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Effects of Overexpression of High Molecular Weight Glutenin Subunit 1Dy10 on Wheat Tortilla Properties

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Wheat (*Triticum aestivum* L.) flour properties necessary for optimal tortilla production have not been identified. Transgenic wheats (*Triticum aestivum* L.) overexpressing high molecular weight glutenin subunit (HMW-GS) 1Dy10 were used to make tortilla and their quality was evaluated. The level of HMW-GS 1Dy10 in flours derived from transgenic wheats was 2.5−5.8-fold greater than in controls. Polymeric proteins in the transgenic samples had a molecular weight distribution shifted toward larger polymers as indicated by increased levels of polymeric proteins present and greater M_w averages of the largest fractions in the insoluble polymeric proteins. Dough derived from transgenic wheats had greater resistance to extension and lower extensibility than controls. Tortilla quality evaluation revealed that tortillas originated from transgenic wheats had decreased diameter, greater thickness and rupture force, and lower rollability scores and stretchability than controls. The presence of 1RS chromosomal translocations from rye (*Secale cereale* L.) in transgenic wheat decreased the negative effects of overexpression of HMW-GS 1Dy10, as tortillas made with this flour mostly exhibited quality properties similar to those made from control flour. Results suggested that the negative effects of overexpression of HMW-GS 1Dy10 on tortilla properties were derived from a nonideal gluten matrix formation.

KEYWORDS: Wheat tortilla quality; high molecular weight glutenin subunit; HMW-GS; protein overexpression

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a unique cereal in its ability to form dough with viscous and elastic properties that allows for the production of a variety of food products such as leavened breads, flat breads, noodles, cookies, and cakes. The viscoelastic properties are derived from the gluten proteins, which are composed of glutenins (high molecular weight [HMW] and low molecular weight [LMW] glutenin subunits) and gliadins. HMW glutenin composition is highly correlated to dough strength and substantially affects end-use functionality of flour for bread-making (*1*−*4*). High molecular weight glutenin subunits (HMW-GS) are coded by two tightly linked genes (x- and y-type) located on the long arms of chromosome 1 on each of the three wheat genomes: A, B, and D. The HMW-GS coded by the D-genome are known to be responsible for significant variability in dough properties, especially strength and elasticity (*5*). HMW-GS 1Dx5 and 1Dy10, coded by x- and y-genes, respectively, generally produce stronger dough than the corresponding subunits 1Dx2 and 1Dy12 (*5*). The individual contribution of subunits 1Dx5 and 1Dy10 on dough properties and final product quality is difficult to assess, since this pair is always expressed together. However, the development of stable transformation of wheat, in which specific HMW-GS are introduced into appropriate genetic backgrounds, have made investigation of their contribution to final product quality possible (*6*).

Introduction of the gene encoding HMW-GS 1Dx5 into hexaploid *Tritordeum*, which does not possess the D genome, resulted in increased dough strength and improved quality of flour to a level suitable for bread-making (*7*). Improvement in mixing properties and dough strength was also observed when HMW-GS 1Dx5 was introduced in the wheat cultivar L88-31, which expresses HMW-GS 1Bx17 and 1By18 only (*8*). However, introduction of extra copies of the gene coding for HMW-GS 1Dx5 in wheat having five HMW-GS (1, 17+18, 5+10), produced excessively strong doughs (*9*). It has been suggested that elevated levels of HMW-GS 1Dx5 form highly cross-linked polymers, with limited expansion potential (*9*−*11*). Transgenic wheat overexpressing HMW-GS 1Dy10 have also been linked to an increase in both mixing time and tolerance to overmixing (*12*). It has been suggested that overexpression of HMW-GS 1Dy10 produces more extensible dough compared to the overexpression of HMW-GS 1Dx5 (*12*, *13*).

Currently, understanding the effects of individual HMW-GS on tortilla quality is a subject of increased interest, because demand for this product is high and the development of ideal cultivars for tortilla production has not been achieved.

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Table 1. Protein Content, Flour Absorption, and Mixing Times of Flours Derived from Control and Transgenic Wheats

experimental population, HMW-GS composition	protein content (%)	flour absorption (mL)	mixing time (min)
1A: 2*, 7 + 8, 5 + 10			
control	10.27	60.1	5.00
transgenic	10.22	60.0	40.00
1B: 2*, 7 + 9, 5 + 10			
control	12.31	63.5	4.63
transgenic	11.85	58.7	>40 min
2: 2*, 7 + 9, 5 + 10			
control	11.89	62.8	4.13
transgenic	11.99	62.9	>40 min
3A: 1, 7 + 9, 5 + 10			
control	11.27	61.7	3.13
transgenic	11.65	62.4	23.50
3B: 1, 17 + 18, 5 + 10			
control	9.90	59.4	5.13
transgenic, without 1RS	10.13	57.8	19.50
transgenic, with 1RS	10.48	60.4	9.50

Tortillas are extensively consumed in the United States, with annual sales exceeding $6 billion (*14*). Desirable characteristics of tortillas include large diameter, high flexibility and opacity, light color, and long shelf stability. The parameters diameter, flexibility, and shelf stability are influenced by wheat gluten proteins (*15*−*18*). To date, only one study, using near-isogenic wheat lines, reported the relationships between tortilla quality characteristics with specific wheat proteins (*19*). It was reported that the HMW-GS coded by *Glu-A1* (HMW-GS 1Ax1) and *Glu-D1* (HMW-GS 1Dx5+1Dy10 or 1Dx2 + 1Dy12) contribute to tortilla stability and flours containing HMW-GS 5+10 produce tortillas with good shelf stability, while absence of HMW-GS 1Dx5 caused a decrease in shelf stability and an increase in tortilla diameter. More investigations are necessary to better understand how individual HMW-GS affect tortilla quality properties.

To determine the effect of overexpression of HMW-GS 1Dy10 on tortilla quality properties, flours derived from sister lines of nontransgenic (control) and transgenic (overexpressing HMW-GS 1Dy10) were used to make tortillas. Subsequently, tortillas were tested by an array of physical and biochemical experiments designed to assess tortilla quality parameters.

MATERIALS AND METHODS

Plant Materials and Experimental Design. Two transgenic wheat lines, designated Dy10-E and B52a-6, were produced via particle gun bombardment, as described by Blechl et al. (*12*), using the hard white spring wheat "Bobwhite" as the recipient. Line Dy10-E was transformed with a construct containing the endosperm-specific promoter, coding and terminating sequences from the native common gene (*Glu-Dy10*) encoding wheat high molecular weight (HMW) glutenin subunit 10 (*20*). This event resulted in the overexpression, in endosperm, of HMW glutenin subunit 10 along with the native accumulation levels of HMW glutenin subunits Ax2*, Bx7, By9, Dx5, and Dy10. B52a-6 also was transformed to overexpress HMW glutenin subunit 10, using the same promoter, coding, and terminating sequences as used to develop Dy10-E. The transformation procedure for B52a-6 was as described in Weeks et al.(*6*), except that 5-day-old excised embryos were incubated on callus induction media containing 0.4 M mannitol 4 h before and 20 h after bombardment. Transformant B52a-6 overexpressed HMW glutenin subunit 10, and, in addition, two novel proteins, which migrated in the zone between native wheat HMW glutenin subunits 5 and 7, were detected by SDS−PAGE. The identities of these proteins have not been determined.

