Pasting properties of transgenic lines of a commercial bread wheat expressing combinations of HMW glutenin subunit genes

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Abstract

Seven transgenic lines of a commercial wheat (*Triticum aestivum* L.) cultivar expressing transgenic subunits 1Ax1, 1Dx5 and 1Dy10, alone or in combination have been developed. Pasting properties were determined in these transgenic lines using a Rapid Visco Analyser (RVA) in order to determine the possible impact of HMW-GS transgene expression on the starch properties. Expression of the HMW-GS transgenes increased the proportions of the corresponding 1Ax, 1Dx and 1Dy subunits affecting significantly the ratios of HMW-GS:LMW-GS and x-type:y-type HMW-GS. Starch granule size distribution varied significantly among all transgenic lines, with the Anza control and transgenic line T616 (expressing subunits 1Ax1 and 1Dy10) showing the highest and the lowest percentage of B granules, respectively. All transgenic lines increased the water binding capacities (WBC) at 25ºC and 90ºC. Line T606 (expressing subunits 1Ax1 and 1Dx5) and line T590 (expressing subunit 1Dy10) showed the lowest and the highest values for peak viscosity, respectively. Notably, lines expressing only transgenic x-type subunits (T580, T581 and T606), with high ratios of x-type:y-type HMW-GS, had low peak viscosities, final viscosities and breakdown viscosities. Line T590 had the highest breakdown viscosity while lines T606 and T581 had the lowest.

Keywords: Cereal; breeding; HMW-GS; Pasting; GM; wheat;

Abbreviations

AACC	American Association of Cereal Chemists
ER	Endoplasmic Reticulum
HMW-GS	High Molecular Weight Glutenin Subunit
ICC	International Association for Cereal Science and Technology
LMW-GS	Low Molecular Weight Glutenin Subunit
LSD	Least Significant Difference
RVA	Rapid Visco Analyser
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
WBC	Water Binding Capacities

Introduction

Cereals are of enormous importance to humankind for food, livestock feed and industrial raw materials. Starch and proteins are major components of the mature wheat (*Triticum aestivum* L.) grain and are responsible for the ability to make many wheat-based food products. Starch accounts for about 60-70% of the wheat grain dry matter. Starch is deposited in partially crystalline granules that vary in morphology and structure between and within plant species. Starch owes much of its functionality in foods to the characteristics of the two constituent glucose polymers, amylose and amylopectin, and to the physical organization of these macromolecules into the granular structure (Annison and Topping, 1994). Amylopectin with its multiple branched chains of (1-4)-α-glucans interlinked by (1-6)-α-linkages is the major component of starch, with the unbranched amylose accounting for the minor fraction. These components are synthesized in amyloplasts in the form of distinct granules. Typically wheat starch is recognized as having at least two different starch granule populations of distinct size and shape (Parker, 1985; Peng et al., 1999). The 'A' granules are large and lenticular, while the 'B' are small and spherical. Variation in starch functionality and pasting properties has been related to differences in starch granule size distribution and composition (Ao and Jane, 2007; Dengate and Meredith, 1984). The B granules have higher contents of lipids than the A granules (Ao and Jane, 2007;Soulaka and Morrison, 1985). Early studies showed that the A and B granules had similar proportions of amylose (about 20-26%) (Evers et al., 1974; Meredith et al., 1981) although a more recent study reported A granules to have higher amylose contents than B granules (Ao and Jane, 2007). Starch granules are insoluble in water below 50^{o}C and when they are heated in water beyond a critical temperature, the granules absorb a large amount of

water and swell to many times their original size. Over a critical temperature range, the starch granules undergo an irreversible process known as gelatinization and loss their granular organization. Numerous studies have indicated that the relationships between the amylose and amylopectin contents and molecular structures of starches, and their water absorption and pasting properties, are complex and vary considerably amongst starches from different genotypes (Dang and Copeland, 2004; Dengate and Meredith, 1984; Peterson and Fulcher, 2001). Differences between the structures and properties of starches among different cereals, and between mutant types of starch which occur within cereal species, also affect their functionality in different food systems, including wheat starch in breadmaking (Stone and Morell, 2009).

