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Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits

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Abstract Wheat HMW glutenin subunit genes 1Ax1 and 1Dx5 were introduced, and either expressed or overexpressed, into a commercial wheat cultivar that already expresses five subunits. Six independent transgenic events were obtained and characterized by SDS-PAGE and Southern analysis. The 1Dx5 gene was overexpressed in two events without changes in the other endosperm proteins. Overexpression of 1Dx5 increased the contribution of HMW glutenin subunits to total protein up to 22%. Two events express the 1Ax1 subunit transgene with associated silencing of the 1Ax2* endogenous subunit. In the SDS-PAGE one of them shows a new HMW glutenin band of an apparent M_r lower than that of the 1Dx5 subunit. Southern analysis of the four events confirmed transformation and suggest that the transgenes are present in a low copy number. Silencing of all the HMW glutenin subunits was observed in two different events of transgenic wheat expressing the 1Ax1 subunit transgene and overexpressing the Dx5 gene. Transgenes and expression patterns were stably transmitted to the progenies in all the events except one where in some of the segregating T_2 seeds the silencing of all HMW glutenin subunits was reverted associated with a drastic lost of transgenes from a high to a low copy number. The revertant T_2 seeds expressed the five endogenous subunits plus the 1Ax1 transgene.

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Introduction

Wheat is the most important crop in the world in terms of the area under cultivation, yield and geographical distribution. It is widely used in food processing (e.g. for bread, pasta, noodles) and as feed for livestock. Nevertheless, the development of transformation technology in wheat has lagged behind that for other major crops (e.g. rice, maize, soybean), with reliable methods based on microprojectile bombardment only becoming available over the past 5 years (Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994; Barcelo and Lazzeri, 1995; Ortiz et al. 1996). Even so the technology is still not routine, with a high variation between the efficiencies achieved with different genotypes. In particular, the cultivar Bob White has proved to be particularly amenable to transformation and regeneration, and has therefore become widely used (Vasil et al. 1993; Weeks et al. 1993).

The availability of transformation technology provides an opportunity to manipulate wheat to enhance its agronomic performance, resistance to abiotic and biotic stresses, yield and end-use quality. However, attention so far has focused on manipulating the composition and properties of the gluten proteins by introducing additional genes for one group of proteins called the high-molecular-weight (HMW) subunits of glutenin.

The gluten proteins are present as a network in dough and confer the visco-elastic properties which allow wheat to be processed into bread, pasta and noodles, as well as in a range of other food products. Although gluten comprises a large number of proteins, the HMW subunits appear to be particularly important in the formation of high M_r polymers, which are highly elastic. Because poor processing quality results from low gluten elasticity, the HMW subunits have been studied in detail to determine their structure and genetics (Payne, 1987;

Shewry et al. 1992, 1995 a). All cultivars of hexaploid bread wheat contain six HMW subunit genes (two each, called x-type and y-type, on chromosomes 1 A, 1 B and 1 D) but specific gene silencing results in the presence of only three, four or five HMW subunit proteins. Each protein accounts, on average, for about 2% of the total extractable grain protein (Halford et al. 1992; Seilmeier et al. 1992) and the differences in gene expression therefore result in quantitative effects on the total amount of HMW subunit protein, which in turn impacts on dough visco-elasticity. Although there are also more subtle effects on quality relating to allelic variation in the composition of the expressed subunits, it is the clear association between the numbers of expressed genes and quality which has made the HMW subunits such an attractive system for manipulation.

Three laboratories have reported the expression of HMW subunit transgenes in wheat, using either Bob White (Altpeter et al. 1996; Blechl and Anderson, 1996) or genetic stocks selected on the basis of their HMW subunit composition (Barro et al. 1997), the latter study also reporting effects on dough functional properties (Barro et al. 1997; Rooke et al. 1999).

Genetic engineering of plants sometimes results in transgene silencing after integration into the genome, which may relate to a defense mechanism against foreign DNA expression (Kumpatla et al. 1998; Vaucheret et al. 1998). Moreover, homologous endogenous genes can also be silenced (Meyer and Saedler, 1996). This phenomenon has attracted considerable interest because it may be detrimental to genetic engineering and also because of its usefulness as a tool to study the mechanisms involved in detecting and inactivating exogenous DNA (Matzke et al. 1996; Kumpatla et al. 1998).