Homozygous plants of each transgenic line were greenhouse-grown, and used as females in controlled matings with hard winter wheat pollen donors. Winter wheats used to produce the F_1 and BC_1F_1 generations were selected at random from a collection of Nebraska and Kansas adapted cultivars and advanced breeding lines. Resultant F_1 plants were seeded in the greenhouse, and, in the spring of 1999, again used as females in backcrosses with hard winter wheat male parents. Twelve plants from each BC_1F_1 were seeded in the greenhouse, and BC_1F_2 populations harvested in-bulk in the spring of 2000. From each population, approximately 100 individual seeds were harvested, and advanced by single-seed-descent to the BC_1F_4 generation. Eight seeds from each plant were ground in a coffee grinder, and composition of gluten proteins were evaluated via SDS−PAGE. On the basis of overexpression of HMW glutenin protein subunits, putative transgenic lines were identified in three populations. Control sister lines, which did not overexpress HMW-GS 1Dy10, were also cultivated along with the transgenic lines in each population. The pedigrees of the populations are Dy10-E/N97S286//TAM202, Dy10-E/W96−495W//N86L177, and B52a-6/Jagger//Heyne for populations designated 1, 2, and 3, respectively. From each population a minimum of eight transgenic, and an equal number of nontransgenic, sister lines were seeded in the greenhouse and harvested in 2003. Twelve seeds were planted from each BC_1F_4-derived line, and plants were harvested in-bulk. In the fall of 2003, lines were seeded in unreplicated 5 sq m plots at Mead, NE. Plots were trimmed to 2.9 sq m before harvest. Following harvest, proteins were extracted from 12 seeds per line for each putative transgenic. In the fall of 2004, the transgenic lines, along with nontransgenic sister lines, and the control lines cited above, were seeded in a three replication randomized complete block design, also at Mead, NE. Plot size at harvest was 8 sq m. The study was replanted at Mead, NE in the autumns of 2005 and 2006, again using three-replication randomized complete block designs. Subsequent to harvest, grain quality traits were evaluated.

Bobwhite carries the 1BL.1RS wheat-rye chromosomal translocation derived from Aurora. In addition, TAM202, a parent used to develop population 1, carries the Amigo 1AL.1RS wheat-rye chromosomal translocation. The presence of 1RS was determined by detection of rye-derived secalins in analysis of ethanol-soluble proteins using SDS−PAGE (*21*).

Flours were tested for protein content and mixing properties at the USDA-ARS Hard Winter Wheat Quality Laboratory (Manhattan, KS) and the results of these tests are shown in **Table 1**. Flours derived from control and transgenic wheats from the three populations were paired by similar protein contents. Two sets of samples were chosen from populations 1 and 3, designated "A" and "B", which originated from the same parents, but have different HMW-GS composition. Transgenic 3B refers to the transgenic line without 1RS translocation, while 3B-1RS refers to the transgenic line with 1RS rye-translocation.

Protein Analysis. *Reverse-phase High Performance Liquid Chromatography (RP-HPLC).* RP-HPLC was performed to determine the level of HMW-GS 1Dy10 overexpression in flours from transgenic wheats relative to controls. Monomeric proteins were extracted twice from flour samples (100 mg) by solubilization in 1 mL of 7.5% *n*-propanol containing 0.3 M NaI and constant vortexing for 30 min at room temperature (*22*). Supernatants were discarded and pellets were washed in 1 mL of deionized water for 5 min. Polymeric proteins were extracted from the pellet twice with 1 mL of 50% *n*-propanol containing 2% β-mercaptoethanol for 30 min at 40 °C. Aliquots (500 μL) of the two

extractions were pooled together and samples (300 μL) were alkylated with 20 μL of 4-vinylpyridine for 15 min at 60 °C. The resulting protein sample was analyzed by RP-HPLC (Agilent 1100 Series, Agilent Technologies, Palo Alto, CA) as described previously (*23*). Briefly, protein samples (1 μL) were injected into a Poroshell 300SB-C8, 2.1 × 75 mm, 5 μm particle size column (Agilent Technologies) maintained at 65 °C. Solvent flow rate was 0.7 mL/min and was composed of a nonlinear gradient of water (A) and acetonitrile (B), both containing 0.1% trifluoroacetic acid (v/v). The gradient was as follows: from 0 to 1 min, 23% B; from 1 to 3 min, the gradient increased from 23 to 30% B; from 3 to 11 min, increased from 30 to 44% B; from 11 to 12 min, the gradient decreased from 44 to 23% B and kept at 23% B until 13 min. Detection was carried out by a UV detector at 206 nm. The level of HMW-GS 1Dy10 overexpression was calculated by the ratio between the area under the curve of peaks derived from transgenic and control, normalized to their respective protein contents.

Polymeric Protein. Monomeric proteins were extracted from flour samples as described above. Supernatants were discarded and the resulting pellets were lyophilized (Labconco Corporation, Kansas City, MO). Pellet protein content was determined following AACC Method 46-30 (*24*), using LECO FP-428 nitrogen determinator (St. Joseph, MI). Polymeric protein percentage (%PP) was calculated by multiplying nitrogen values by a conversion factor of 5.7 and dividing by total flour protein (*25*).

Size-Exclusion High Performance Liquid Chromatography (SE-HPLC) and Multiangle Laser Light Scattering (MALLS). To determine the molecular weight distribution of the largest protein polymers in the samples, proteins from "insoluble fractions" of flours derived from control and transgenic wheats were characterized using a combination of SE-HPLC and MALLS. Soluble polymeric proteins (SPP) were first removed by extracting flours (100 mg) twice with a 50 mM sodium phosphate (1 mL, pH 7), containing 1% SDS and vortexing for 5 min at room temperature. After centrifugation, the supernatants were discarded. Pellets were resuspended in 1 mL of the same solvent and insoluble polymeric proteins (IPP) were extracted from pellets via sonication (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) for 30 s at power setting 10 W. Resulting protein extracts were analyzed by SE-HPLC, coupled to a MALLS detector (DAWN EOS, Wyatt Technology Corp. Santa Barbara, CA) as described by Bean and Lookhart. (*26*). To determine the molecular weight, average molar mass of protein polymers, a Dn/Dc value for wheat proteins of 0.31 was used (*26*).