The protein fraction of wheat grain represents about 8-15% of the whole grain dry matter. The prolamin storage proteins, which correspond to the gluten proteins, are the most important protein fraction present in the wheat grain. The gluten proteins are synthesized on the rough endoplasmic reticulum (ER), co-translationally transported into the lumen of the ER and subsequently either deposited within protein bodies derived directly from the ER or transported to the vacuole to form a second population of protein bodies (Kumamaru et al., 2007; Tosi et al., 2009). When the cells of the starchy endosperm dry and die during the later stages of grain maturation, the protein deposits fuse to form a proteinaceous network in each endosperm cell. These networks are brought together when flour is mixed with water to form a continuous network in the dough. The high molecular weight glutenin subunits (HMW-GS) of wheat play an important role in determining the functional properties of this protein network and hence of wheat dough. Allelic differences in the HMW-GS composition result in effects on the structures and properties of the glutenin polymers and thus on breadmaking

quality (Payne, 1987; Shewry et al., 2003a). Therefore, genes for HMW-GS, in particular those encoding subunits 1Ax1, 1Dx5 and 1Dy10, have been identified as targets for expression in transgenic wheat (Altpeter et al., 1996; Barro et al., 1997; Blechl and Anderson, 1996; Blechl et al., 2007; León et al., 2009a) to develop cultivars with new HMW-GS combinations and hence with improved or novel functional properties. As the expression of these HMW-GS transgenes in transgenic wheat alters the proportions of glutenins to gliadins, the ratio of polymeric to monomeric gluten proteins, the ratio of HMW-GS to LMW-GS and the contents of individual subunits, they also affect the size, composition and functional properties of the glutenin polymers formed in each line. Consequently, differential effects of transgenes encoding HMW-GS 1Ax1, 1Dx5 and 1Dy10 on gluten properties have been reported (Barro et al., 2003a; 2003b; Blechl et al., 2007; Darlington et al., 2003; León et al., 2009a). The presence of different HMW-GS can also affect the interactions of the gluten matrix with starch and hence the pasting properties of wheat. In fact, differential effects on peak viscosity between subunits from the *Glu-1B* locus, and between subunits from *Glu-1D* locus have been reported (Batey, 2000).

We have developed a set of seven transgenic lines expressing HMW-GS 1Ax1, 1Dx5 and 1Dy10 of a commercial wheat cultivar Anza, which endogenous HMW-GS composition is 1Bx7*, 1By8, 1Dx2 and 1Dy12. Three lines were single transformants while in the other four lines the HMW-GS genes were combined by conventional crossing. This set of lines represents an excellent background in which to evaluate the effects of HMW-GS transgene combinations on the functional properties including the pasting properties of wheat flour.

1. Materials and Methods

1.1. Plant material

Seven transgenic lines expressing the transgenes encoding HMW-GS 1Dx5, 1Ax1 and 1Dy10, single or in combination, were used in this work. Lines T580, T581 and T590 were obtained by genetic transformation as described by León et al. (2009a) while lines T606, T616, T617 and T618 were obtained by conventional crossing using former lines as parents (León et al., 2009b). The control line was the bread wheat cv Anza as the parent of the transgenic lines, which which endogenous HMW-GS composition is 1Bx7*, 1By8, 1Dx2 and 1Dy12. Lines were analysed over two years, using a randomized complete block design with two replicates, as described by Barro et al. (2002).

1.2. Protein and SDS-PAGE analysis

The protein content of whole flour was calculated from the Kjeldahl nitrogen content (%N x 5.7) and expressed on a dry matter basis. Seeds were crushed into a fine powder which was used to extract the endosperm storage proteins. Gliadins were extracted in 60% (v/v) aqueous ethanol using a rotary shaker for 40 min. Samples were centrifuged at 13000xg for 5 min and the supernatant collected. Glutenins were extracted as described by Shewry et al. (1995). For densitometry, glutenins from thirty individual seeds per line and year (60 seeds in total) were separated by SDS-PAGE gels and analysed using a Kodak Image Station 440CF and Kodak 1D Image Analysis Software using the SDS-PAGE Molecular Weight Standards from Bio-Rad as reference.

1.3. Starch and amylose determinations

The starch content of whole flour was determined according to standard ICC method no. 123/1 (ICC, 1994). Four samples per genotype and year were used.

