Prolamin Aggregation, Gluten Viscoelasticity, and Mixing Properties of Transgenic Wheat Lines Expressing 1Ax and 1Dx High Molecular Weight Glutenin Subunit Transgenes

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The composition of high molecular weight (HMW) subunits of glutenin determines the gluten strength and influences the baking quality of bread wheat. Here, the effect of transgenes coding for subunits 1Ax1 and 1Dx5 was studied in two near-isogenic wheat lines differing in their HMW subunit compositions and mixing properties. The subunits encoded by the transgenes were overexpressed in the transformed lines and accounted for 50−70% of HMW subunits. Overexpression of 1Ax1 and 1Dx5 subunits modified glutenin aggregation, but glutenin properties were much more affected by expression of the 1Dx5 transgene. This resulted in increased cross-linking of glutenin polymers. In dynamic assay, the storage and loss moduli of hydrated glutens containing 1Dx5 transgene subunits were considerably enhanced, whereas expression of the 1Ax1 transgene had a limited effect. The very high strength of 1Dx5 transformed glutens resulted in abnormal mixing properties of dough. These results are discussed with regard to glutenin subunit and glutenin polymer structures.

Keywords: *Gluten; glutenin; HMW subunits; transformation; viscoelasticity; wheat*

INTRODUCTION

Variation in the composition of the high molecular weight (HMW) glutenin subunits of wheat is associated with large differences in the bread-making properties of different cultivars (*1*). Studies of near-isogenic lines of wheats differing only in their HMW glutenin subunit compositions have demonstrated that the HMW glutenin subunits determine glutenin aggregation and gluten rheological behavior. These effects are related to the relative amounts of the HMW subunits in the glutenin polymers and to structural features of individual subunits (*2*−*4*). Rheological analyses of gluten and gluten fractions showed a strong correlation between viscoelasticity, or network connectivity, and the relative amount of glutenin polymers with the highest molecular masses (*4*−*8*). In this respect, HMW subunits 1Dx5 and 1Dy10, which are encoded by chromosome 1D, give better baking performances than the homeoallelic subunits 1Dx2 and 1Dy12 (*1*, *4*, *9*, *10*). In the same way, a double-null line, expressing only subunits 1Dx5 and 1Dy10, had higher gluten strength than a line expressing only the chromosome 1B-encoded subunits 1Bx17 and 1By18 (*6*, *11*, *12*).

The effect of HMW subunits on dough mixing properties has also been assessed by addition of purified subunits to flours (*13*), but this approach involves partial reduction of the gluten and reoxidation, treatments that could alter the gluten's original structure and functionality. The creation of transgenic lines differing in their HMW subunit composition allows us to study the effects of individual subunits on glutenin structure and on the technological properties of wheat gluten (*14*) and thus to relate structure more closely to functionality. In this work, we compared the effects of expression of trangenes coding for subunit 1Ax1 (containing two cysteines available for intermolecular disulfide bonds) or for subunit 1Dx5 (with three cysteines available for intermolecular disulfide bonds) on glutenin aggregation, gluten viscoelasticity, and the mixing properties of doughs.

Table 1. Characteristics of Lines Grown in Rothamsted (RES) and Long-Ashton (LARS)

genotype	line	HMW subunit composition	transgene subunit	growing location	no. of plots
L88-31	control	17+18		RES	4
				LARS	4
	B72-8-11b	17+18	1Dx5	RES	2
				LARS	3
	B102-1-2	17+18	1Ax1	RES	4
				LARS	4
L88-6	control	1, 17+18, 5+10		RES	4
				LARS	4
	B73-6-1	1, 17+18, 5+10	1Dx5	RES	4
				LARS	4

MATERIALS AND METHODS

Transgenic Lines. Transgenic lines were produced at IACR−Rothamsted as described by Barro et al. (*15*). Seeds were grown in replicate field plots at IACR−Rothamsted (Harpenden, Herts, U.K.) and IACR−Long Ashton (Bristol, U.K.) in 1998 (Table 1).