Microscale Dough Extensibility Test. Microscale dough extension test was performed using a texture analyzer equipped with a Kieffer rig (model TA.XT.Plus, Texture Technology Corp., Scarsdale, NY). Dough was prepared using a 10 g mixograph (National Manufacturing Mixograph, Lincoln, NE), using optimal water absorption and mixing time parameters that had been previously determined (**Table 1**). A constant mixing time of 40 min was used for flours derived from transgenic wheats that exhibited mixing time greater than 40 min without achieving a peak. Dough preparation and all procedures involved in this test were performed as recommended by the manufacturer. Dough resting was performed in a chamber at 35 °C with 70% relative humidity (RH). From each dough sample, extensibility (mm) and resistance to extension (Rmax) were measured.

Tortilla Formulation and Processing. Tortillas were made by the hot-press method, adapted to a research laboratory setting as described previously (*27*). Dough development time was constant (4 min) for control flours, while doughs originated from transgenic wheats were developed within a range of 6−19 min. Dough samples were placed in a closed plastic container, rested for 5 min at room temperature, divided into 40 g pieces, and rolled into balls using an automatic rounder (Round O Matic dough rounder, AM Manufacturing, Dolton, IL). Additional resting in a proof chamber (Oliver Products Company, Grand Rapids, MI) at 35 °C with 70% RH was maintained for 30 min.

Dough balls were pressed using a tortilla dough press (TXA-SS Tortilla Press, DoughXpress, Pittsburgh, KS) with both top and bottom platens set at 71 °C for 10 s under the "thin" setting. Immediately after pressing, tortillas were baked on a griddle (DoughPro, model 1520) at 160 °C, for 30 s on each side, followed by an additional 10 s on each side. Tortillas were allowed to cool on a metal baking rack for about 5 min, packaged into plastic bags, and stored at room temperature, protected from light.

In an additional experiment, tortillas were prepared with a mixture of flours derived from control (50%) and transgenic (50%) wheats of experimental populations 1A, 2, and 3B (with and without 1RS translocation). This test was not performed in populations 1B and 3A due to insufficient availability of flours. The processing was the same as above-described, with the exception of the mixing time, which was adjusted to 4 min plus half of the time used to mix the respective dough obtained from transgenic wheat.

Tortillas were also prepared with addition of cysteine at concentrations of 0, 6, 50, and 300 μg/g. Because of limited flour availability, a different pair of flours obtained from control and transgenic wheats derived from population 1 was used. The flour protein contents were 11.21% (control) and 10.71% (transgenic). Cysteine was added only to formulations made with transgenic wheats. The processing was identical as described above, with a mixing time of 9 min for the dough made with transgenic wheat.

Tortilla Quality Tests. Tortilla quality parameters measured were diameter, thickness, and texture. All tests were performed 2 h after baking, designated day 0 (d 0). Tortilla texture tests were performed at days 2, 4, 7, and 14. Two diagonal measurements of each tortilla diameter were measured with a ruler and averaged. Tortilla thickness was determined using a digital caliper. Tortilla texture was measured subjectively using rollability test and objectively by the extensibility test using a texture analyzer. In the subjective rollability test (*28*), determinations were assigned according to a scale ranging from 1 (impossible to roll due to breakage) to 5 (no cracking or breaking). Objective extensibility tests (*27*) were performed using a texture analyzer. Values of rupture force (Fr) and stretchability (distance at Fr) were derived from the force−distance graph.

Statistical Analysis. Means, standard error of the means, and *t*-tests were derived with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA), and one way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) were derived with SAS statistical software package (SAS Institute, Cary, NC).

Protein analysis (determination of level of overexpression of HMW-GS 1Dy10, %PP and molecular weight of protein polymers in the insoluble fraction) were all done in duplicate samples and the reported values are the means of these two measurements. For the microscale dough extensibility test, dough samples were made in triplicate for each flour and from each dough sample, four dough strips were analyzed. The values reported are the mean of triplicates. The means of diameter and thickness were obtained on 23 tortillas in each experimental group, which were the total tortillas made in one single recipe of tortilla. The rollability scores were determined on one tortilla from each experimental group at d 0, and this was the value reported and on three tortillas from each experimental group at d 2, d 4, d 7 and d 14, in which scores were averaged. Parameters obtained from the texture analyzer were evaluated on two tortillas from each experimental group on all days of analysis. Each tortilla provided four strips and the mean of eight measurements was determined for each experimental group and for each day of analysis. In order to identify significant differences between samples derived from control and transgenic wheats, *t*-test was performed in groups 1A, 1B, 2, and 3A. In group 3B, since three samples were compared (control, transgenic, and transgenic-1RS), one way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) at level of 0.05 were used (SAS statistical software package, SAS Institute, Cary, NC). The statistical analysis of the parameters measured over a period of time (rupture force and stretchability) was performed within each time point.

In the experiment in which tortillas were made with a mixture of flours derived from control and transgenic wheats, the mean of diameter and thickness was obtained on three tortillas, rollability was determined on one tortilla at days 0, 2, and 7 (value reported), and texture properties were obtained from one tortilla, which provided three strips per day of analysis and the values were averaged. These values were compared to tortilla quality properties obtained from the original tortilla recipe, which was made with only flour derived from control and transgenic wheats. ANOVA and Fisher's LSD was used to determine significant differences among the samples.

The analysis of diameter and thickness of tortillas made with the addition of cysteine were performed on three tortillas, rollability was determined on one tortilla at days 0, 2, 7, and 14, and texture was determined on one tortilla per day of analysis, which provided three strips. ANOVA and Fisher's LSD was used to determine significant differences among the samples.

RESULTS AND DISCUSSION

Flours obtained from transgenic wheats were used in this study to investigate the effects of increased expression of HMW-GS 1Dy10 on dough and tortilla quality properties. Flours derived from control and transgenic wheats in each experimental group were paired by similar total protein content, as it is known that protein content modulates tortilla quality (*15*−*18*, *29*).

The level of overexpression of HMW-GS 1Dy10 in transgenic wheats was determined by RP-HPLC. Integration of the chromatograms revealed the flours derived from transgenic wheats to have a 5.84, 4.22, 5.76, 4.82, 3.55, and 2.5-fold increase in HMW-GS 1Dy10, compared to controls in populations 1A, 1B, 2, 3A, 3B, and 3B-1RS, respectively. HMW-GS 1Dy10 levels in flours obtained from transgenic wheats without 1RS translocation were on average 4.8-fold greater than the respective control flours, while flour derived from wheat in population 3B-1RS had a reduced expression of HMW-GS 1Dy10 when compared to other transgenic samples, determined to be 2.5-fold increased over that of its respective control. The overexpression of HMW-GS 1Dy10 resulted in significant negative effects on mixing behavior, as well as on dough and final product properties. Flours originated from transgenic wheats exhibited longer times to achieve dough peak development than controls, as observed by mixing times (**Table 1**). Flours from transgenic wheats from populations 1B and 2 did not achieve dough development in the mixograph analysis by 40 min and interruption of mixing had to be conducted before a dough peak was achieved. Lines with the greatest level of HMW-GS 1Dy10 (5.8 fold increase in populations 1A and 2) showed the longest mixing times, while the line with the lowest level (2.5 fold in the transgenic with 1RS translocation in population 3B) showed the shortest mixing time. Those observations are in agreement with several other studies describing increased mixing time in the presence of elevated amount of one or more HMW-GS (*9*, *10*, *12*, *30*). In the study of Blechl et al. (*12*), it was demonstrated that flours with increased in HMW-GS 1Dx5 and/or 1Dy10 levels had mixing times at least twice as long as controls, and flours with increases in HMW-GS 1Dy10 had mixing curves typical of strong doughs, with increased mixing tolerance, but with increasingly longer dough development time. In their study, as the level of HMW-GS 1Dy10 increased, the mixing time increased, the peak resistance declined and bandwidths after the peak increased.

