were frequent. In addition to the basic knowledge gained on the individual effects of the Bx6 and By8 subunits and their interactions, the genetic stocks developed in this study provide useful tools to study the effects of natural or synthetic HMW-GS on pasta quality parameters in a background lacking endogenous HMW-GS.

1. Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husn.) is an important food crop used primarily for pasta, but also for couscous and various types of bread in the Middle East, parts of Italy and North Africa. Premium prices are paid for durum wheat varieties that produce high-quality pasta products with bright yellow color, high protein content, and firm pasta with minimal cooking loss (Troccoli et al., 2000). Many of these pasta quality parameters are affected by the composition of the grain storage proteins, which include gliadins and glutenins. Gliadins are monomeric molecules, while glutenins form polymers containing subunits connected by intermolecular disulfide bonds. These polymers play a major role on gluten strength, viscosity, pasta firmness and cooking loss (Gianibelli et al., 2001). Based on their mobility on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), researchers have classified glutenins into high- (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) (Branlard et al., 1989; Edwards et al., 2007).

HMW-GS account for 9–11% of the total storage proteins (De Santis et al., 2017) and the role of different allelic forms on gluten technological properties have been extensively studied (Edwards et al., 2007; Sissons et al., 2005). HMW-GS proteins are encoded by pairs of closely

Abbreviations: AACC, American Association of Cereal Chemists; A-PAGE, acid polyacrylamide gel electrophoresis; CWC, California Wheat Commission; HMW-GS, High-molecular-weight glutenin subunits; LMW-GS, Low-molecular-weight glutenin subunits; L, alveograph length; P, alveograph tenacity; RVA, rapid visco analyzer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TILLING, Targeting Local Lesions IN Genomes; UC, University of California; WT, wildtype.

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linked genes (designated as *x*- and *y*-type), located at the *Glu1* loci on the long arms of chromosomes from homeologous group 1 (Lafiandra et al., 2007). In a survey of 502 durum wheat varieties from 23 countries, more than 80% of the varieties carried a null-allele in the A genome copy, designated *Glu-A1c* (Branlard et al., 1989). In this allele, the *Glu-A1x* gene has a point mutation that generates a premature stop codon and the *Glu-A1y* has an insertion of a repetitive element that disrupts the coding region (Gu et al., 2004). In the same survey, three *Glu-B1* alleles, designated as *Glu-B1b* (Bx7+By8), *Glu-B1d* (Bx6+By8) and *Glu-B1e* (Bx20+By20), accounted for more than 85% of the accessions (Branlard et al., 1989). The *Glu-B1d* and *Glu-B1b* alleles have been associated with superior pasta quality parameters compared to the *Glu-B1e* allele (Sissons et al., 2005), but the individual contributions of the *Glu-B1x* and *Glu-B1y* genes to the different quality parameters remain unknown.

In this study, we used a Targeting Local Lesions IN Genomes (TILLING) mutant population generated in the tetraploid durum wheat variety Kronos (Uauy et al., 2009) to study the individual contributions and interactions of the *Glu-B1x* and *Glu-B1y* genes to semolina functionality and pasta quality. In addition to this new basic information, this study provides a publicly available tetraploid stock lacking all endogenous HMW-GS, which can be used to test the effect of synthetic HMW-GS and also to develop novel strategies to improve pasta quality.

2. Material and methods

2.1. Experimental materials and growth conditions

Kronos is a durum wheat variety that has been extensively grown in California and Arizona since the early 1990's (https://smallgrains.ucdavis.edu/), becoming a standard for high-quality pasta. Kronos carries the null *Glu-A1c* allele in the A genome and the *Glu-B1d* (Bx6+By8) in the B genome. To study the individual effects of the two functional genes at the *Glu-B1d* locus on pasta quality, we screened an ethyl methane sulfonate (EMS) mutagenized population of Kronos, initially using a *Cel*I screen (Uauy et al., 2009).

We used primers *Bx6*-F (5′ TCAGCATGCACACAACCATGTCCTGAAC 3′) and *Bx6*-R (5′ GCGTTTTCCCTTTCCCTTGCTTGGATGA 3′) to detect mutations in the Bx6 subunit, and *By8*-F (5′ GGCAAATTGTGCTTTTCCAACTGACTTTATTCC 3′) and *By8*-R (5′ GCATGCATACCAAGCACCATGCA 3′) to detect mutations in the By8 subunit by sequencing. We validated the mutations identified in this screen using the exome capture data generated later for the complete Kronos mutant population (Krasileva et al., 2017), and confirmed the absence of the Bx6 and By8 subunits using SDS-PAGE.

We backcrossed each of the selected mutants with the non-mutagenized Kronos twice to reduce the number of background mutations. Using molecular markers, we then selected all four possible homozygous BC_2F_2 combinations. These lines are referred hereafter as WT (both Bx6 and By8 wildtype alleles), ΔBx6 (Bx6 mutant and By8 wildtype), ΔBy8 (Bx6 wildtype and By6 mutant) and ΔBxy (both Bx6 and By8 mutant). It is important to emphasize that the mutations in the double mutant are independent of the mutations in the single mutants and that, therefore, serve as a control that the observed phenotypes are not the result of linked mutations persisting after the two backcrosses. It is extremely unlikely that the same background mutation is fixed in independent mutant backgrounds.

We increased seeds for the four genotypes and organized a field experiment using 6-m^2 plots (1.5 m × 4 m) in a completely randomized design with six replications. Plots were sown at a 3 million seeds ha^{-1} seeding rate in November 2018 (fall planting) at the UC Experimental Field Station in Davis, CA (38° 32′ N, 121° 46' W) in a Yolo loam soil (fine-silty, mixed, superactive, nonacid, thermic Mollic Xerofluvent). Fertilization consisted of a 112 kg ha^{-1} N at pre planting and an additional 67 kg ha^{-1} N at tillering. Plots were flood irrigated three times.