Gluten Extraction. A dough mixed from 30 g of flour and 18 g of water was suspended in 400 mL of deionized water by

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10.1021/jf001015j CCC: $20.00
Published on Web 12/09/2000

Table 2. Glutenin Subunit Composition of Transgenic Lines of Wheat Determined by Capillary Electrophoresis

line	total protein (% of dry wt)	total glutenin (% of total protein)	HMW subunits (% of total glutenin subunits)	*Glu-1A* subunits (% of total HMW subunits)	*Glu-1B* subunits (% of total HMW subunits)		*Glu-1D* subunits (% of total HMW subunits)	
				1Ax1	1Bx17	1By18	1Dx5	1Dy10
L88-6 control	13.7	44	36	16	33	16	26	10
B73-6-1	13.7	53	44	4	8	4	73	4
L88-31 control	12.7	33	18	0	75	25	0	0
B102-1-2	12.7	37	31	50	38	12	0	0
B72-8-11b	12.6	44	28	0	21	8	71	0

mechanical stirring. This suspension was centrifuged at 5000*g* for 15 min. The gluten-rich top layer was collected and washed with water until no starch was released. After washing, the gluten samples were placed in an excess of water and centrifuged at 500*g* for 15 min before rheological analysis. The protein content of the glutens was 83.7 ± 3% of the dry weight.

Protein Content and Composition. Grain was milled with a Brabender Quadrumat Junior laboratory mill, and the protein contents of flours were determined by Kjeldhal digestion and colorimetric determination of N content. The glutenin subunit composition was determined by SDS-PAGE in the presence of reducing agent and by capillary electrophoresis carried out according to the method of Werner et al. (*16*) in a capillary tube (separation length = 22 cm, diameter = 55 μm) filled with a solution of cross-linked acrylamide Prosort (Applied Biosystem) containing 5% (v/v) glycerol and 5% (v/v) methanol. Migration was performed at −12 kV under 11 μA at 25 °C, in a CE Ultra instrument (Thermo Separation Products SA, Les Ulis, France). Proteins were detected at 214 nm.

Protein Extractability and Size Distribution. The size distribution and aggregation of prolamins in flours from control and transgenic lines was determined by SE-HPLC on a Superose 6 column (1 × 30 cm; flow rate = 0.3 mL/min; detection at 220 nm) equilibrated in 0.0125 M sodium borate buffer containing 0.1% SDS. Samples were prepared by a three-step extraction. The first step comprised stirring in 0.0125 M sodium borate buffer, pH 8.5, containing 0.5% (v/v) SDS: the solubilized proteins constituted "extractable" prolamins. A second protein fraction was then extracted by sonication under controlled conditions (6 W, 30 s) in 0.0125 M sodium borate buffer, pH 8.5, with 2% (v/v) SDS. A third extraction step was then carried out with 0.0125 M sodium borate buffer, pH 8.5, containing 2% (v/v) SDS and 1% (v/v) dithiothreitol (DTT). All three protein fractions were analyzed by SE-HPLC. The chromatographic patterns were divided into three peaks, P1, P2, and P3, corresponding to large-size glutenin polymers (MW > 500K), medium-size glutenin polymers (500K > MW > 70K), and gliadin monomers (or glutenin subunits in the case of the third reduced extract), respectively. Each peak was quantified by measurement of the surface area.

Determination of Gluten Viscoelastic Properties. Rheological measurements were performed on glutens fully hydrated with water using a Carri-Med CSL 100 constant-stress rheometer (cone-plate geometry; cone angle = 4°, diameter = 2 cm). Freshly extracted glutens were analyzed. After loading into the measuring device, the sample was left to rest for 1 h to allow dissipation of stress. The sample was then submitted to a frequency scan covering the 0.001−36 Hz frequency window range at 20 °C, and the storage (G') and loss (G'') moduli were recorded. The amplitude of strain at all frequencies was kept close to 3% to ensure that the viscoelastic behavior remained within the linear region under test conditions (*5*). Mechanical spectra were analyzed as described previously (*12*) to determine the height of the elastic plateau (G_N^0), which estimates the connectivity of the protein network.

Mixing Test. A 2 g Micromixograph (National Manufacturing Division, Lincoln, NE) was used. Two grams of flour was hydrated with 1.2 mL of distilled water and mixed for 10 min at 88 rpm and 20 °C. The mixing parameters were calculated with Mixsmart 3.4 software.

Statistical Analysis. When necessary, a variance analysis was performed and the results were compared according to Fisher's least significant difference (LSD) procedure.