In the present paper we report on the transformation of a currently grown commercial variety of spring wheat with two HMW subunit genes, using microprojectile bombardment. Six independent events are characterized, four of which result in partial or complete gene silencing. This high level of silencing contrasts with the previous studies reported above, although it is not unexpected considering the homology of the transgenes introduced with the endogenous genes (Meyer and Saedler, 1996).

Materials and methods

Plant material and wheat transformation

Immature embryos were prepared from wheat plants (*Triticum aestivum* L., cv Pro INTA Federal) grown in the field or in growth chambers with a 12-h photoperiod and a day/night temperature of 22/18°C and cultured for 7–9 days before bombardment, as described by Ortiz et al. (1996). Wheat transformation was achieved essentially as reported (Ortiz et al. 1997) using a helium-driven gene gun and a mixture of the following plasmids: pHMW1Dx5 (Halford et al. 1989) containing a 8.7-kb *Eco*RI genomic fragment including the complete coding sequence of the 1Dx5 gene (Anderson et al. 1989), flanked by approximately 3.8 kb and 2.2 kb of 5' and 3' sequences, respectively; pHMW1Ax1 (Halford et al. 1992) containing a 7.0-kb *Eco*RI fragment including the complete coding sequence of the 1Ax1 gene, flanked by approximately 2.2 and 2.1 kb of 5' and 3' sequences, respectively. In both cases, the HMW glutenin subunit genes were driven by their own endosperm-specific promoters. In addition, a third plasmid was added carrying a selectable marker gene which was either the *bar* gene [plasmid pAHC25 (Christensen and Quail 1996)] or the *hpt* gene [plasmid pGL2 (Shimamoto et al. 1989)]. Plasmid DNA (an equimolar mixture of the three plasmids) was co-precipitated onto gold particles (1.5–3 µm, Bio Rad) at a rate of 2 µg of DNA per mg of particle as described (Klein et al. 1988). After bombardment calli were cultured as described (Ortiz et al. 1996) in the presence of the selective agent (either hygromycin at 25 mg/l or glufosinate 1 mg/l). Plantlets arising from calli were recovered and transferred to Murashige-Skoog medium without hormones. Rooted plants were acclimated and grown to maturity in a Percivall growth chamber.

DNA isolation and Southern-blot hybridization

Southern blots were carried out with total genomic DNA isolated from wheat leaves by the CTAB method (Saghai-Maroof et al. 1984). DNA was digested with *Eco*RV, *Bam*HI or *Xba*I and the resulting fragments were separated by electrophoresis in a 0.7% (w/v) agarose gel and transferred by capillary blotting to Hybond N+ membranes according to the manufacturer's instructions (Amersham, Little Chalfont, UK). Membranes were hybridized with ^{32}P-radioactive probes generated by PCR (Mertz and Rashtchian 1994) using primers for the 1Ax1 HMW glutenin subunit gene (upper primer 5′ AGATGACTAAGCGGTTGGTTC 3′; lower primer 5' CCTTGTCCTGGTTGCTGTCTTTGT 3') and the 1Dx5 subunit gene with p1Ax1 or p1Dx5, respectively, as the DNA template (D'Ovidio and Anderson 1994). Hybridization was performed at 65°C for 18 h, and signals were visualized by autoradiography.

Electrophoresis of total wheat endosperm proteins

Protein fractions were extracted by grinding mature single half-grains with a mortar and pestle. The resultant flour from each seed was vortexed with 25 µl of total protein extraction buffer [0.0625 M Tris-HCl pH 6.8; 2% (w/v) SDS; 1.5% (w/v) dithiothreitol; 10% (v/v) glycerol; 0.002% (w/v) Bromophenol blue], per mg of material, and allowed to stand for 2 h at room temperature. The extracts were centrifuged (15 min, 14 000 rpm) and the supernatant boiled for 5 min. The proteins were separated by SDS-PAGE using a Tris-borate buffer system and 10% (w/v) acrylamide gels (Shewry et al. 1995 b), and run until the dye front reached the bottom of the 15-cm gel.