Further characterization of protein structure was investigated by determining the %PP, and results are shown in **Table 2**. Control samples ranged from 59.37 to 63.61%PP, while flours derived from transgenic wheats exhibited 66.01 to 74.48%PP. *T*-tests performed within each experimental population revealed that the %PP was consistently and significantly greater in samples derived from transgenic wheats versus controls ($P < 0.05$), except in population 3B. Flour from transgenic wheat in the population 3B-1RS revealed smaller %PP than the respective control flour.

SE-HPLC, coupled with a MALLS detector, was used to characterize the molecular weight (M_w) distribution of the protein polymers in the insoluble fraction. On the basis of the pattern of molecular weight curve, the excluded peak from SE-HPLC was split into two peaks, designated IPPE1 and IPPE2. The IPPE1 correspond to the polymers with the largest molecular weight (**Table 2**). M_w of protein polymers in the IPPE1 region of control samples ranged from 10.69×10^6 to 17.22×10^6 Da, while samples obtained from transgenic wheats exhibited values from 12.44×10^6 to 23.31×10^6 Da. Although samples from transgenic wheats exhibited greater M_w than that of control samples in this region, significant differences were observed only in population 2 ($P < 0.05$). The M_w of protein polymers from samples derived from transgenic wheats were also greater than control in the IPPE2 region; however, significant differences were observed in population 1B only ($P < 0.05$). The increases in M_w along with increases in the %PP demonstrate that in general the transgenic lines contained proteins with a larger molecular weight distribution than those of the controls.

Table 2. Percentage of Polymeric Protein (%PP) and Molecular Weight of Protein Polymers in the Insoluble Fraction of Flours Derived from Control and Transgenic Wheats[a]

populations	%PP	molecular Weight (Da)	
		IPPE1	IPPE2
control-1A	$61.78^{b} \pm 0.96$	$11.65 \times 10^{6\ a}$	$1.92 \times 10^{6\ a}$
transgenic-1A	$72.11^{a} \pm 0.28$	$12.44 \times 10^{6\ a}$	$2.05 \times 10^{6\ a}$
control-1B	$63.61^{d} \pm 0.22$	$10.69 \times 10^{6\ b}$	$2.01 \times 10^{6\ c}$
transgenic-1B	$74.48^{c} \pm 0.25$	$23.31 \times 10^{6\ b}$	$3.42 \times 10^{6\ b}$
control-2	$59.37^{f} \pm 0.58$	$10.81 \times 10^{6\ d}$	$2.05 \times 10^{6\ d}$
transgenic-2	$73.77^{e} \pm 0.60$	$14.33 \times 10^{6\ c}$	$2.88 \times 10^{6\ d}$
control-3A	$60.56^{h} \pm 1.15$	$12.29 \times 10^{6\ e}$	$1.99 \times 10^{6\ e}$
transgenic-3A	$67.99^{g} \pm 0.22$	$14.58 \times 10^{6\ e}$	$2.66 \times 10^{6\ e}$
control-3B	$62.47^{i} \pm 2.61$	$17.22 \times 10^{6\ f}$	$2.08 \times 10^{6\ f}$
transgenic-3B, without 1RS	$66.01^{i} \pm 1.34$	$19.04 \times 10^{6\ f}$	$2.73 \times 10^{6\ f}$
transgenic-3B, 1RS	$57.29^{j} \pm 2.66$	$15.62 \times 10^{6\ f}$	$2.23 \times 10^{6\ f}$
LSD*	10.28	5.09×10^{6}	0.58×10^{6}

* LSD: least significant difference for population 3B, determined at 0.05 level of significance. [a] Values are expressed as mean ± standard error of the mean. Within a column and each population, mean values exhibiting the same letter are not significantly different ($P < 0.05$).

Data from dough microextension tests showed that HMW-GS 1Dy10 overexpression influenced dough properties (**Table 3**). Doughs from populations 1A, 3A, and 3B had a similar pattern of Rmax for flours derived from control and transgenic wheats. Rmax ranged from 23.27 to 31.93 g in control doughs and from 55.76 to 82.36 g in doughs from transgenic wheats. Flours derived from transgenic wheats produced doughs with significantly greater resistance to extension than control flours in these three experimental pairs ($P < 0.05$). In populations 1B and 2, flours originated from transgenic wheats showed lower resistance to extension, which was statistically significant in population 2 only ($P < 0.05$). In population 3B, the flour derived from 1RS-transgenic wheat produced dough with Rmax of 33.16 g, which was not statistically different from its control ($P < 0.05$). Extensibility of doughs made with control flours ranged from 42.05 to 83.39 mm, while doughs made with flours from transgenic wheats exhibited 18.91 to 33.64 mm. These results revealed that transgenic wheats produced doughs significantly less extensible than controls in all populations ($P < 0.05$). In population 3B, the extensibility of the dough originated from 1RS-transgenic wheat was 43.84 mm, while its control was 54.06 mm. This apparent difference did not prove statistically significant ($P < 0.05$). In summary, doughs produced with flours derived from transgenic wheats were less extensible (except in group 3B-1RS), with a greater resistance to extension (except groups 1B and 2) than control flours. These observations are in agreement with a study in which incorporation of isolated HMW-GS 1Dy10, or all HMW-GS, to flour led to a decrease in dough extensibility and an increase in resistance to extension (*31*). On the other hand, the lack of all HMW-GS in wheat flour has been linked to a decrease in both dough resistance to extension and extensibility (*32*). The greater resistance to extension and lower extensibility observed in doughs containing an increased amount of HMW-GS 1Dy10 as presented here suggest the development of a very strong gluten network in these doughs. The development of strong dough using a flour with an elevated amount of HMW-GS 1Dy10 has been previously reported (*12*). Therefore, it is likely that the negative