2.2. Gliadins and glutenins analyses

For the extraction of storage proteins, we used 50 mg of whole flour and separated the glutenin and gliadin fractions based on their different solubility in 65% alcohol. The extracted glutenin proteins were resolved using 4–12% NuPAGE protein gels (ThermoFisher Scientific; Waltham, MA) as per manufacturer's standard protocol. The extracted gliadins were detected using 12% acid polyacrylamide gel electrophoresis (A-PAGE) as described before (Watry et al., 2020). Densitometric analysis of LMW-GS and gliadin proteins in SDS- and A-PAGE gels, respectively, was performed using ImageJ (https://imagej.nih.gov/ij/index.html). We performed two experiments using samples from the six field replications, the first one including 6 gels and the second one including 12 gels. Eight smeared lanes out of 72 were treated as missing data.

2.3. Evaluation of grain and semolina quality

We performed all quality analyses at the California Wheat Commission Milling and Baking Laboratory (CWC, http://californiawheat.org/) following methods approved by the American Association of Cereal Chemist International (AACC Intl., Approved methods of analysis, 11th ed. AACC Intl., St. Paul http://methods.aaccnet.org/). Grain quality methods included test weight (AACC 55–10.01), moisture (AACC 44–15.02), and protein (AACC 46–30.01).

We produced semolina by tempering the grain to final moisture of 16% and milled the durum wheat grains following AACC method 26–42.01. We determined percent semolina extraction and moisture using method AACC 44–15.02, protein with method AACC 46–30.01, semolina ash content with method AACC 08–01.01 and falling number with method AACC 56–81.04. The sodium dodecyl sulfate (SDS) micro sedimentation test was performed using method AACC 56–63.01.

2.4. Gluten, dough and starch viscosity tests

We evaluated gluten content and gluten index using method AACC 38–12.02 in a Glutomatic® (Hägersten, Sweden). Dough strength and dough quality parameters were evaluated using an alveograph (CHOPIN Technologies, Villeneuve-la-Garenne Cedex, France) and a mixograph (National Manufacturing Company, Lincoln, Nebraska, USA) equipment, following methods AACC 54–30.02 and AACC 54–40.02, respectively.

We evaluated starch viscosity using a Rapid Visco Analyser (RVA) following the manufacturer's RVA Durum Method (Newport Scientific Method 11, Version 5, December 1997, AACC Method 61–02.01). We analyzed the RVA data as described in previous studies (Ravi et al., 1999). Amylose levels were determined using the AMYLOSE/AMYLOPECTIN kit developed by Megazyme International (Wicklow, Ireland; catalogue number K-AMYL) as described before (Hazard et al., 2015).

2.5. Evaluation of pasta quality

We used AACC method 66–42.01 for micro-scale pasta processing with 1-kg sample of semolina. We adjusted the water added to the semolina based on the P values from the alveograph results and mixed them for 5 min using a Hobart mixer. We determined extrusion rate by dividing the extruded weight in grams by the time in seconds for each sample. We dried the extruded spaghetti in a pasta dryer for 20.5 h at 40 °C and a maximum relative humidity of 95% following a modified Buhler low-temperature drying cycle (Yue et al., 1999).

Pasta quality evaluations included cooked weight, cooking loss, firmness, and color. Cooked weight was determined by cooking 10 g of spaghetti for 12 min in 350 mL of distilled water. We drained the cooked spaghetti and recorded weights. For cooking loss, we collected the cooking water from each sample and evaporated to dryness overnight in a forced-air oven at 130 °C. We weighed the remaining residue and reported it as a percentage of the original sample. We measured firmness

of five pasta strands using a TA.XTplus Texture Analyzer (Stable Micro Systems, Godalming, UK) following method AACC 66–50.01 and pasta color using a Minolta Chroma Meter CR-310 (Minolta, Osaka, Japan) following method AACC 14–22.01. We measured each sample three times and averaged the three values.

2.6. Statistical analyses

We analyzed quality data for each parameter separately using a two-by-two factorial ANOVA, with genes as factors and alleles as levels (SAS version 9.4, SAS Institute, Cary, NC). We tested normality of residuals using the Shapiro-Wilks test and homogeneity of variance using the Levene's test. When necessary, we transformed the data to satisfy the assumptions of the ANOVA. Mean comparisons were performed using the Tukey test.

3. Results

3.1. Description of the selected mutants

Since both the *Glu-A1x* and *Glu-A1y* alleles of Kronos are not functional, we searched for mutations in genes from *Glu-B1* locus. For the *Glu-B1x* gene, we identified mutation C1198T (1st letter is the wildtype allele, last letter the mutant allele and the number indicated the position from the initial ATG) in line T4-0865. This mutation generates a premature stop codon (Q400*) in the encoded protein (Fig. 1A). Since the Bx6 subunit has 824 amino acids (aa), the Q400* mutation is predicted to eliminate the C-terminal half of the protein (Fig. 1A). For the *Glu-B1y* gene, we identified mutation C1351T in line T4-2197. This mutation was also predicted to generate a premature stop codon (Q451*) that eliminates 37% of the C-terminal part of the By8 protein (720 amino acids, Fig. 1A).

We were very fortunate to find one mutant line, T4-1280, which carried truncation mutations in both *Glu-B1x* and *Glu-B1y*. The C349T mutation in *Glu-B1x* results in a premature stop codon (Q117*) that eliminates 86% of the C-terminal part of the protein. The C190T mutation in *Glu-B1y* originates an early stop codon (R64*) that eliminates 91% of the protein (Fig. 1A).

To test the effect of the different mutations at the protein level we analyzed the HMW-GS of the wildtype and three mutant lines by SDS-PAGE (Fig. 1B). In the wildtype Kronos, we observed the predicted Bx6 and By8 bands. The upper band was missing in ΔBx6 and ΔBxy, whereas the lower band was missing in ΔBy8 and ΔBxy. Mutant line T4-1280 carrying mutations in both genes (ΔBxy) showed no functional HMW-GS. The smaller bands predicted from the Q400* in ΔBx6 (~42.7 kDa) and Q451* in ΔBy8 (~48.7 kDa) were not observed in the gel suggesting that these smaller predicted transcripts or proteins were likely degraded.