Quantification of HMW glutenin subunits

Densitometric analysis of SDS-PAGE gels was carried out using a Hewlett Packard ScanJet 3c scanner together with the image analyzer software, Image-Pro Plus for Windows. Lane and peak values were corrected by interband background subtraction. Although proteins of molecular weights <20 kDa are lost under the conditions used to separate subunits 1Dx5 and 1Ax2*, the other storage proteins that constitute the bulk of the endosperm remain on the gels and were used to normalize expression of the HMW-GS within a given lane.

Results

A commercial spring wheat cultivar, Federal, was co-transformed by microparticle bombardment with the 1Ax1 and 1Dx5 HMW glutenin subunit genes plus a selectable marker gene which was either the *bar* or the *hpt*

Table 1 Six independent transgenic events expressing either one or both glutenin subunit transgenes

Transgenic event	Transgenes		Gene(s) silenced	Transgene(s) copy number
	1Ax1	Dx5		
A	–	+	None	Low (3)
B	–	+	None	nd[a]
C	+	–	1Ax2	Low (3)
D	+	+	1Ax2	Low (3)
E	+	?	All HMW glu	High (20)
F	+	+	All HMW glu	High (50)

[a] nd, not determined

gene as described (Ortiz et al. 1996). In nine experiments involving 1348 explants at least six independent transgenic events, expressing either one or both glutenin subunit transgenes, were recovered (Table 1) giving an overall transformation efficiency of 0.45%. Not all recovered plants were analysed for the selectable marker gene and it is possible that some transformants containing only this gene, or the subunit transgenes with low or no expression, were not scored as transformants.

The wheat cultivar selected for transformation expresses five HMW glutenin subunits: 1Ax2*, 1Dx5, 1Bx7, 1By9 and 1Dy10 (Fig. 1, W.T.). Thus, the expression of the 1Ax1 subunit transgene was easily detected by SDS-PAGE in four out of the six transgenic events (events C, D, E, F in Table 1). Although subunit 1Dx5 was present in the control lines, the 1Dx5 subunit transgene was expressed at a sufficient level to give an increased proportion of the encoded protein in at least two events (events A and B in Table 1 and Fig. 1). The progeny of eight T_1 plants of event A were obtained and analysis of the T_2 seeds revealed that two families were homozygous for the 1Dx5 transgene, one of these being shown in Fig. 1. No major changes were observed in the endogenous HMW subunits nor in the contents of LMW glutenins or gladins in events A and B (Fig. 1, AT_2), but complete silencing of subunit 1Ax2* was observed with the expression of the 1Ax1 transgene in events C and D (Table 1) and of all the HMW glutenin subunits in events E and F (Table 1).

Transformation in event A was confirmed by the Southern hybridization (Fig. 2 A lanes 1–3) of DNA from T_1 plants restricted with *Eco*RV or *Xba*I. Both enzymes cut only once within the plasmid but do not cut the transgenes. Restriction of wild-type DNA with *Eco*RV results in four main bands of 5.8, 6, 7.6 and 8-kb, but the 5.8- and 6-kb bands are often not resolved giving a single broad band of 5.9 kb. The DNA from the transgenic T_1 plant shown in Fig. 1 A gave an extra hybridization band of 4.2 kb in addition to a stronger 5.9-kb band (Fig. 2 A, lane 3). The T_2 progeny of this plant (Fig. 1, AT2) gave a similar hybridization pattern (Fig. 2A, lanes 1 and 2). Similarly DNA from the T_1 plant restricted with *Xba*I gave an extra hybridization band of 12.8-kb and a stronger 22.3-kb endogenous band data (not shown) . The copy number of the 1Dx5 transgene,