Table 3. Properties of Dough and Tortilla Quality Made with Flours Derived from Control and Transgenic Wheats[a]

	dough properties		tortilla quality properties	
populations	resistance to extension (g)	extensibility (mm)	thickness (mm)	diameter (cm)
control-1A	$31.93^{b} \pm 1.47$	$49.62^{a} \pm 7.22$	$2.526^{b} \pm 0.05$	$15.9^{a} \pm 0.12$
transgenic-1A	$59.15^{a} \pm 3.81$	$18.91^{b} \pm 0.66$	$2.709^{a} \pm 0.07$	$13.7^{b} \pm 0.11$
control-1B	$46.79^{c} \pm 2.44$	$42.05^{c} \pm 3.14$	$2.339^{d} \pm 0.07$	$16.0^{c} \pm 0.11$
transgenic-1B	$46.53^{c} \pm 1.88$	$19.31^{d} \pm 0.65$	$2.643^{c} \pm 0.07$	$13.5^{d} \pm 0.10$
control-2	$30.71^{d} \pm 1.26$	$71.72^{e} \pm 5.62$	$2.233^{f} \pm 0.07$	$16.1^{e} \pm 0.11$
transgenic-2	$15.01^{e} \pm 0.48$	$33.64^{f} \pm 0.69$	$2.804^{e} \pm 0.04$	$13.8^{f} \pm 0.08$
control-3A	$23.27^{g} \pm 0.14$	$83.39^{g} \pm 0.54$	$2.200^{h} \pm 0.07$	$16.9^{g} \pm 0.10$
transgenic-3A	$82.36^{f} \pm 3.04$	$20.73^{h} \pm 0.97$	$2.622^{g} \pm 0.09$	$14.1^{h} \pm 0.16$
control-3B	$30.50^{i} \pm 1.76$	$54.06^{i} \pm 3.46$	$2.109^{j} \pm 0.05$	$16.2^{i} \pm 0.12$
transgenic-3B, without 1RS	$55.76^{h} \pm 2.05$	$19.15^{j} \pm 1.10$	$2.313^{i} \pm 0.04$	$14.8^{k} \pm 0.07$
transgenic-3B, 1RS	$33.16^{i} \pm 2.08$	$43.84^{i} \pm 5.10$	$2.257^{i,j} \pm 0.07$	$15.9^{j} \pm 0.13$
LSD[b]	6.81	12.52	0.16	0.31

[a] Values are expressed as mean ± standard error of the mean. Within a column and each population, mean values exhibiting the same letter are not significantly different ($P < 0.05$). [b] LSD: least significant difference for population 3B, determined at 0.05 level of significance.

Table 4. Pearson's Correlation of Tortilla Diameter with Polymeric Protein (PP) and Dough Properties (Extensibility and Resistance to Extension)

	diameter	PP	extensibility	resistance to extension
diameter	1			
PP	**−0.919**	1		
extensibility	**0.867**	−0.726	1	
resistance to extension	−0.466	0.294	−0.659	1

effects observed on dough properties are derived from the formation of very strong gluten network, which in turn, affected final tortilla quality.

Tortilla quality was evaluated by diameter, thickness and texture. Results from tortilla diameter and thickness are summarized in **Table 3**. Tortilla diameter from control samples ranged from 15.9 to 16.9 cm, while tortillas made from transgenic wheats ranged from 13.5 to 14.8 cm. Tortillas made with flours containing elevated amounts of HMW-GS 1Dy10 exhibited significantly smaller diameter than controls in all populations ($P < 0.05$) (**Table 3**). Small bread loaf volume has also been previously reported with an increase in the level of HMW-GS 1Dx5 (*10*). Pearson's correlation analysis was carried out to investigate the relationship between diameter and %PP, as well as diameter and dough properties (**Table 4**). Results indicated that diameter had a high negative correlation ($R = -0.919$) with %PP and a high positive correlation ($R = 0.867$) with dough extensibility. The % PP represents the large glutenin polymers that are left behind after removal of monomeric proteins. A number of studies have reported directly correlations between the amount of polymeric protein and functional properties of flour, such as mixing time, dough strength, and loaf bread volume (*33*−*36*). However, as flour for tortilla production is different from bread, this high negative correlation between diameter and %PP indicates that flours producing overstrong doughs, as observed in transgenic wheats, are more likely to produce tortillas with a small diameter. The balance between the viscosity and elasticity in dough modulates the final product properties (*37*). In order to obtain large diameter tortillas, extensible dough with low elasticity is required. Since dough originated from transgenic wheats exhibited lesser extensibility, tortillas from those flours had reduced diameters. In addition, since glutenins modulate dough elasticity (*38*), increased amount of HMW-GS 1Dy10 caused excessive elasticity and shrinkage of tortillas after hot-pressing, resulting in tortillas with decreased diameter and irregular shapes. This is in agreement with the findings of Uthayakumaran et al. (*32*), who demonstrated that flours lacking all HMW-GS produced dough with very low elasticity, tortillas did not shrink after hot-press and large diameters were obtained. However, flour lacking all HMW-GS also produced dough with very low extensibility and tortillas still exhibited large diameters, an observation that contradicts the high and positive correlation between dough extensibility and diameter derived from data presented here. Therefore, results presented here combined with previously published data (*32*) suggest that a balance in HMW-GS is important to obtain desirable dough extensibility and elasticity, and consequently, tortillas with increased diameter. Because of the long mix times of flours derived from transgenic wheats, the dough was not mixed to complete development. This most likely detrimentally contributed to lower tortilla quality. Srinivasan et al. (*39*) found that undermixed doughs resulted in smaller diameter tortillas with a shorter shelf stability compared to tortillas made from optimum mixed dough. The extremely long mixing times of flours derived from transgenic wheats (20 to >40 min) makes them unsuitable for commercial bakeries, although this flour has potential to be added to very weak flour to increase dough strength.

Tortilla thickness from control flours ranged from 2.1 to 2.5 mm. On average, tortillas derived from transgenic wheats were 15% thicker than control tortillas. ANOVA indicated that tortillas made from flours with increased amount of HMW-GS 1Dy10 were significantly thicker than their respective controls in all populations, except in 3B-1RS ($P < 0.05$). Adequate tortilla thickness ranges from 1 to 5 mm (*40*). Under normal conditions, an increase in tortilla thickness is largely derived from increased moisture retention and puffing, which occurs when air bubbles incorporated by mixing expand during baking (*40*). However, data presented here reveals that tortillas made with flours derived from transgenic wheats exhibited greater thickness and a rough, nondesirable appearance. These observations associated with high dough elasticity and reduced tortilla diameter suggest that increased moisture retention was not the only underlying factor, and most likely greater thickness resulted from shrinkage of the tortillas. Evidence to support this statement came from the results of dough properties, in which doughs made from transgenic wheats exhibited higher gluten strength than controls. It was demonstrated that stronger doughs produced by addition of vital wheat gluten (VW gluten) produced small diameter tortillas due to high elasticity (*41*). Therefore, the greater thickness observed in tortillas made from transgenic wheats might have originated from highly elastic dough that shrank after being hot-pressed.