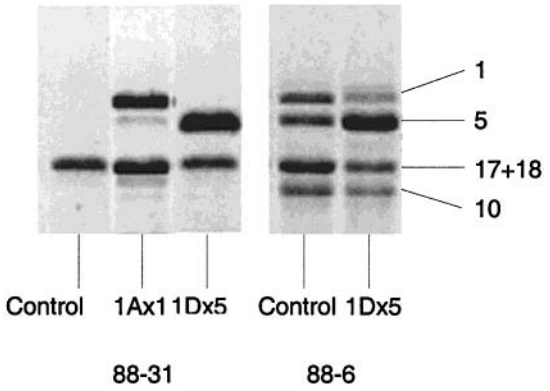

Figure 1. SDS-PAGE pattern of high molecular weight glutenin subunit compositions of control and transformed lines of wheat: 88-6C and 88-31C, control lines; 88-6 1Dx5 and 88-31 1Dx5, transformed lines expressing 1Dx5 subunit transgene; 88-31 1Ax1, transformed line expressing 1Ax1 subunit transgene. Numbers on the right identify HWM subunits according to Payne et al. (*1*).

RESULTS

The production of a series of transgenic wheat lines has been described by Barro et al. (*15*). Two near-isogenic lines derived from crossing mutants of the Australian spring cultivars Olympic and Gabo were used as recipients, expressing either subunits 1Bx17 and 1By18 only [i.e., null at the *Glu-1A* and *Glu-1D* loci (L88-31)] or subunits 1Ax1, 1Dx5, 1Dy10, 1Bx7, and 1By18 (L88-6). Transgenic lines expressing transgenes for subunits 1Ax1 (B102-1-2) and 1Dx5 (B72-8-11b) in L88-31 and subunit 1Dx5 in L88-6 (B73-6-1) were grown in replicate field trials (2, 3, or 4 × 1 m^2 plots) on two sites in the United Kingdom in 1998, together with the control parental lines (Table 1).

SDS-PAGE of total grain proteins confirmed that the proteins encoded by the transgenes were expressed (Figure 1) and that their molecular masses were similar to those of the endogenous wild-type subunits.

Glutenin Subunit Composition. Analysis of replicate plots of the lines showed similar biochemical and technological properties (see below). Consequently, only one sample of each line (from a plot grown at Long Ashton) was analyzed for glutenin content and composition. The total glutenin content, determined by SE-HPLC of the total protein extract, depended on the number of HMW subunits expressed (Table 2), with the L88-31 control line (1A, 1D null) containing less glutenin than the L88-6 control line, which contained 1A, 1B, and 1D subunits. As expected, the insertion of transgenes increased the proportions of HMW subunits. However, the total protein contents were not affected by the expression of transgenes, although the 1Ax1 and 1Dx5

Table 3. SE-HPLC Analysis of Proteins Extracted from Control and Transgenic Lines[a]

genotype	line	growing location	expressed subunit	% of total protein extracted in: % 0.5 SDS P1	% 0.5 SDS P2	% 0.5 SDS P3	2% SDS + sonication	2% SDS + DTT + sonication
L88-31 (1Bx17,1By18)	control	LARS		7.5 (0.4) e	13.6 (0.9) d	66.3 (4.1) f	10.6 (4.9) b	2.0 (0.2) a
		RES		5.0 (0.9) d	14.4 (0.3) d	68.2 (1.4) f	10.3 (1.3) b	2.9 (0.6) a
	B72-8-11b	LARS	1Dx5	4.8(0.6) d	11.1 (0.9) b	57.5 (0.05) c	8.3 (0.9) a	18.4 (0.7) b
		RES		3.2 (0.4) c	11.0 (0.4) b	53.2 (0.8) b	14.4 (0.8) c	18.2 (0.2) b
	B102-1-2	LARS	1Ax1	8.0 (0.6) e	15.0 (1.3) d	63.3 (1.7) d	11.8 (3.5) b	2.0 (0.08) a
		RES		3.6 (0.7) c	12.8 (0.7) c	61.6 (1.4) d	19.4 (1.3) d	2.9 (0.3) a
L88-6 (1Ax1, 1Bx17 By18, 1Dx5 1Dy10)	control	LARS		3.2 (0.2) c	9.7 (0.7) b	53.4 (2.7) b	29.9 (1.9) e	3.1 (0.5) a
		RES		2.6 (0.4) b	10.6 (0.4) b	58.7 (1) c	25.4 (0.8) e	2.5 (0.6) a
	B73-6-1	LARS	1Dx5	2.8 (0.1) b	8.7 (0.2) a	47.0 (2.3) a	12.1 (0.9) b	29.4 (1.3) c
		RES		1.9 (0.3) a	8.5 (0.4) a	46.2 (2.8) a	14.8 (1.6) c	28.8 (1.5) c

[a] Figures correspond to the mean values of the different plots of each line (standard deviation in parentheses). In each column, numbers with different following letters differ at the $P = 0.05$ level.

subunits encoded by the transgenes were overexpressed and accounted for 50 and >70% of HMW subunits, respectively, in the transformed lines.