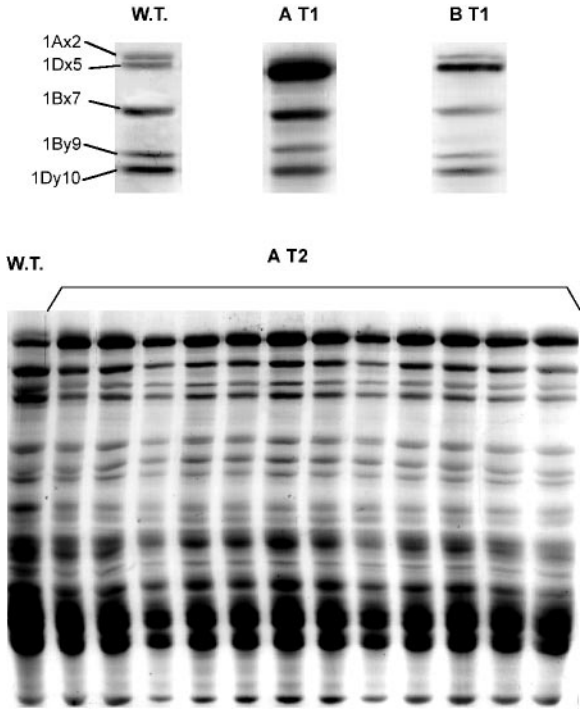

Fig. 1 SDS-PAGE of endosperm proteins of single seeds from events A and B. Upper panels, HMW glutenin subunits of a control wild type (W.T.) and T_1 seeds from events A (AT_1) and B (BT_1). *Lower panel,* endosperm proteins of 12 T_2 seeds from a single T_1 from event A (AT_2) and a wild-type seed

calculated by densitometric analysis of the Southern blots obtained with both restriction enzymes, gave a value of about three, indicating that the transgene was present in low copy number.

In order to determine whether overexpression of 1Dx5 in events A and B affected the contribution of HMW glutenins to total endosperm proteins, densitometric analyses of the gels were carried out (Table 2). These showed that the total amounts of HMW glutenin subunits expressed as a percentage of the total protein in the gel increased from about 11% to 13.4% and 12.1% for events A and B, respectively (Table 2). These increases correlate with increases in the amount of subunit 1Dx5, from 2% to 4.8% and 2.7% of total protein for events A and B (Table 2), respectively. It should be noted that the levels of the remaining four subunits did not change significantly in any of the experiments, suggesting that the greater contribution of the HMW glutenins to total endosperm protein is due solely to the expression of the 1Dx5 subunit transgene.

Figure 3 shows analyses of protein fractions from T_1 and T_2 seeds of event C. Expression of the 1Ax1 transgene is observed in all T_2 progeny but is associated with complete silencing of the endogenous 1Ax2* gene. Densitometric analysis of the gels shows that the contributions of HMW glutenin subunits to the total endosperm protein increased from 11% to 13.3% (Table 2, event C), with the 1Ax1 transgene product accounting for about 2% of the total. Southern-blot analysis of DNA from a