Results from the subjective rollability test are shown in **Figure 1**. Tortillas made from both transgenic and control wheats from populations 1A, 1B, and 2 showed similar rollability scores

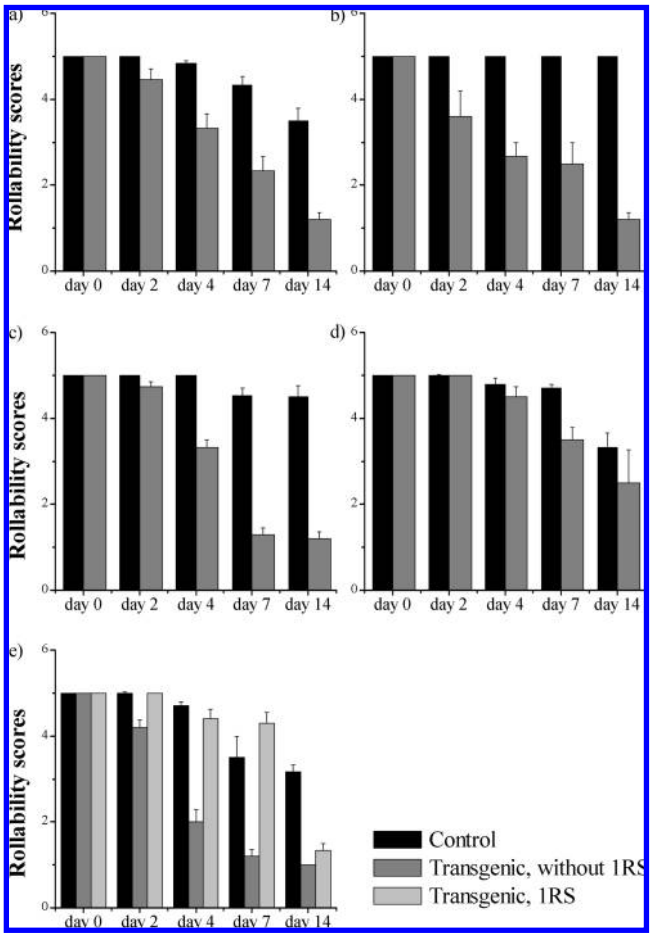

Figure 1. Subjective rollability of tortillas made with flours derived from control and transgenic wheats: (a) Population 1A, (b) Population 1B, (c) Population 2, (d) Population 3A, and (e) Population 3B. Values reported are the mean and the standard error of the mean.

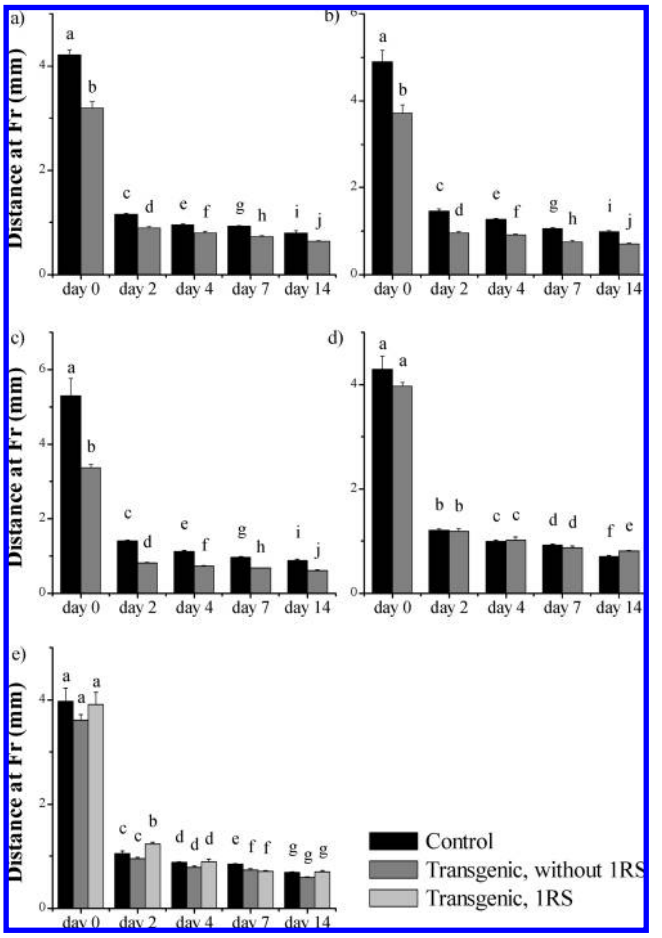

Figure 2. Stretchability of tortillas made with flours derived from control and transgenic wheats: (a) Population 1A, (b) Population 1B, (c) Population 2, (d) Population 3A, and (e) Population 3B. Values reported are the mean and the standard error of the mean. Within each time point, mean values exhibiting the same letter are not significantly different ($P < 0.05$). In population 3B, the LSD are 0.60, 0.13, 0.11, 0.05, and 0.05 for days 0, 2, 4, 7, and 14, respectively.

over time. In these populations, substantial lower rollability scores were measured in tortillas made with flours derived from transgenic wheats after d 4. In population 3A, control tortillas also exhibited better rollability scores than tortillas made with flour from transgenic wheat over time, although the decline in rollability scores in the tortillas from transgenic wheats was not as intense as in transgenic from the previous three populations. In population 3B, tortillas made with flour derived from 3B-transgenic wheat had substantial lower rollability scores than control tortillas and tortillas made with flour derived from 3B-1RS-transgenic wheat at all time points after d 2. Tortillas made with flour derived from control and 3B-1RS transgenic wheat were similar in most days of analysis, except at d 14.

One of the parameters measured by the objective extensibility test was stretchability (**Figure 2**). Tortillas from control and transgenic wheats from populations 1A, 1B, 2 and 3A had a similar pattern of stretchability, in which tortillas exhibited the highest value at d 0, a decrease in their stretchabilities by ~73% at d 2, and smaller decreases after d 2. Control tortillas had significantly greater stretchabilities than tortillas made from flours with elevated amount of HMW-GS 1Dy10 in populations 1A, 1B, and 2 ($P < 0.05$) at all time points. In population 3A, there was no significant difference in stretchability between control and tortillas made from flours derived from transgenic wheats at all time points, with the exception of d 14 ($P < 0.05$). In population 3B, significant differences in stretchability between control and tortillas made from transgenic wheats (with and without 1RS translocation) were not observed at d 0, d 4, and d 14. Rupture force (Fr) was the second parameter measured by the extensibility test and the results are exhibited in **Figure 3**. Fr of tortillas made from flours in populations 1A, 1B, 2, and 3A were also similar, with small differences in mean values. Tortillas made with transgenic wheats had significantly greater rupture forces than control tortillas at all time points ($P < 0.05$). In population 3B, ANOVA revealed that tortillas made with flour derived from transgenic wheat without 1RS translocation had significantly greater Fr than control tortillas and tortillas made with flour derived from 3B-1RS-transgenic wheat at all time points. Rupture forces of tortillas made with flour originated from 3B-1RS-transgenic wheat were not significantly different from control tortillas, except for d 2 and d 7 ($P < 0.05$).