A faint band observed below subunit By8 in the wildtype was missing in the ΔBy8 and ΔBxy mutants (Fig. 1B asterisk) suggesting that it was also encoded by By8. We observed a similar faint band in other durum varieties carrying the By8 subunit (Aguiriano et al., 2008; Brites and Carrillo, 2001). Previous studies using mass spectrometry demonstrated that this lower band designated By8*, is a truncated form of By8 that lacks the C-terminal region, resulting in faster electrophoretic mobility than the complete By8 subunit (Mamone et al., 2009). Since there is only one gene encoding By8 in Kronos, By8* is likely the result of a partial proteolytic cleavage of the full-length protein.

3.2. Effect of the Glu-B1 mutation on grain and semolina quality

Table 1 describes the grain yields and the effects of the individual and combined mutations in the *Glu-B1x* and *Glu-B1y* genes on grain and semolina quality. The field plots produced grain yields typical for durum trials at UC Davis (6708–8097 kg/ha), which generated sufficient seed for the quality analyses. A Tukey test showed a slightly higher yield for ΔBy8 that was marginally significant ($P < 0.05$) and no significant differences among the other three genotypes (Fig. 1C).

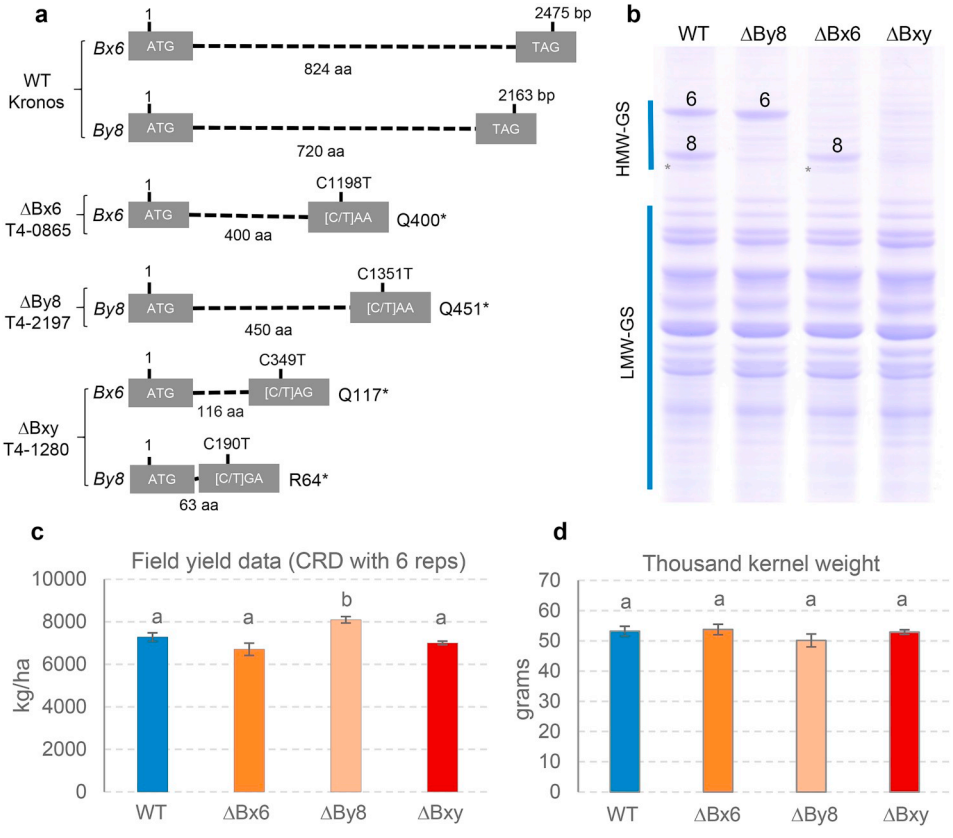

Fig. 1. Kronos wildtype and mutant lines. (**a**) Schematic representation of wildtype and mutant *Glu-B1x* and *Glu-B1y* genes encoding HMW-GS Bx6 and By8, respectively. DNA mutations are indicated above the genes, with the first letter indicating the wildtype allele, the last letter the mutant allele and the number the position in the gene from the start codon. The effect of the mutation in the protein is presented to the right of the gene. The predicted number of amino acids (aa) excluding the stop codon are listed below the gene model. (**b**) HMW-GS (top) and LMW-GS (bottom) proteins extracted and run in SDS-PAGE from Kronos homozygous line WT (both functional Bx6 and By8), ΔBy8 (WT Bx6 and mutant By8), ΔBx6 (mutant Bx6 and WT By8) and ΔBxy double mutant (truncation mutations in both genes). A faint band that co-segregated with By8 in all our gels is marked with an asterisk. This is a C-terminal truncated version of By8 designated as By8* (see text). (**c**) Grain yield in the field experiment used to generate the seeds for the quality test. (**d**) Thousand Kernel Weight from the same experiment. (c–d). Bars represent averages with standard errors of the means (n = 6), and different letters on top of the bars significant differences in Tukey tests (α = 0.05).

Table 1
Grain and semolina quality parameters. The first four columns are averages of six field replications. They are followed by the percent effect of ΔBx6, ΔBy8 and ΔBxy relative to the wildtype, and by the *P* values for the ΔBx and ΔBy main effects and their interaction from the 2 × 2 factorial ANOVA.