Extractability and Aggregation of Gluten Proteins. Little difference was observed among plots of the same line and only mean values of the plots from each growing location were considered below. The effects of overexpression of subunits 1Ax1 or 1Dx5 in the two different genotypes are compared in Table 3. Initial comparison of the two control lines showed that L88-6 contained a higher proportion of highly aggregated glutenin polymers (extracted by 2% SDS with sonication) than did L88-31, which is consistent with the presence of five HMW subunits in L88-6 but only two (1Bx7 and 1By18) in L88-31. This agrees with previous studies of near-isogenic lines differing in their numbers of HMW glutenin subunits (*4*, *6*). It is notable that only small proportions of the total protein (≅2%) were extracted from these lines in the third step (sonication with 2% SDS and DTT), demonstrating that sonication in the absence of a reducing agent is generally very efficient at solubilizing glutenin.

Overexpression of subunit 1Ax1 increased the proportion of glutenin extractable by sonication with 2% SDS in the L88-31 background but the amount extracted by sonication with SDS and DTT did not change. This indicated that the transgenic line contained a higher proportion of more highly aggregated glutenin polymers but that these had solubility properties similar to those in the control line. Overexpression of subunit 1Dx5 modified glutenin solubility more extensively. The proportions of proteins extractable with 0.5% SDS and with 2% SDS with sonication decreased, but the proportion of proteins extracted only in the presence of DTT increased considerably in both backgrounds. The difference between the effects of the 1Ax1 and 1Dx5 transgenes is clearly observed when the L88-31 lines are compared, because their quantitative compositions of glutenin subunits and their total glutenin contents are similar.

The modification of glutenin extractability was also greater when subunit 1Dx5 was expressed in the L88-6 background, because the proteins extracted in the presence of DTT accounted for ∼29% of total flour proteins compared with ∼18% in the L88-31 background. Nevertheless, subunit 1Dx5 accounted for about the same proportion of the HMW glutenin subunits (∼70%) in the two lines (see Table 2). The greater effect on glutenin extractability may, therefore, be due to the higher content of HMW glutenin subunits in L88-6. SDS-PAGE analyses of the extracted fractions indicated that subunit 1Dx5 was more concentrated in the DTT-extractable fractions of both lines (not shown), suggesting that it increased the extent of intermolecular covalent (disulfide) bonding, resulting in very large and insoluble glutenin polymers. The specific effect of subunit 1Dx5 on intermolecular disulfide bonding may