The amylose content was determined using the Megazyme Amylose/Amylopectin Assay (Megazyme International Ireland Ltd, Bray, Ireland) according to the manufacturer's recommendations. The Megazyme standard of 64% amylose content was used as a control. The amylose content was expressed in percentage of starch dry weight. Four different replicates per genotype and year were used.

1.4. Starch granule size analysis

Starch granules were isolated from wheat genotypes according to Caballero et al. (2008) using 90 mg of flour. Starch granule size was measured using an Axioskop 2 MOT microscope (Carl Zeiss Vision GmbH, Germany). Four biological replicates per genotype and year were used and, for each sample, fifteen fields were counted.

1.5. Water binding capacity (WBC)

The water binding capacity was measured as the amount of water that one gram of material will retain after centrifugation using AACC method 56-30 (AACC, 1999) and also after heating at 90ºC. Briefly, the wheat flour suspension was kept in a water bath at 90ºC for 10 min. After cooling for one hour, the water binding capacity of the samples was determined using the standard method.

1.6. Rapid visco-analysis

The pasting profiles were obtained using whole flour with a Rapid Visco Analyser (RVA-4, Newport Scientific, Warriewood, Australia) according to the ICC standard method 162 (ICC, 1996). Two biological replications per year and genotype were analysed.

1.7. Statistics

Data were analysed using the SPSS version 11.0 statistical software package (SPSS Inc., Chicago, Illinois, USA). Arcsine transformation was carried out on variables expressed as percentages before analysis. The general analysis of variance and the least significant difference (LSD) pairwise comparisons of means were used to determine significant differences. Simple correlations was performed using Statgraphics V.7.1 program (Bitstream, Cambridge, MN).

2. Results and discussion

2.1. Protein properties of transgenic lines

Table 1 compares the characteristics of the transgenic lines with the control line Anza, which expresses four endogenous HMW-GS: 1Bx7*+1By8 and 1Dx2+1Dy12. Consequently, the total number of HMW-GS ranged from four for control line Anza to seven for line T618. Although transformation and further crossing of the transgenic lines resulted in lines containing increasing numbers of HMW-GS, this did not have a significant effect on the total flour protein content compared to the Anza control (Table 1). The expression of transgenic subunits significantly affected the proportions of the

two major glutenin protein fractions in the endosperm of wheat lines. Thus, the HMW-GS:LMW-GS ratio and proportions of HMW-GS in the glutenin fraction were increased in all lines with respect to the Anza control, with the increases being significant for five of the lines but not for lines T590 and T606, expressing the 1Dy10, and the 1Ax1 and 1Dx5 transgenes, respectively (Table 1). Notably, this increase was greatest in line T618, which expressed all three HMW-GS transgenes, and was associated with a correspondingly lower proportion of LMW-GS. The expression of the HMW-GS transgenes also resulted in increased proportions of the corresponding 1Ax, 1Dx and 1Dy subunits. However, differential effects on the proportions of individual endogenous HMW-GS were also observed. The proportion of subunit 1By8 was decreased in most lines but not in line T581, expressing subunit 1Dx5. In contrast, the proportion of subunit 1Bx7* was reduced significantly in two lines, (T606 and T616) that both expressed the subunit 1Ax1 transgene. The ratio of x-type:y-type HMW-GS varied significantly with the different combinations of transgenes. In particular, the expression of x-type subunits (lines T580, T581 and T606) significantly increased the ratio of x-type:y-type with respect to the Anza control, whereas the expression of y-type subunits (line T590) decreased this ratio (Table 1). However, when one x-type and one y-type subunit were expressed together (lines T616 and T617), the x-type:y-type ratios were more balanced and similar to that of the Anza control. Again, the expression of two x-type and one y-type subunits in line T618 resulted in a higher x-type:y-type ratio than in the T616 and T617 lines and equal to that of the line expressing only 1Dx5.