	WT	ΔBx6	ΔBy8	ΔBxy	%Bx	%By	%Bxy	*P* Bx	*P* By	*P* int.
Grain										
Total yield kg/ha	7280^{a}	6708^{a}	8097^{b}	7003^{a}	−7.9	+11.2	−3.8	ns	*	**
Protein 12% Mb	13.0^{bc}	13.4^{ab}	12.8^{c}	13.9^{a}	+3.5	−1.7	+7.2	****	ns	**
Ash 12% Mb	1.86^{a}	1.81^{ab}	1.68^{b}	1.89^{a}	−2.3	−9.7	+1.8	*	ns	**
Test weight kg/hl	80.0^{a}	79.3^{a}	80.4^{a}	79.2^{a}	−0.9	+0.5	−1.1	*	ns	ns
TKW g	53.2^{a}	53.8^{a}	50.1^{a}	52.9^{a}	+1.1	−5.7	−0.5	ns	ns	ns
Falling No. sec.	549^{a}	667^{a}	643^{a}	678^{a}	+21	+17	+23	ns	ns	ns
Semolina										
Protein 14% Mb	11.9^{bc}	12.3^{ab}	11.6^{c}	12.7^{a}	+3.3	−2.8	+6.4	****	ns	*
Ash 14% Mb	0.85^{b}	0.87^{ab}	0.79^{b}	0.95^{a}	+3.0	−7.2	+12.0	***	ns	**
Extraction %	61.1^{a}	60.8^{a}	62.3^{a}	61.5^{a}	−0.5	+2.0	+0.6	ns	ns	ns

ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. Different letters after the means indicate significant differences ($P < 0.05$) using a stringent Tukey test.

None of the mutants showed significant effects on falling number or thousand kernel weight, and small reductions were detected in test weight (−0.9%, $P = 0.011$) and ash content (−2.3%, $P = 0.040$) for the ΔBx6 mutant (Table 1). We detected a significant interaction between the two genes for ash content ($P = 0.004$). The most significant effect of the HMW-GS mutations on grain quality ($P < 0.0001$) was a 3.5% increase in grain protein content in the ΔBx6 mutant, which increased to 7.2% in the ΔBxy double mutant. The synergistic effect of the two mutations on grain protein content was reflected in a highly significant interaction between the two genes ($P = 0.009$, Table 1).

The results for semolina quality were correlated to those observed for grain quality with significant effects of ΔBx6 and significant interactions between the two genes on ash and protein content (Table 1). For both parameters, the ΔBxy double mutants showed stronger effects than the ΔBx6 mutant alone (Table 1). We did not detect significant effects for any of the *Glu-B1* mutations on semolina extraction compared with the Kronos wildtype (Table 1).

To see if the significant increase in grain and semolina protein content observed in ΔBx6 and ΔBxy was correlated with the increase of any particular LMW-GS or gliadin subunits, we prepared six SDS- (Fig. 1B) and A-PAGE (Supplementary Fig. 1) gels using the same amount of protein for each genotype. We did not observe any obvious change in relative intensity for specific gliadin or LMW-GS band, but we detected some significant effects when we quantified the combined intensity of all the bands in each of these groups using ImageJ.

A factorial ANOVA revealed no significant increases in overall band intensity for the LMW-GS. For the gliadins, we observed a significant effect of ΔBx6 in two separate experiments (combined $P = 0.0004$, Supplementary Fig. 1), no significant effect of ΔBy8 and no significant interaction. The gliadin bands in ΔBx6 and ΔBxy were 9.3% and 12.3% more intense than in the WT, respectively. These results paralleled the significant effect of ΔBx6 on grain and semolina protein (Table 1), and suggested that the increase in gliadin levels may have contributed to the overall increase in grain and semolina protein content in the lines carrying the two independent ΔBx6 mutations.

3.3. Effect of the Glu-B1 mutations on gluten strength and dough properties

The *Glu-B1* mutations had highly significant effects on gluten strength (Table 2). Wet gluten, gluten index, alveograph W values and sedimentation volume were all highly correlated with each other ($R \geq 0.90$). All these parameters showed negative and highly significant ($P < 0.0001$) effects associated with both ΔBx6 and ΔBy8. The effects were of similar magnitude for both mutants, but the double mutant showed effects that were larger than the addition of the effects of the individual genes, resulting in highly significant interactions in all parameters except for W (Table 2).

Associated with the negative effect of the *Glu-B1* mutations on gluten strength, we found highly significant negative effects of ΔBx6 and ΔBy8 on mixograph peak time, peak height and peak integral (Fig. 2A) and on alveograph parameters P (maximum pressure), L (extensibility) and W (deformation energy) (Fig. 2B, Table 2). For these parameters, the interactions between genes were significant only for mixogram peak height and integral and for alveograph P/L ratio (Table 2). Since reductions in L values relative to the wildtype (ΔBx6 = 27%, ΔBy8 = 23%,

Table 2
Gluten strength and dough properties parameters. The first four columns are averages of six field replications. They are followed by the percent effect of ΔBx6, ΔBy8 and ΔBxy relative to the wildtype, and by the *P* values for the ΔBx and ΔBy main effects and their interaction from the 2 × 2 factorial ANOVA.

	WT	ΔBx6	ΔBy8	ΔBxy	%Bx	%By	%Bxy	*P* Bx	*P* By	*P* int.
Gluten strength										
Sedimentation cc	50.7^{a}	44.0^{b}	44.3^{b}	30.5^{c}	−13.2	−12.5	−39.8	****	****	****
Wet Glu. 14% Mb	32.1^{a}	30.1^{b}	30.0^{b}	26.6^{c}	−6.2	−6.4	−17.0	****	****	**
Gluten Index	52.9^{a}	38.6^{b}	41.0^{b}	1.4^{c}	−27.1	−22.5	−97.3	****	****	****
Mixograph										
Absorption %	61.5^{bc}	62.1^{ab}	61.0^{c}	62.5^{a}	1.0	−0.8	1.7	****	ns	*
Peak Time min	2.65^{a}	2.12^{bc}	2.21^{b}	1.86^{c}	−20.0	−16.5	−29.9	****	***	ns
Peak height	51.7^{a}	38.3^{c}	44.5^{b}	37.4^{c}	−26.0	−13.9	−27.7	****	***	**
Peak Integral	103.9^{a}	64.4^{bc}	76.3^{b}	54.1^{c}	−38.0	−26.5	−47.9	****	****	*
Alveograph										
P mmH2O	103.3^{a}	88.5^{b}	93.2^{ab}	69.5^{c}	−14.4	−9.8	−32.7	****	****	ns
L mm	40.7^{a}	29.7^{b}	31.2^{b}	15.3^{c}	−27.0	−23.4	−62.3	****	****	ns
P/L ratio	2.62^{b}	3.05^{b}	3.00^{b}	4.56^{a}	+16.4	+14.5	+74.2	****	****	*
W 10^{-4} J	154.5^{a}	102.7^{b}	110.5^{b}	49.3^{c}	−33.5	−28.5	−68.1	****	****	ns

ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. Different letters after the means indicate significant differences ($P < 0.05$) using a stringent Tukey test.

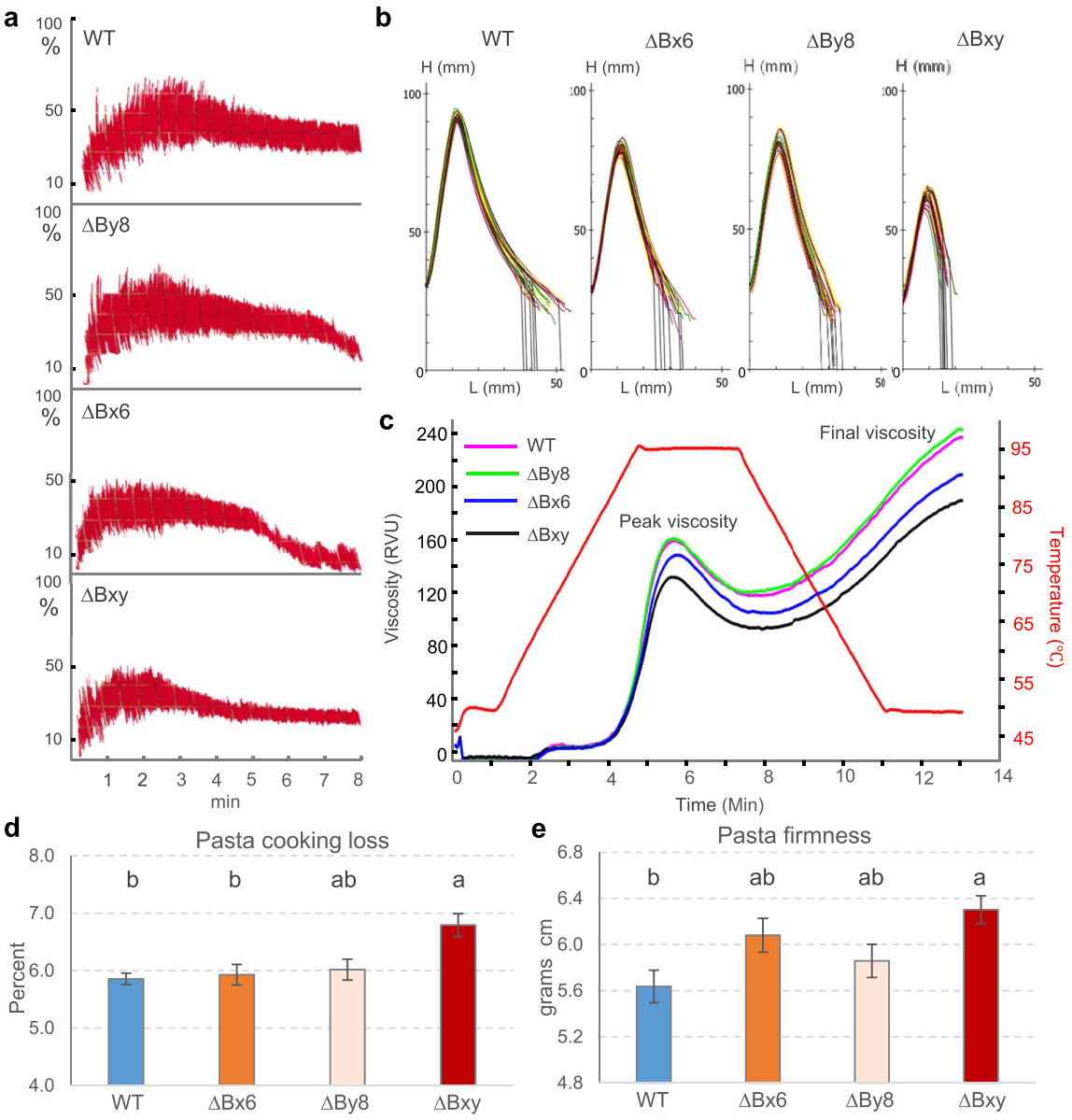

Fig. 2. A. Effect of mutations in the *Glu-B1* on mixograph curves, farinograph, RVA profiles and pasta quality. (**a**) Mixograph curves for Kronos WT, ΔBx6, ΔBy8 and ΔBxy. (**b**) Alveograph curves for the same genotypes as (a). Different line colors indicate the six different replicates. (**c**) RVA curves for semolina extracted from the same four genotypes. The red curve indicates the changing temperature and the pink, blue, green, and black lines indicate the WT, ΔBx6, ΔBy8 and ΔBxy genotypes, respectively. (**d**) Pasta cooking loss (**e**) Cooked pasta firmness. (**d-e**) Bars are averages of 6 replications ± s.e.m. Different letters above the bars indicate significant differences in Tukey tests ($P < 0.05$). ANOVAs for these parameters are presented in Table 4.

and ΔBxy = 62%) were larger than in P (ΔBx6 = 14%, ΔBy8 = 10% and ΔBxy = 33%), the P/L ratio showed a significant increase in the mutants relative to the wildtype (Fig. 2B).