The texture parameters as measured by rollability, stretchability, and Fr are related because they derive from a common biochemical factor: the gluten protein network. The HMW-GS gluten was shown to be very important for tortilla shelf stability, since tortillas made from flours lacking some or all of the HMW-GS exhibited low rollability (*19*, *32*). However, data presented here demonstrated that excess of HMW-GS 1Dy10 also caused a decrease in rollability scores. This outcome may be associated with the formation of a nonoptimum gluten protein structure for tortillas, caused by excessive incorporation of HMW-GS 1Dy10

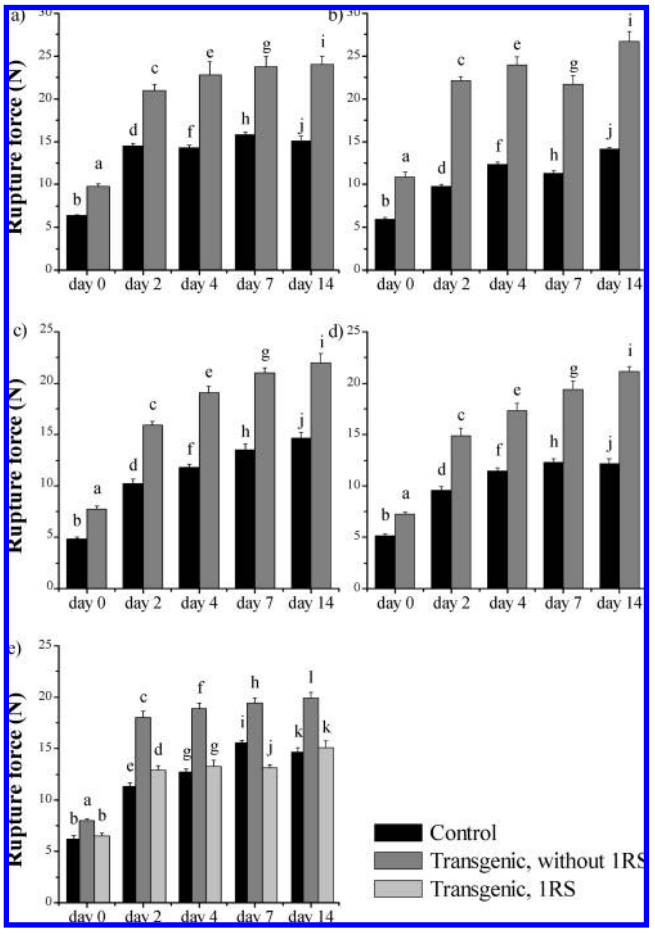

Figure 3. Rupture force of tortillas made with flours derived from control and transgenic wheats: (a) Population 1A, (b) Population 1B, (c) Population 2, (d) Population 3A, and (e) Population 3B. Values reported are the mean and the standard error of the mean. Within each time point, mean values exhibiting the same letter are not significantly different ($P < 0.05$). In population 3B, the LSD are 0.90, 1.43, 1.49, 1.04, and 1.68 for days 0, 2, 4, 7, and 14, respectively.

into the protein polymer. Evidence supporting that HMW-GS 1Dy10 was excessively added to protein polymers and formed a highly cross-linked structure is the data presented in this study that revealed greater %PP and larger M_w polymer size in flours derived from transgenic wheats when compared to controls. Increased incorporation of HMW-GS 1Dy10 among other gluten proteins promoted an increase in the size of protein polymers and higher levels of polymeric proteins. These findings are in agreement with a previous report in which overexpression of HMW-GS 1Dx5 caused an increase in cross-linking of gluten proteins (*11*). Wheats overexpressing HMW-GS 1Dx5 produced dough with limited expansion potential and with very low volume bread. These findings were also linked to high cross-linking among proteins (*10*). It has been demonstrated that larger protein polymers are formed and decreased protein solubility is observed when the number of HMW-GS increases in flour (*42*). Excessive polymerization formed in tortillas made with flours originated from transgenic wheats could also justify the significantly higher rupture force and decreased stretchability observed in tortillas made from transgenic wheats.

Overexpression of HMW-GS 1Dy10 in group 3B-1RS did not exhibit the same negative effects on dough and tortilla properties as the transgenic without 1RS translocation. The results were mostly comparable to control. The relative level of HMW-GS 1Dy10 in flour derived from 3B-1RS-transgenic wheat was 2.5-fold of control, while flours derived from transgenic wheats without 1RS translocation revealed ~5-fold increase. The results from tortilla properties of the 3B-1RS flour most likely derive from the fact that 1RS translocation changes the protein composition of flour. Normally, the 1RS translocation introduces genes to the wheat genome that promote deleterious effect on dough and bread properties, as observed by low loaf volumes, production of sticky doughs, reduced dough strength, lack of tolerance to overmixing, and low SDS sedimentation volumes (*43*−*49*). Wheat cultivars containing 1RS translocations have an increase in hydrophilic monomeric proteins and a decrease in hydrophobic monomeric and polymeric proteins (*46*, *47*, *50*−*52*). A combination of factors such as lower overexpression (2.5-fold instead of 5-fold) of HMW-GS 1Dy10, differences in protein composition in the 1RS-transgenic wheat, and the association of 1RS translocation with low dough strength might form the basis for the similarities between dough and tortilla properties of transgenic in group 3B-1RS and control. Polymeric protein formation in the 3B-1RS line did not appear to be similar to the non-1RS transgenic lines possibly due to the lower amount of HMW-GS 1Dy10 present in the 1RS transgenic line and the absence of some LMW glutenins in this sample. Three pieces of evidence presented here support this statement. First, the 1RS-transgenic line exhibited the lowest %PP among all experimental samples tested and it was comparable to the respective control. Second, the M_w of protein polymers in the insoluble fraction of 3B-1RS transgenic was very similar to its control line. Third, the mixing time of this flour was substantially shorter (9 min) than the non-1RS transgenic that had a mix time of 19.5 min. This was much closer to that of the control mix time of 5.13 min. Proper mixing is essential for flour components to become hydrated and allow formation of the gluten network. Results on the dough and tortillas prepared from flours derived from transgenic wheats are similar to that obtained from underdeveloped (undermixed) dough. Underdevelopment results in poor dough extensibility and smaller bread loaf volumes with poor crumb structure (*10*). Additionally, overly strong dough results in longer mix times. Methods to overcome the effects of strong doughs include mixing at higher speed or blending with flours that have normal properties (*30*).