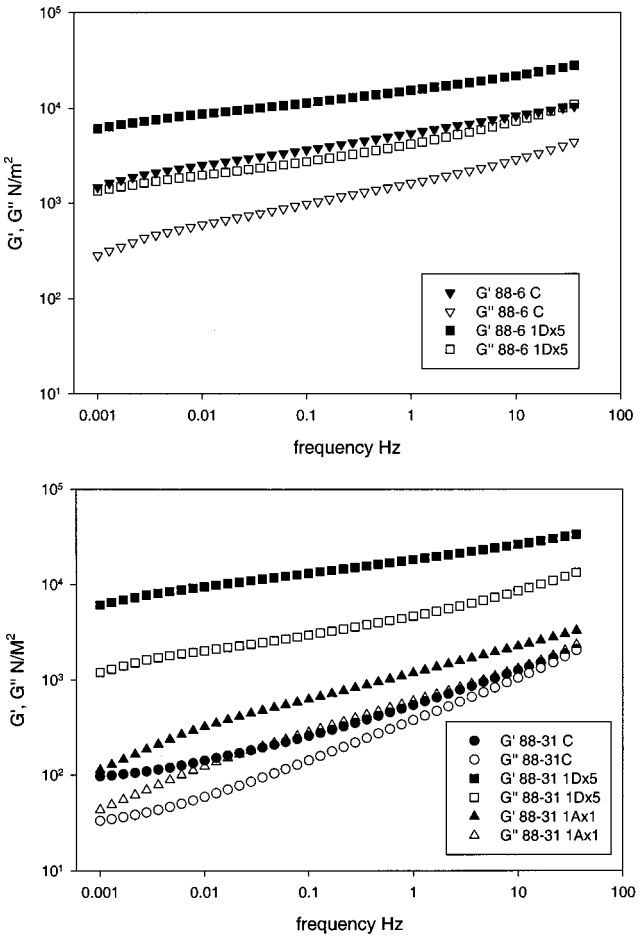

Figure 2. Mechanical spectra of fully hydrated glutens extracted from control and transgenic lines showing storage (G') and loss (G'')moduli as a function of frequency: 88-6C and 88-31C, control lines; 88-6 1Dx5 and 88-31 1Dx5, transformed lines expressing 1Dx5 subunit transgene; 88-31 1Ax1, transformed line expressing 1Ax1 subunit transgene.

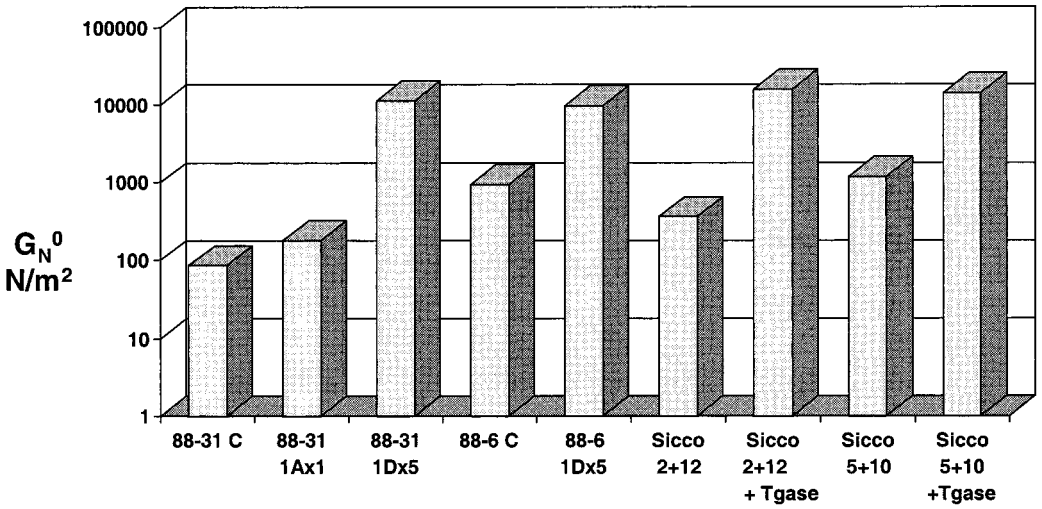

Figure 3. Height of the viscoelastic plateau (G_N^0) of glutens extracted from control and transgenic lines of wheat (this work) compared with glutens from near-isogenic lines with or without covalent cross-linking by transglutaminase (from ref *18*): 88-6C and 88-31C, control lines; 88-6 1Dx5 and 88-31 1Dx5, transformed lines expressing 1Dx5 subunit transgene; 88-31 1Ax1, transformed line expressing 1Ax1 subunit transgene.

Table 4. Mixograph Analysis of Flours from Control and Transgenic Lines[a]

genotype	line	growing location	inserted subunit	peak time (min)	peak value torque (%)	peak width torque (%)	midline at 10 min torque (%)	width at 10 min torque (%)
L88-6	control	LARS		4.9 (0.4) e	43.7 (2.5) e	20.6 (2.0) d	34.3 (0.6) g	8.6 (0.7) c
		RES		5.0 (0.2) e	47.7 (0.8) f	22.2 (1.7) e	34.2 (1.0) g	7.2 (0.8) b
	B73-6-1	LARS	1Dx5	1.3 (0.3) a	16.8 (1.7) a	21.0 (4.0)) d	13.4 (0.1) b	7.9 (0.4) b
		RES		1.5(0.3) a	18.0 (0.5) a	18.6 (2.3) c	16.0 (0.1) c	7.8 (0.3) b
L88-31	control	LARS		2.6 (0.2) b	27.7 (0.9) b	11.2 (0.6) b	20.0 (1.2) d	5.3 (0.4) a
		RES		3.1 (0.2) c	31.6 (3.1) c	11.9(0.8) b	23.2 (2.4) e	4.9 (0.4) a
	B72-8-11b	LARS	1Dx5	4.5 (0.1) d	16.0 (0.4) a	7.8 (0.1) a	8.7 (2.0) a	4.9 (0.5) a
		RES		5.0 (0.1) e	18.2 (0.2) a	8.9 (0.2) a	13.9 (2.5) b	4.5 (0.7) a
	B102-1-2	LARS	1Ax1	3.1 (0.1) c	36.3 (3.1) d	16.5 (1.6) c	26.5 (4.6) f	6.5 (0.2) b
		RES		3.3 (0.1) c	48.7 (2.2) f	22.2 (2.9) e	34.4 (1.4) g	7.4 (0.7) b