The differences in mixogram water absorption were small (<2%) but significant, and larger in the ΔBxy than in the ΔBx6 mutant, suggesting an effect of By8 in increasing water absorption in the absence of Bx6 (Table 2). The average values of water absorption in the four genotypes were highly correlated with semolina protein content ($R = 0.999$), suggesting a strong effect of the differences in protein content on water absorption.

3.4. Effect of the Glu-B1 mutations on semolina and pasta color

The *Glu-B1* mutations showed a more limited effect on semolina and pasta color than on gluten strength (Table 3). No significant differences were detected for the L* value. For the a* value, the effect was not significant for pasta and only marginally significant ($P = 0.02$) for semolina. However, the negative effect of the *Glu-B1* mutations on the b* values (yellow pigments) was significant in both semolina (−4.8 to −11.6%) and pasta (−1.5 to −5.6%) analysis, and the effects were mainly additive (no significant interactions, Table 3).

3.5. Effect of the Glu-B1 mutations on pasta quality and starch viscosity

The presence of the ΔBx6 and ΔBy8 mutations was associated with positive effects on extrusion flow rate (Table 4). The effects of the two mutants were similar to each other and additive, resulting in a 9.3% increase in the ΔBxy double mutant. We interpreted the increased flow rate in the mutants as an indirect effect of their reduced gluten strength. Extrusion of the stronger wildtype dough requires more force than extrusion of the weaker dough from the mutants, so at constant force, the dough from the mutant extrudes faster than the dough from the

Table 3
Semolina and pasta color. The first four columns are averages of six field replications. They are followed by the percent effect of ΔBx6, ΔBy8 and ΔBxy relative to the wildtype, and by the *P* values for the ΔBx and ΔBy main effects and their interaction from the 2 × 2 factorial ANOVA.

	WT	ΔBx6	ΔBy8	ΔBxy	%Bx	%By	%Bxy	*P* Bx	*P* By	*P* int.
Semolina color										
L*	84.6^{a}	84.6^{a}	85.1^{a}	84.8^{a}	0.0	+0.6	+0.2	ns	ns	ns
a*	2.58^{a}	2.31^{ab}	2.42^{ab}	2.02^{b}	−10.4	−6.2	−21.7	*	ns	ns
b*	26.2^{a}	24.8^{b}	24.9^{ab}	23.2^{c}	−5.4	−4.8	−11.6	****	***	ns
Pasta color										
L*	52.9^{a}	53.2^{a}	52.8^{a}	52.3^{a}	+0.6	−0.2	−1.1	ns	ns	ns
a*	2.52^{a}	2.93^{a}	3.07^{a}	3.34^{a}	+16.4	+22.0	+32.7	ns	ns	ns
b*	37.1^{a}	36.3^{a}	36.6^{a}	35.1^{b}	−2.4	−1.5	−5.6	****	**	ns

ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. Different letters after the means indicate significant differences ($P < 0.05$) using a stringent Tukey test.

Table 4
Pasta extrusion, pasting and cooking quality. The first four columns are averages of six field replications. They are followed by the percent effect of ΔBx6, ΔBy8 and ΔBxy relative to the wildtype, and by the *P* values for the ΔBx and ΔBy main effects and their interaction from the 2 × 2 factorial ANOVA.

	WT	ΔBx6	ΔBy8	ΔBxy	%Bx	%By	%Bxy	*P* Bx	*P* By	*P* int.
Extrusion										
Flow rate g/s	3.17^{b}	3.36^{a}	3.33^{ab}	3.47^{a}	6.0	5.0	9.3	**	**	ns
Pasta analyses										
Cooking loss %	5.58^{b}	5.93^{b}	6.02^{ab}	6.79^{a}	+1.2	+2.8	+16.0	*	**	ns
Firmness g cm	5.64^{b}	6.08^{ab}	5.86^{ab}	6.30^{a}	+7.9	+3.9	+11.8	**	ns	ns
RVA										
Peak viscosity RVU	166^{a}	154^{b}	167^{a}	139^{c}	−7.1	+0.9	−16.0	****	**	***
Final viscosity RVU	241^{a}	222^{b}	243^{a}	206^{c}	−7.9	+1.0	−14.3	****	ns	*
Starch										
Amylose %	28.5^{b}	29.7^{ab}	28.4^{b}	30.2^{a}	+4.4	−0.3	+6.2	**	ns	ns

ns = not significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. Different letters after the means indicate significant differences ($P < 0.05$) using a stringent Tukey test.

wildtype.

The simultaneous presence of the two mutations was also associated with a significant 16% increase in cooking loss, which was still within acceptable pasta quality parameters (Fig. 2D, Table 4). In spite of the increased cooking loss and reduced gluten strength (Table 2), the *Glu-B1* mutations were associated with a significant increase in cooked pasta firmness, which reached 11.8% in the double mutant (Fig. 2E, Table 4). We found no significant differences in pasta cooked weight among genotypes, which suggests no differences in pasta water absorption.

Since starch properties can also affect cooking firmness (Hazard et al., 2015), we used a Rapid Visco Analyser (RVA) to determine the pasting properties of the semolina starch from the four genotypes. We found no significant differences among genotypes for peak time and pasting temperature, but the ΔBx6 mutant showed highly significant ($P < 0.0001$) reductions in peak viscosity (−7.1%) and final viscosity (−7.9%, Table 4). The reductions were even larger in the ΔBxy double mutant (−16% and −14.3%, respectively, Table 4), which suggest that in the absence of Bx6 the ΔBy8 mutation also contributed to reduced viscosity. In the RVA graph, the ΔBxy curve was consistently below the ΔBx6 curve, which was below the WT and the ΔBy8 curves. The WT and ΔBy8 curves showed no differences from each other (Fig. 2C).

Since changes in starch composition can affect viscosity and cooking firmness, we determined the % amylose in the 24 samples. We detected a significant increase in amylose content in the ΔBx6 (+4.4%) and ΔBxy (+6.2%) relative to the wildtype and no changes in the ΔBy8 (Table 4).