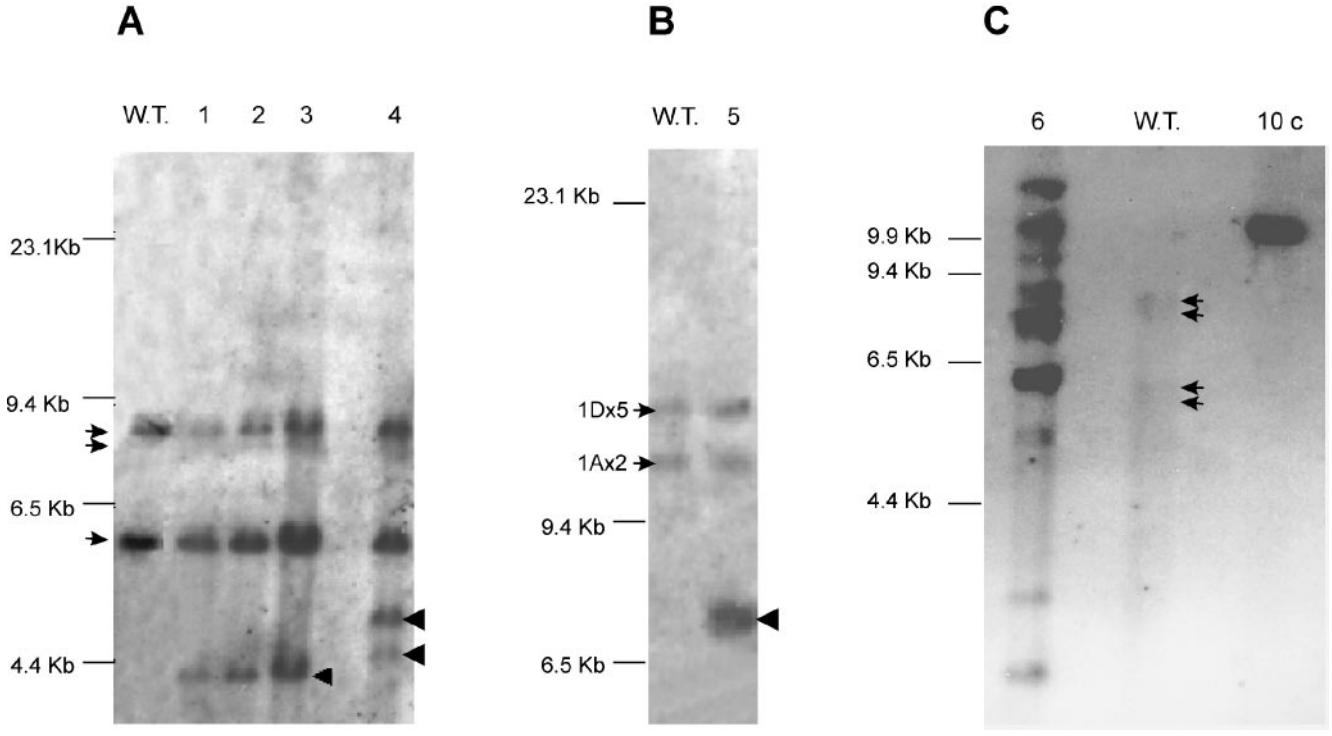

Fig. 2 A–C Southern blot analysis of transgenic lines from events A, C, D and E. Genomic DNA was digested with *Eco*RV (**panels A and C**) or *Bam*H1 (**panel B**). *Lanes 1,2 and 3* in **panel A** are from T_2, T_2 and T_1 plants, respectively, from event A; *lane 4* is from a T_2 plant of event D; *lane 5* in **panel B** is from a T_2 plant of Event C and *lane 6* in **panel C** is from a T_3 plant of event E. W.T., wild-type; *large arrowhead* extra transgenic band; *smaller arrows* endogenous bands; *10 c*, ten copies of p1Ax1

T_2 plant restricted with *Bam*HI (Fig. 2 B, lane 5) confirms transformation and suggests that the copy number was about three.

Figure 4 shows protein fractions from single seeds from transgenic event D (Table 1). Six HMW glutenin bands are present including one corresponding to the subunit 1Ax1 transgene product and a new band, labelled X, with an apparent M_r lower than that of the 1Dx5 subunit. On the other hand, the endogenous 1Ax2* subunit was partially silenced in the hemizygous T_1 which shows seven bands and was completely silenced in the homozygous T_2 as well as in the T_3 progeny, as shown in Fig. 4 (lanes 1–7). In this event the contribution of HMW glutenin subunits to the total protein increased from about 11% to 12.3% but there was little or no effect on the expression of the other glutenin subunits. Two extra bands of 4.5 and 4.9 kb are shown in the Southern blot of a T_2 plant (Fig. 2 A lane 4) which suggests a low copy number of about three.

In addition to the specific silencing of the subunit 1Ax2* gene in events C and D, strong suppression of all the HMW glutenin subunits was observed in two events (Table 1, events E and F, and Figs. 5 and 6). Figure 5, panel T_1, lane 2, shows endosperm proteins from a T_1 seed in which the expression of all the HMW glutenin subunits was strongly reduced when compared with lane 1 corresponding to a null T_1 seed. In spite of the low level of expression, the transgenic 1Ax1 subunit is evident (arrowhead). Moreover, the 1Dx5 band remains visible suggesting that it may also correspond to a transgene product (Fig. 5, panel T_1, lane 2). The phenomenon was genetically stable since the T_2 and T_3 progenies showed a similar pattern or even a stronger silencing (Fig. 5, panels T_2 and T_3). The three T_2 seeds shown in Fig. 5 reached maturity and set T_3 seeds all of which also showed silencing (two seeds from each plant are shown in Fig. 5, panel T_3). The silencing of the HMW glutenin subunits in this transgenic event is associated with a high