219 *2.2. Starch and Pasting properties of transgenic lines*

220 The starch and amylose contents did not show differences among the transgenic
221 lines (Table 2). In particular, the amylose contents of the lines agreed with those
222 reported for hexaploid wheat (Soulaka and Morrison, 1985). The starch granule size
223 distribution for the transgenic lines reported in this work is given in Table 2. A cut-off
224 diameter of 10 μm for the A and B granule size classes was selected based on results
225 reported by other researches (Dengate and Meredith, 1984; Peterson and Fulcher, 2001;
226 Salman et al., 2009). Notably, the B granules made up the major portion of the total
227 percentage of granules. Even though starch granule size varied only within a 3.0%
228 range, these differences were statistically significant (Table 2). The control line Anza
229 showed the highest percentages of B granules, and lines T616 (expressing subunits
230 1Ax1 and 1Dy10) and T606 (expressing subunits 1Ax1 and 1Dx5) the lowest . It has
231 been calculated that the total surface of the B granules is about three times greater than
232 that of the A granules and suggested that this contributes to endosperm cohesiveness
233 and strength (Konopka et al., 2005). Starch granule size distribution has been related to
234 the pasting and mixing properties of wheat (Dengate and Meredith, 1984; Park et al.,
235 2009; Peterson and Fulcher, 2001). However, other researchers suggest that the
236 composition and structure of the starch rather than the size of the starch granules
237 determine the pasting properties of wheat (Ao and Jane, 2007).

238 Water-binding capacity was determined in all transgenic lines at 25ºC and also after
239 heating at 90ºC (Table 2), in order to determine the water uptake of the starch granules
240 before and after gelatinization. The expression of HMW-GS transgenes increased the
241 WBC of all transgenic lines at both 25ºC and 90ºC in comparison to Anza control. Lines

T606 (expressing subunits 1Ax1 and 1Dx5) and T616 (expressing subunits 1Ax1 and 1Dy10) showed the highest WBC at 25ºC and 90ºC, respectively (Table 2).

The pasting properties of transgenic lines expressing different combinations of HMW-GS transgenes were determined using the Rapid Visco Analyser (RVA). The RVA is an effective instrument for determining the viscous properties of flour as a function of processing temperature and stirring rate, and for relating functionality to structural properties (Collar et al., 2006). However, these relationships are complex, and the results influenced by factors include genetics, processing, and environmental conditions (Batey et al., 2001; Becker et al., 2001). In the work described here, the transgenic lines are derived from the same genetic background. This allows the comparative study of the effect of different combinations of HMW-GS transgenes on the pasting properties of wheat flour (Table 3). When flour is tested in the RVA, it is subjected to a heat-hold-cool cooking cycle which gives a pasting curve for the sample (Figure 1). The most important parameters measured include pasting temperature, peak time, peak viscosity, breakdown, final viscosity and setback (Table 3). The pasting temperature gives an indication of the minimum temperature required to cook a given sample, and also indicates energy costs. Though the pasting temperatures of the lines studied in this work ranged from 82.2^{o}C to 86.8^{o}C, these differences were not statistically significant (Table 3), suggesting that all of the lines had comparable gelatinization properties. After reaching the pasting temperature, the temperature is increased further and peak viscosity is reached (Figure 1). At this point, there is an equilibrium between starch granule swelling and rupture, with amylose leaching out into solution. Line T606 (expressing subunits 1Ax1 and 1Dx5) showed the lowest value for the peak time, while there were no significant differences among the other lines

(Table 3). Line T606 also showed the lowest value for peak viscosity and line T590 (expressing subunit 1Dy10) together with Anza the highest. The peak viscosity is related to the water uptake capacity of the starch, and it is often correlated with final product quality. In fact, high viscosities during pasting and low viscosities after the holding period at 95ºC of wheat dough slurries are considered valuable predictors of bread firming behaviour during storage (Collar, 2003). The three lines expressing only transgenic x-type subunits (T580, T581 and T606), with high x-type:y-type ratios (Table 1), also had low peak viscosities. The decrease in peak viscosity has been associated with a lower degree of swelling of the starch granules and also lower starch contents (Symons and Brennan, 2004). However, no significant correlation was found between peak viscosity and the starch content or granule size distribution (Table 3, 4).

During the holding period at constant high temperature, a breakdown in viscosity to a holding strength or trough, takes place (Figure 1). The transgenic lines showed significant differences in the breakdown viscosities (Table 3). Line T590 (expressing subunit 1Dy10) had the highest breakdown viscosity while line T606 had the lowest (Table 3). Line T590 expresses the transgenic HMW-GS 1Dy10 while line T606 expresses transgenic HMW-GS 1Ax1 and 1Dx5 (Table 1).