4. Discussion

For more than 30 years, numerous studies have reported the contributions of different natural combinations of *Glu-B1x* and *Glu-B1y* genes to pasta quality and semolina functionality (Branlard et al., 1989; Lafiandra et al., 2007; Sissons et al., 2005). However, we have a limited knowledge of the contributions of the individual x- and y-type linked genes to pasta quality. Using additions of individual HMW-GS to bread wheat flour, a previous study showed that the addition of Bx7 resulted in higher dough strength and stability than the incorporation of Bx20 (Shewry et al., 2003). Instead of artificial additions of HMW-GS, we directly modified the HMW-GS genes to generate semolina with different HMW-GS composition and test their individual contributions to different pasta quality parameters. Our results showed that both genes make positive contributions to multiple quality parameters, and that in general the Bx6 subunit had stronger effects than the By8 subunit. In addition, we detected significant interactions between the two genes for multiple parameters, usually because of the stronger effects of each gene in the mutant background than in the wildtype background of the other gene.

4.1. Effect of Bx6 and By8 subunits on grain protein content

The significant increases in grain protein content observed in the ΔBx6 and ΔBxy relative to Kronos cannot be attributed to differences in total grain yield or grain size because these genotypes showed no significant differences in total grain yield (Fig. 1C) or thousand kernel weight (Fig. 1D).

Although the individual ΔBy8 mutation showed no significant effect on grain or semolina protein content, the increase in protein content in the ΔBxy mutant was 2-fold higher than in ΔBx6, suggesting that in the absence of Bx6 the By8 subunit also contributed to increase protein content. This hypothesis was supported by the significant interaction between the two genes for grain and semolina protein content detected in the factorial ANOVA (Table 1).

Our quantification of the gliadin and LMW-GS band intensities suggest that the increase in grain protein content in the lines carrying the ΔBx6 mutation was associated with an increase in the gliadin fraction. Other studies have also shown that changes in dosage of homeologous group 1 chromosomes storage proteins can be compensated by changes

in storage proteins encoded by other chromosomes (Dumur et al., 2004; Galili et al., 1986). Similarly, studies using ion beam deletions of linked x- and y-type *Glu-B1* genes on chromosome 1A, 1B and 1D in hexaploid wheat showed compensatory increases of the gliadin fraction, with the highest increases in lines with the *Glu-D1* deletion (Yang et al., 2014). Our *Glu-B1* mutants showed not only a compensatory effect to the missing HMW-GS, but a slight net increase in grain protein content. These results suggest that either the synthesis of HMW-GS is energetically more costly than the synthesis of other storage proteins or that the HMW-GS exert a repressive effect (direct or indirect) on the synthesis of other storage proteins.

The simultaneous elimination of the HMW-GS and the increase in gliadin content in the ΔBxy mutant limited our ability to separate their effects on quality. To understand the specific effects of the gliadins, we reviewed previous studies that used reconstituted durum flours with different proportions of gliadins. These studies have shown that the addition of gliadins reduces mixing time and generates weaker doughs (Edwards et al., 2003; Sissons et al., 2007), suggesting that the reduced gluten strength in the mutants described in this study is likely a combined effect of the absence of the HMW-GS and the increase in the relative proportion of gliadins.

4.2. Effect of Bx6 and By8 on gluten strength and associated parameters

The most consistent effect of the *Glu-B1* mutations was a significant reduction in gluten strength (Table 2). Not only did we detect highly significant reductions in gluten index, wet gluten and sedimentation values for both ΔBx6 and ΔBy8, but we also observed highly significant interactions between these two genes for these three parameters. These interactions were synergistic, with the ΔBxy double mutant showing reductions that were larger than the combined effect of the individual mutations. This result suggests that future attempts to engineer pasta quality using transgenic approaches may benefit more by using both Bx6 and By8 subunits than from including multiple copies of one of the subunits.

Gluten Index values were extremely low in the ΔBxy double mutants because the gluten ball went easily through the centrifuge sieve after the washing step. As a result, very little material was left on top of the sieve, resulting in a very small numerator in the GI calculation [top weight/(top + bottom weight) x 100]. Possibly, a combination of low gluten strength and increased gliadin content facilitated the rapid flow of the gluten ball through the centrifugation sieve resulting in the unusually low values in the double mutant.

The reduced gluten strength of the *Glu-B1* mutants was associated with highly significant reductions in mixograph peak time, height and integral values and alveograph P, L and W values. In the Chopin-Alveograph, the parameter P measures the pressure required to expand a piece of dough like a balloon, and therefore, the reduced P values observed in the *Glu-B1* mutants are a reflection of their weaker gluten strength. This reduced gluten strength also resulted in a premature bursting of the dough balloon resulting in reduced L values. However, since the reductions in L values relative to the wildtype were approximately two-fold higher than the reductions in P, the P/L ratios in the mutants showed a significant increase relative to the wildtype. These *Glu-B1* mutant samples have a low P and very low L, indicating that they are very weak, with low elasticity and very low extensibility.

The weak gluten found in the ΔBxy double mutant highlights the importance of the HMW-GS on gluten strength, but not necessarily negates the known importance of the LMW-GS to pasta quality (Brites and Carrillo, 2001). If strong gluten requires both LMW- and HMW-GS, the absence of either of them would result in weak dough.

4.3. Effect of Bx6 and By8 subunits on pasta quality

In addition to the negative effect on gluten strength, the *Glu-B1* mutations were associated with a negative effect on pasta yellow color. Both ΔBx6 and ΔBy8 showed reduced b* values, with the ΔBxy double mutant showing an additive effect. The average b* values of the different genotypes showed a negative correlation with semolina ash content ($R = -0.79$), suggesting the possibility that the increase in ash content was partially responsible for the decrease in b* values. Negative correlations between yellow color and ash content have been reported previously (Joubert et al., 2018). An even higher negative correlation was observed between pasta b* values and amylose content ($R = -0.89$), an effect that has been reported before in high amylose pasta wheat (Hazard et al., 2015). Based on these correlations we speculate that the increases in amylose content and ash contributed to the reduced b* values.