[a] Figures correspond to the mean values of the different plots of each line (standard deviations in parentheses). In each column, numbers with different following letters differ at the $P = 0.05$ level.

relate to the presence of an additional cysteine residue in its sequence [as discussed by Shewry et al. (*17*)].

Rheological Properties of Glutens Extracted from Control and Transformed Lines. The mechanical spectra of gluten fractions from the lines were determined (Figure 2). The L88-31 control, which contained only subunits 1Bx17 and 1By18, showed the lowest values of G' and G''. Furthermore, the high-frequency limit of the elastic plateau could be observed within the frequency range. The spectrum is similar to that reported for the same line studied previously (*12*). Furthermore, the G' and G'' values of L88-31 were lower than the corresponding values for the L88-6 control line (1Ax1, 1Bx17, 1By18, 1Dx5, and 1Dy10), confirming that gluten elasticity and viscosity are affected by the number of HMW glutenin subunits expressed in the genotype.

Expression of the 1Ax1 transgene in L88-31 resulted in a moderate increase in the storage and loss moduli, and the upper limit of the plateau was clearly shifted toward higher frequencies. This is related to the limited change observed in glutenin aggregation. On the other hand, expression of subunit 1Dx5 drastically increased gluten storage and loss moduli in both backgrounds. Both moduli were enhanced, but the tangent delta (G''/G') was decreased (not shown), the storage modulus being affected more than the loss modulus. Remarkably, the viscoelastic plateau (G_N^0) of gluten from the L88-31 1Dx5 line was higher than that of gluten from the L88-6 control line, which contains 1A-, 1B-, and 1D-encoded HMW glutenin subunits (Figure 3). This is related to the lower gliadin content and the higher proportion of covalently cross-linked (or tightly aggregated) glutenin polymers in glutens where subunit 1Dx5 is overexpressed. The spectra of glutens from the two lines expressing the 1Dx5 transgene were also similar, with the initial difference between the rheological behavior of glutens from the two parents lines (L88-31 and L88-6) being abolished.

Figure 3 compares also the viscoelastic properties of glutens from the five lines described here with those of normal and transglutaminase-modified glutens from near-isogenic lines of Sicco reported in a previous study (*18*). The present study showed that expression of the 1Dx5 transgene resulted in a very large increase in the connectivity of the gluten network, expressed as G_N^0, which was increased 10 times in the L88-6 background and $\times 100$ times in the L88-31 background. These increased values are very similar to those obtained when glutens from Sicco near-isogenic lines were modified by transglutaminase, an enzyme catalyzing the formation of interchain lysyl−glutamyl bonds. As in the subunit 1Dx5 transgenics, the initial differences between the rheological behavior of glutens from the near-isogenic lines were abolished by the covalent cross-linking of the gluten proteins by transglutaminase. Furthermore, it was also shown that the HMW subunits were preferentially cross-linked by transglutaminase treatment