The breakdown in viscosity is caused by rupture of the swollen granules (Rani and Bhattacharya, 1995). The lower breakdown viscosity for lines T606 and T581 may therefore be related to a decrease in the rate of rupturing of the starch granules during RVA processing. This parameter has been linked to the cooking or hot stability or the easiness of cooking the starch (Rojas et al., 1999), thus, differences observed in this parameter revealed different heating stability of the starch granules of different lines.

289 The last phase in the pasting curve (Figure 1) is the setback region, and corresponds
290 to the gelling process of the starch, in which the amylose chains are prompted to
291 recrystalize, yielding the formation of a gel structure. In the cereal slurries, low values
292 of setback indicate low rates of starch retrogradation and low syneresis (Rojas et al.,
293 1999). Setback is measured as the difference between final viscosity and holding
294 strength or trough, while the final viscosity indicates the ability of the flour to form a
295 viscous paste after cooking and cooling. Lines T590 and Anza control showed the
296 highest values for final viscosity (Table 3) while line T606 had the lowest. Notably,
297 lines T606, T580 and T581, which express only transgenic x-type subunits, with high x-
298 type:y-type ratios, showed lower values for final viscosity than the other lines (Table
299 3).

300 The lines expressing only transgenic x-type subunits had low peak viscosities and
301 final viscosities. This is particularly marked for line T606 which expresses the 1Ax1
302 and 1Dx5 HMW-GS transgenes. In addition, the decrease in the breakdown viscosity
303 was also less for line T606 (Figure 1). Lower values for pasting viscosities are an
304 indication of a reduction of the available starch for gelatinization (Collar et al., 2006).
305 This reduction is unlikely to be due to a reduction in the starch content of this line as all
306 of the lines had similar levels of starch (Table 2).

307

308 **3. General discussion**

309 We have developed a series of seven transgenic lines of a commercial wheat cultivar
310 expressing single, double or, in the case of line T618, triple combinations of the HMW-
311 GS 1Ax1, 1Dx5 and 1Dy10 transgenes. The expression of the HMW-GS transgenes

312 increased the proportions of the corresponding 1Ax, 1Dx and 1Dy subunits, and this is
313 expected to affect the size and structure of the different glutenin polymers formed in
314 each transgenic line. Notably, the expression of transgenic x-type subunits (lines T580,
315 T581 and T606) increased the ratio of x-type:y-type HMW-GS in comparison to that of
316 Anza control and lines which expressed transgenic subunit 1Dy10 alone. All transgenic
317 lines had higher HMW-GS:LMW-GS ratios than the Anza control, except lines T590
318 and T606, which didn´t show significant differences in comparison to Anza. Although
319 all of the transgenic lines had comparable levels of starch and amylose, they had lower
320 proportions of B granules than the Anza control. It is difficult to establish the cause of
321 these differences as the A and B granules differ in their time of biogenesis during grain
322 filling. The A granules start to form at about 4-5 days after anthesis and continue until
323 physiological maturity. On the other hand, B granules are initiated at 10-12 days after
324 anthesis and continue to accumulate until about 35 days after anthesis (Bechtel et al.,
325 1990; Parker, 1985). However, the proportions of A granules correlated well with WBC
326 at 25 ^{o}C as lines with higher WBC at 25 ^{o}C had higher contents of A granules. Although
327 the lines studied in this work did not significantly differ in protein content, there were
328 clear differences in the glutenin subunit and polymer composition of in the transgenic
329 lines in comparison with the Anza control. These changes in protein composition
330 appeared to be associated with changes in protein:starch interactions and in turn, in the
331 pasting properties of the transgenic wheat lines. Notably, peak viscosity, final viscosity,
332 and breakdown viscosity were lower in transgenic lines expressing only x-type subunits
333 (lines T580, T581 and T606). In contrast, line T590 (expressing subunit 1Dy10) had the
334 lowest ratio of x-type:y-type HMW-GS and also had the highest values for peak
335 viscosity, final viscosity and breakdown viscosity. Our results also showed a negative
336 correlation between WBC at 25ºC and peak viscosity, final viscosity and setback (Table

4). Flour from line T606 (expressing subunits 1Ax1 and 1Dx5) had a higher water binding capacity at 25ºC than the other lines. However, at 90ºC line T616 (expressing subunits 1Ax1 and 1Dy10) showed the highest value for WBC. This result clearly indicates that the effects of the expression of the HMW-GS on the gluten matrix significantly modifies the WBC and influences the pasting properties of transgenic lines.