Grzybowski and Donnelly (1979) reported positive and significant correlations ($P < 0.01$) between cooking firmness and both protein content ($R = 0.44$) and gluten strength ($R = 0.44$), and negative correlations ($P < 0.01$) between cooking loss and both protein content ($R = -0.37$) and gluten strength ($R = -0.40$). They also observed a strong negative correlation between firmness and cooking loss ($R = -0.93$) (Grzybowski and Donnelly, 1979). Surprisingly, we found that the *Glu-B1* mutations characterized in this study were associated with significant increases in both cooking firmness and cooking loss, resulting in a positive correlation between these two parameters ($R = 0.89$).

A previous study in Kronos mutants for the *Starch Branching Enzyme II* (*sbeII*) genes (Hazard et al., 2015) revealed a similar positive correlation between cooking firmness and cooking loss, which was associated with reduced starch viscosity and increased amylose content. To test if similar changes could explain the positive correlation between cooking loss and firmness in the *Glu-B1* mutants, we measured the starch pasting properties in these mutants using a Rapid-Visco-Analyzer (RVA) and determined their grain amylose content. Both final viscosity and amylose content showed a significant effect of the ΔBx6 mutation and showed a very strong negative correlation ($R = -0.99$, Table 4). These results suggest that the changes in amylose content likely contributed to the observed differences in starch viscosity.

The soluble nature of amylose and its ability to leach off during cooking may explain the positive correlation detected in this study between amylose content and cooking loss ($R = 0.73$). In addition, the ability of starch granules with higher amylose content to resist rupture and deformation on swelling may explain the positive correlation between amylose content and firmness ($R = 0.93$). Similar positive correlations were observed in the high-amylose *sbeII* mutants in Kronos (Hazard et al., 2015), and may contribute to the unusual positive correlation between cooking loss and cooking firmness observed in both studies.

In addition to the starch changes, the higher grain and semolina protein content observed in the ΔBx6 and ΔBxy mutants (Table 1) may have contributed to increased firmness by retaining water and strengthening the structure that limits starch swelling and solubilization into the cooking water. Since the increased protein content in the *Glu-B1* mutants is expected to reduce cooking loss, we hypothesize that the effects of the changes in amylose content and starch viscosity on cooking loss were stronger than the effects of the increased protein content on this parameter (Table 4).

4.4. Potential applications for the HMW-GS mutant developed in this study

In addition to the basic knowledge gained on the individual effects of the Bx6 and By8 subunits and their interactions on pasta quality, the genetic stocks developed in this study may be useful tools for future studies of HMW-GS. In particular, the ΔBxy mutants lacking all HMW-GS may be useful to test the effect of different natural or synthetic HMW-GS on different pasta quality parameters. The tetraploid wheat variety Kronos used to develop the *Glu-B1* mutants is easy to transform (Richardson et al., 2014), which can accelerate the development of transgenic plants. Possible projects using the ΔBxy mutants include the testing of HMW-GS from common wheat (e.g. Dx5 and Dy10) that confer

high gluten strength, as reported recently in a genetic stock of the variety Svevo lacking the endogenous 7 + 8 HMW-GS (Sissons et al., 2019).

The ΔBxy mutants can be also used to test the effect on quality of synthetic HMW-GS without the epitopes associated with celiac disease or gluten intolerance. Using a published list of epitopes we found six copies of the QGYYPTSPQ epitope (Sollid et al., 2012) in Bx6 and two in By8. The Kronos Bx6 subunit, but not the By8 subunit, also carried epitopes QGQQGYYPISPQQSGQGQQP, GQLQQPAQGQQPAQGQQSAQ, QLVYYPTSPQQPGQLQQPAQ, and QPGYYPTSPQQSGQGQQSGQ, which have been associated with intermediate levels of allergenicity (Juhasz et al., 2018). Synthetic HMW-GS similar to Bx6 and By8 but with altered epitope sequences and driven by known wheat HMW-GS promoters (Lamacchia et al., 2001), can be synthesized and tested in the ΔBxy mutants to assess their effect on pasta quality.

Although the ΔBxy mutant lacks the HMW-GS coded epitopes for celiac disease, it still has many other epitopes in gliadins and LMW-GS that are toxic to celiac patients. This is just one step in a much larger and complex project that will required the elimination or editing of all the sequences encoding epitopes for celiac disease. A non-allergenic pasta wheat model would be useful to test the allergenicity of natural or engineered storage proteins and their effect on pasta quality. To facilitate these future uses we have deposited the genetic stocks generated in this study in the USDA-National Small Grain Collection under numbers PI 692251 (T4-0865, ΔBx6), PI 692253 (T4-2197, ΔBy8) and PI 692252 (T4-1280, ΔBxy).

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Declaration of competing interest

The authors of the manuscript “Contributions of individual and combined Glu-B1x and Glu-B1yhigh-molecular-weight glutenin subunits to semolina functionality and pasta quality”, Yazhou Zhang, André Schönhofen, Wenjun Zhang, Joshua Hegarty, Claudia Carter, Teng Vang, Debbie Laudencia-Chingcuanco, and Jorge Dubcovsky declare that they have no conflict of interest.

CRediT authorship contribution statement

Yazhou Zhang: Investigation, Methodology, Writing - original draft. **André Schönhofen:** Resources, Writing - review & editing. **Wenjun Zhang:** Investigation, Writing - review & editing. **Joshua Hegarty:** Investigation, Writing - review & editing. **Claudia Carter:** Supervision, Writing - review & editing. **Teng Vang:** Investigation, Methodology, Writing - review & editing. **Debbie Laudencia-Chingcuanco:** Visualization, Investigation, Writing - review & editing. **Jorge Dubcovsky:** Conceptualization, Formal analysis, Supervision, Project administration, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

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