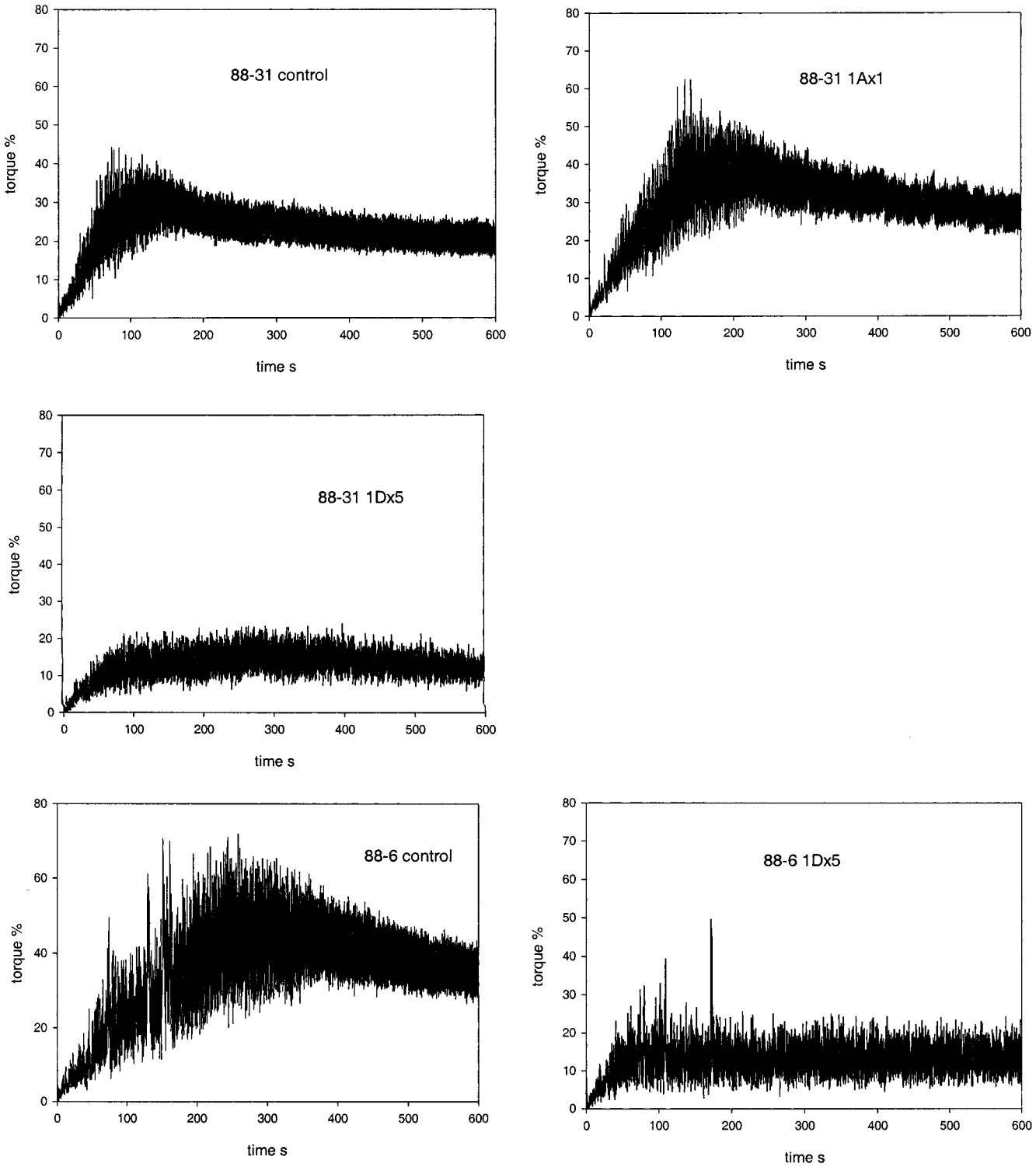

Figure 4. Small-scale (2 g) Mixograph curves for dough of control and transgenic lines of wheat: 88-6C and 88-31C, control lines; 88-6 1Dx5 and 88-31 1Dx5, transformed lines expressing 1Dx5 subunit transgene; 88-31 1Ax1, transformed line expressing 1Ax1 subunit transgene.

(*18*). It is thus reasonable to draw an analogy between the effects on gluten structure of overexpression of the 1Dx5 subunit and of covalent cross-linking by transglutaminase. Similarly, this also supports the hypothesis that expression of the transgene 1Dx5 increases the covalent cross-linking of glutenin.

Mixing Properties. Typical mixograms of the control and transgenic lines are shown in Figure 4. For a given line, the mixing behavior was almost independent of the plot considered, as shown by the standard deviations of measurements, and only small differences were observed between samples of the same line grown at the two sites (Table 4).