It is clear that water plays an important part in wheat flour processing, with gluten, starch and cell wall polysaccharides all competing for water absorption, ultimately defining the technological functionality of the wheat flour (Rosell and Collar, 2009).The HMW-GS present in transgenic lines differ in their number and distributions of cysteine residues and these transgenic lines would therefore be expected to differ in the extent and pattern of cross-linking of the gluten matrix. In particular, HMW-GS 1Dx5 has an additional cysteine residue in the repetitive domain, and it has been suggested to provide an additional site for inter-chain bonds between HMW-GS (Shewry et al., 2003b). The extent of cross-linking of the gluten matrix would in turn be expected to affect its WBC and hence its competition for water with starch and other components and flour pasting properties. Results of WBC support this explanation as all transgenic lines had higher WBC at 25ºC and 90ºC than Anza control. Notably, line T606 (expressing subunits 1Ax1 and 1Dx5) had significantly higher WBC at 25ºC than the rest of lines. The WBC at 25ºC was also positively correlated with the content of A granules and negatively correlated with the content of B granules, peak viscosity, final viscosity, and setback (Table 4). Finally, all transgenic lines had low contents of B granules which have been reported to contain higher proportions of lipids (Whattam and Cornell, 1991) and surface proteins (see review Baldwin, 2001) than A granules. High

levels of starch lipids are thought to have a negative impact on granule swelling due to the inhibition of amylose mobility (Morrison et al., 1993). The results agree with our findings, since the transgenic lines had lower contents of B granules and higher WBC at 25ºC.

In conclusion, differences in the pasting properties of bread wheat are associated with the expression of single and multiple HMW-GS transgenes. Lines expressing only transgenic x-type subunits (T580, T581 and T606), with high ratios of x-type:y-type HMW-GS, had differences in pasting properties with reduced peak and final viscosities, and breakdown viscosity. In contrast, line T590 (expressing subunit 1Dy10), with the lowest ratio of x-type:y-type HMW-GS, showed the highest values for peak viscosity, final viscosity and breakdown viscosity.

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Table 1. Protein properties of transgenic lines.

Line	Transgenic HMW-GS	Total protein (N x 5.7)	SDS PAGE							
			HMW/ LMW	x/y	HMW % glutenin	Ax % glutenin	Bx % glutenin	By % glutenin	Dx % glutenin	Dy % glutenin
Anza	NA	12.0 a	0.39 d	1.41 e	27.8 c	NA	8.45 bc	5.03 a	7.71 d	6.56 c
T580	1	13.3 a	0.59 bc	2.69 b	36.1 b	10.50 a	7.84 bc	4.07 b	7.34 d	5.71 c
T581	5	13.2 a	0.67 ab	2.21 c	38.8 b	NA	9.39 a	5.37 a	17.36 a	6.72 c
T590	10	12.5 a	0.44 d	1.03 f	30.6 c	NA	8.69 ab	3.90 b	6.82 d	11.17 a
T606	1, 5	13.3 a	0.45 d	3.72 a	31.0 c	6.86 c	5.41 e	2.82 c	12.01 c	3.92 d
T616	1, 10	12.7 a	0.56 c	1.60 d	35.8 b	8.55 b	6.83 d	4.03 b	6.52 d	9.84 b
T617	5, 10	12.4 a	0.61 bc	1.61 d	37.6 b	NA	8.29 bc	3.78 b	14.71 b	10.81 a
T618	1, 5, 10	12.0 a	0.74 a	2.21 c	42.3 a	9.05 b	7.66 cd	2.84 c	12.36 c	10.55 ab

NA - Not applicable. Values within the same column followed by the same letter are not significantly different ($P < 0.05$). All lines have the endogenous HMW-GS 1Bx7*, 1By8, 1Dx2 and 1Dy12.