To confirm that the observations detected in tortilla quality were influenced by the overexpression of HMW-GS 1Dy10, tortillas were also prepared with a mixture of flours derived from control (50%) and transgenic (50%) wheats. HMW-GS 1Dy10 levels in the resultant mixtures were slightly greater than the half overexpression of HMW-GS 1Dy10 in the transgenic wheat alone in all populations analyzed (data not shown). Flour mixtures produced tortillas with characteristics that were either intermediate to those made with flour derived only from control or only from transgenic wheat, or resembled tortillas made with only control flour. Tortillas made with a mixture of control and flour derived from transgenic wheats from populations 1A, 2, 3B, and 3B-1RS had thickness between 2.23 and 2.37 mm and diameters between 15.15 and 15.92 cm. Results from texture analysis are shown in **Table 5**. Rollability scores of tortillas made with flour mixture were lower than those of control and transgenic at d 2 (populations 3B and 3B-1RS) and d 7 (populations 1A and 3B-1RS). Rupture force values of tortillas made with flour mixtures were intermediate between control and transgenic, largely resembling the results derived from control tortillas at almost all time points. Stretchability was either in between that of control and transgenic or significantly greater. These results additionally support that greater HMW-GS 1Dy10 levels in flours originated from transgenic wheats underlie the differences

Table 5. Quality Properties of Tortillas Made with Flours Derived from Control, Transgenic, and Mixture of Control and Transgenic Wheat (50% each)[a]

	rollability			rupture force			stretchability		
population	day 0	day 2	day 7	day 0	day 2	day 7	day 0	day 2	day 7
control-1A	5.00	5.00	4.30	6.36^{c}	14.51^{b}	15.87^{b}	4.21^{a}	1.16^{b}	0.93^{b}
transgenic-1A	5.00	4.47	2.30	9.81^{a}	21.02^{a}	23.75^{a}	3.20^{b}	0.90^{c}	0.73^{c}
mixture-1A	5.00	5.00	1.00	7.93^{b}	16.36^{b}	18.88^{b}	3.34^{b}	1.33^{a}	1.06^{a}
control-2	5.00	5.00	4.50	4.86^{f}	10.24^{e}	13.49^{d}	5.31^{c}	1.40^{d}	0.96^{e}
transgenic-2	5.00	4.70	1.30	7.74^{d}	15.92^{c}	21.00^{c}	3.37^{d}	0.81^{e}	0.67^{f}
mixture-2	5.00	5.00	4.00	6.57^{e}	11.83^{d}	14.60^{d}	$4.45^{c,d}$	1.53^{d}	1.13^{d}
control-3B	5.00	5.00	3.50	6.16^{h}	11.31^{h}	15.57^{f}	3.97^{e}	1.05^{f}	0.85^{h}
transgenic-3B	5.00	4.20	1.20	7.97^{g}	18.03^{f}	19.45^{e}	3.60^{e}	0.95^{f}	0.74^{i}
mixture-3B	5.00	1.00	2.00	6.56^{h}	15.32^{g}	19.18^{e}	3.00^{e}	1.13^{f}	1.02^{g}
control-3B-1RS	5.00	5.00	3.50	6.16^{i}	11.31^{i}	15.57^{g}	3.97^{f}	1.05^{g}	0.85^{k}
transgenic-3B-1RS	5.00	5.00	4.30	6.48^{i}	12.88^{j}	13.12^{i}	3.91^{f}	1.23^{h}	0.72^{l}
mixture-3B-1RS	5.00	1.00	1.00	5.96^{i}	$12.98^{i,j}$	14.32^{h}	3.84^{f}	$1.15^{g,h}$	0.95^{j}

[a] Within a column and each population, mean values exhibiting the same letter are not significantly different ($P < 0.05$). No statistical evaluation was performed for rollability test.

in dough and tortilla properties presented by the control and transgenic wheats. To some extent, the negative effect of HMW-GS 1Dy10 overexpression was absent in tortillas derived from flour mixtures.

To further investigate the role of disulfide bond formation in tortilla production, cysteine (which prevents the formation of or disrupts disulfide bonds) was used at different concentrations in tortilla formulation made with flours derived from transgenic wheats. Tortilla thickness tended to decrease as cysteine concentration increased. Tortillas made without addition of cysteine had thicknesses of 2.00 and 2.33 mm for control and transgenic wheat, respectively. The addition of 300 μg/g of cysteine to the tortilla made with the transgenic wheat decreased the thickness from 2.33 to 2.13 mm, although this difference was not significant different ($P < 0.05$). The diameter of tortilla made from transgenic wheat significantly increased from 14.70 cm (no cysteine) to 15.38 cm (300 μg/g). Rupture forces of tortillas made with transgenic wheat without cysteine were 9.76, 19.08, 20.35, and 25.86 N at days 0, 2, 7, and 14, respectively, while rupture forces of tortillas made with 300 μg/g were 7.52, 17.15, 20.16, and 23.57 N, respectively. Therefore, a decrease in rupture force was observed with the addition of 300 μg/g, while addition of 6 and 50 μg/g did not significantly change rupture force in most time points (data not shown). Cysteine at any concentration tested did not significantly influenced stretchability of transgenic tortillas in almost all time points ($P < 0.05$). Tortillas derived from transgenic wheat without cysteine had poor rollability scores over time when compared to control tortillas. Addition of cysteine in the formulation did not improve the rollability scores of transgenic tortillas, as tortillas containing cysteine at any of the concentrations tested had a rollability score of 1.0 at d 7. These results indicated that addition of cysteine may have influenced the cross-linking among proteins, decreasing the molecular weight distribution/polymeric protein composition in the transgenic lines as tortilla parameters shifted toward those of control. However, these effects were observed at 300 μg/g only, which is a concentration ~100 times greater than that found in commercially available tortillas.

Ultimately, data presented here demonstrates that HMW-GS 1Dy10 overexpression in transgenic wheat caused a broad negative effect on dough properties and tortilla quality properties. Doughs produced from flours derived from transgenic wheats exhibited greater resistance to extension and lesser extensibility than control doughs. Tortillas derived from transgenic wheats exhibited an undesirable rough appearance with decreased diameter and greater thickness. In addition, tortillas made from flours containing greater levels of HMW-GS 1Dy10 exhibited lower rollability scores, lower stretchability, and greater rupture force over time. Data presented here also support that the changes in the dough and tortilla properties induced by increased amounts of HMW-GS 1Dy10 derive from a nonoptimum formation of the gluten network through increased cross-linking of the gluten proteins and increased levels of polymeric proteins. Overexpression of HMW-GS 1Dy10 in a wheat line containing 1RS-rye-translocation did not promote the same deleterious effects on dough and tortilla properties as it did in transgenic lines without 1RS translocation. This finding might derive from a lower level of HMW-GS 1Dy10 overexpression in this line and to defined protein composition differences in 1RS-translocated lines.

ABBREVIATIONS USED

HMW-GS: high molecular weight glutenin subunits; IPP: insoluble polymeric proteins; LMW-GS: low molecular weight glutenin subunits; %PP: percentage of polymeric protein; SPP: soluble polymeric protein; MALLS: multi-angle laser light scattering; RP-HPLC: reverse-phase high performance liquid chromatography; SE-HPLC: size-exclusion high performance liquid chromatography.

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