The control line L88-31 exhibited weaker mixing properties than the control line L88-6, with lower peak time, peak value, and peak width and lower width at 10 min (Table 4). This is related to the differences in HMW subunit composition and glutenin aggregation, and similar results were reported on another set of near-isogenic lines (*6*). Expression of the subunit 1Ax1

transgene enhanced the mixing properties of line L88-31. Although there was little effect on the peak time, the bandwidth, which is related to resistance to the extension of dough (*19*), was increased. The effect of the expression of subunit 1Dx5 transgene was unexpected. In L88-31, this increased the peak time but decreased all of the other mixogram characteristics. In line L88-6, which was initially much stronger than L88-31, the expression of subunit 1Dx5 decreased all of the mixogram characteristics. In fact, the transgenic lines expressing subunit 1Dx5 failed to form a cohesive dough under hydration and mixing in the mixograph bowl, as observed previously (*14*). In the present sudy, however, increasing mixing speed and changing the dough hydration did not allow the "normal" mixing properties to be recovered (results not shown). This unusual behavior was related to the very high strength of the gluten, as shown by rheological analyses. Under the mixograph mixing conditions the gluten particles may not have associated but rested divided, so that the dough could not exhibit the usual extensibility. It should also be noted that isolation of gluten from the transgenic lines with subunit 1Dx5 required a centrifugation step to recover a cohesive viscoelastic material without loss.

DISCUSSION

It has been shown from extractability experiments that glutenin aggregation depended on the number of HMW subunits expressed in the lines. Clear differences were observed between the functional properties of subunits 1Ax1 and 1Dx5, emphasizing that small differences in subunit structure can influence their functionality and contribution to gluten structure and rheology. Insertion of the subunit 1Ax1 transgene increased glutenin aggregation but did not appear to result in extensive cross-linking by disulfide bonds. Gluten viscoelasticity was only moderately altered by expression of subunit 1Ax1, which mainly increased the dough resistance to elongation during mixing. In contrast, overexpression of subunit 1Dx5 considerably increased the aggregation of glutenin, probably through covalent cross-linking of polymers, because this generated very insoluble proteins that were extracted only by reducing disulfide bridges. The connectivity of the gluten network was considerably increased. This effect can be attributed to the presence of an additional cysteine residue available for intermolecular cross-linking in subunit 1Dx5 [see Shewry et al. (*17*)]. This very high gluten strength resulted in abnormal mixing behavior, such as the absence of a real peak of torque and very low torque, because proteins failed to form a cohesive mass under the action of the mixing head. It can be postulated that an excess of subunit 1Dx5 modified the glutenin (gluten) structure and hindered the formation of a homogeneous protein network. Subunit 1Dx5 is always expressed as a pair with 1Dy10, and there is evidence that dimers between these two subunits, and between other x-type and y-type subunits, are present as "building blocks" in the glutenin polymers (*20*−*24*). Overexpression of subunit 1Dx5 in the absence of additional subunit 1Dy10 (or other y-type subunit) could therefore result in extensive restructuring of the glutenin polymers with important consequences for gluten strength and for the mixing and baking properties of the dough. The results reported here show that transformation can indeed be used to modify the technological properties of gluten proteins. Furthermore, the drastic effects obtained by expression of subunit 1Dx5 may facilitate the development of new uses of wheat requiring very strong glutens, either in the food industry or for nonfood applications.

ACKNOWLEDGMENT

We are grateful to colleagues at IACR—Rothamsted and IACR—Long Ashton for assistance with designing and running the field trials, notably Dr. Paul Lazzeri, Dr. Pilar Barcelo (both currently at DuPont Wheat Transformation Laboratory, c/o Rothamsted), Dr. Huw Jones, Mr. Colin Peters, Dr. Lee Rooke, Dr. Martin Cannell, Ms. Gillian Arnold, Mr. Bob Hughes, Mr. Jason Hughes, and Ms. Sheila Dixon. We thank L. Linossier (ULICE, Riom, France), who performed capillary electrophoresis determinations. Finally, we are indebted to Dr. Francisco Barro (CSIC, Cordoba, Spain) for production of the transgenic lines and to Dr. Rudi Appels and Dr. Greg Lawrence (CSIRO, Canberra, Australia) for providing L88-6 and L88-31.

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Received for review August 14, 2000. Accepted October 20, 2000. IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. This work is part the program Eurowheat "Improving the quality of EU wheats for use in food industry" FAIR CT96-1170 supported financially by the European Community, DG XII.

JF001015J