Table 2. Starch content and composition and water binding capacity (WBC) properties of transgenic lines

Line	Transgenic HMW-GS	Starch (%)	Amylose (%)	Granule size distribution (%)		WBC (g H_2O/ g flour)	
				A	B	25 (^oC)	90 (^oC)
Anza	NA	62.2 a	25.8 a	3.9 d	96.1 a	0.760 e	3.192 e
T580	1	60.0 a	27.7 a	6.1 ab	93.9 cd	0.796 c	3.436 d
T581	5	57.5 a	30.0 a	5.4 bc	94.5 bc	0.798 c	3.744 ab
T590	10	58.6 a	30.1 a	4.6 cd	95.4 ab	0.783 d	3.590 bcd
T606	1, 5	59.5 a	29.4 a	6.8 a	93.2 d	0.877 a	3.510 cd
T616	1, 10	62.9 a	27.5 a	7.1 a	92.9 d	0.825 b	3.837 a
T617	5, 10	62.9 a	29.4 a	5.4 bc	94.6 bc	0.802 c	3.695 abc
T618	1, 5, 10	62.8 a	28.4 a	5.3 bc	94.7 bc	0.794 cd	3.732 ab

NA - Not applicable. Values within the same column followed by the same letter are not significantly different ($P < 0.05$)

Table 3. RVA properties of transgenic lines

Line	Transgenic HMW-GS	RVA					
		Pasting T. (^{o}C)	Peak time (min)	Peak viscosity (cP)	Breakdown (cP)	Final viscosity (cP)	Setback (cP)
Anza	NA	85.2 a	5.75 a	2054 a	676 ab	2701 a	1322 a
T580	1	83.3 a	5.70 a	1712 b	566 bc	2276 b	1130 cd
T581	5	82.2 a	5.65 ab	1637 b	507 cd	2284 b	1153 c
T590	10	83.9 a	5.72 a	2103 a	720 a	2731 a	1338 ab
T606	1, 5	83.5 a	5.50 b	1289 c	371 d	1871 c	953 d
T616	1, 10	86.8 a	5.73 a	1859 ab	653 abc	2350 ab	1144 bc
T617	5, 10	84.9 a	5.73 a	1768 b	569 bc	2360 ab	1162 bc
T618	1, 5, 10	85.1 a	5.73 a	1938 ab	632 abc	2514 ab	1207 abc

NA - Not applicable. Values within the same column followed by the same letter are not significantly different ($P < 0.05$)

Table 4. Correlation matrix between starch and water binding capacity at 25ºC (WBC25) and 90ºC (WBC90) and RVA properties of transgenic lines.

	WBC25	WBC90	Starch (%)	Amylose (%)	A-granules	B-granules	Peak viscosity	Breakdown	Final viscosity	Setback
WBC25	1									
WBC90	0.3496	1								
Starch (%)	0.0087	0.0279	1							
Amylose (%)	0.2761	0.4733	-0.5873	1						
A-granules	0.8561**	0.4957	0.0401	0.1559	1					
B-granules	-0.8517**	-0.5065	-0.0198	-0.1694	-0.9995***	1				
Peak viscosity	-0.7774*	-0.1386	0.3277	-0.3462	-0.6706	0.6798	1			
Breakdown	-0.6988	-0.0892	0.3481	-0.3586	-0.5420	0.5526	0.9848***	1		
Final viscosity	-0.8482**	-0.2173	0.2173	-0.2915	-0.7914*	0.7977*	0.9825***	0.9404***	1	
Setback	-0.8758**	-0.2738	0.1180	-0.2532	-0.8308**	0.8347**	0.9569***	0.9086**	0.9923***	1

Correlations indicated by R^2 values. *** *P*-value < 0.001. ** *P*-value < 0.01. * *P*-value < 0.05.

Figure legends

Figure 1. Plots of pasting and gelling of whole flour doughs recorded with the Rapid Visco Analyser (RVA). **(A)**. single transgenic lines expressing the HMW-GS subunits 1Ax1, 1Dx5 and 1Dy10. **(B)**. transgenic lines expressing combinations of the 1Ax1, 1Dx5 and 1Dy10 HMW-GS transgenes. Temperature is shown as the solid colored lines.