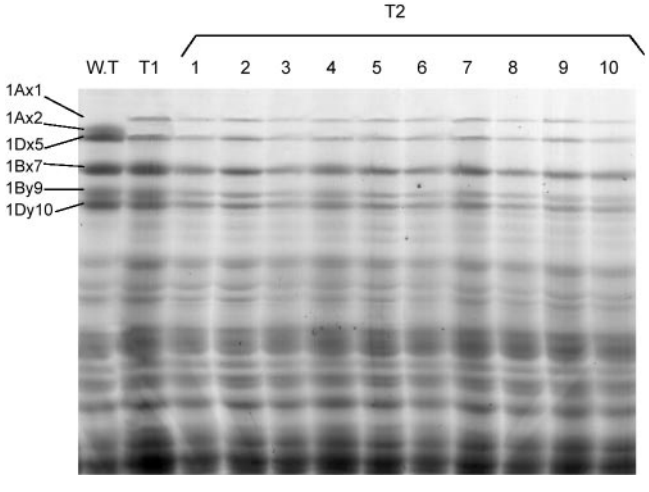

Fig. 3 SDS-PAGE of endosperm polypeptides of a T_1 seed (*T_1*) and ten of its T_2 progeny from event C (*lanes 1–10*). W.T. wild-type

Table 2 Densitometric analysis of SDS-PAGE of endosperm protein from transgenic wheat. The values given are averaged ± standard error of densitometric scans of SDS-PAGE gels of the stated amounts of seeds carried out as described in the text. The sums of all HMW-GS peaks or single peaks were divided by the total scan of the same lane. S: seeds with silencing of endogenous HMW-GS genes. (†) seeds showing normal pattern of five endogenous HMW-GS expression and an extra band belonging to 1Ax1 transgene. (‡) seeds with reversion of the silencing phenomenon (all of them came from a silenced T1 seed)

Event	Seeds	Total HMW-GS/ total protein (%)	Transgenic HMW-GS 1Ax1/ total protein (%)	HMW-GS 1Ax2*/ total protein (%)	HMW-GS 1Dx5/total protein (%)	Band X/ total protein (%)	HMW-GS 1Bx7 /total protein (%)	HMW-GS 1By9/total protein (%)	HMW-GS 1Dy10/total protein (%)
Control	25	11.0 ± 0.19	0.0 ± 0.0	1.2 ± 0.06	2.0 ± 0.05	–	2.9 ± 0.07	2.0 ± 0.05	2.8 ± 0.05
A	11 T1	13.4 ± 0.28	0.0 ± 0.0	1.4 ± 0.07	4.8 ± 0.24	–	2.9 ± 0.09	1.8 ± 0.08	2.6 ± 0.12
	12 T2	13.1 ± 0.29	0.0 ± 0.0	1.5 ± 0.08	4.8 ± 0.11	–	2.6 ± 0.10	1.9 ± 0.10	2.4 ± 0.09
B	21 T1	12.1 ± 0.20	0.0 ± 0.0	1.3 ± 0.08	2.7 ± 0.05	–	3.0 ± 0.06	2.3 ± 0.06	2.9 ± 0.09
C	1 T1	12.7 ± 0.29	1.9 ± 0.19	0.0 ± 0.0	2.4 ± 0.04	–	3.5 ± 0.07	2.0 ± 0.03	2.9 ± 0.01
	24 T2	13.3 ± 0.24	2.0 ± 0.05	0.0 ± 0.0	2.5 ± 0.06	–	3.3 ± 0.09	2.3 ± 0.05	3.2 ± 0.08
D	1 T1	12.3	1.4	1	1.8	1.6	2,3	2.2	2.2
	1 T2	12.1	1.6	0	1.8	1.4	2,5	1.8	3
	13 T3	12.3 ± 0.23	1.8 ± 0.05	0.0 ± 0.0	1.7 ± 0.07	1.6 ± 0.06	2.8 ± 0.08	1.8 ± 0.06	2.9 ± 0.06
E	1 T1 S	4.0 ± 0.12	0.5 ± 0.03	0.0 ± 0.0	1.0 ± 0.03	–	0.9 ± 0.06	0,6 ± 0,5	1.1 ± 0.4
	13 T2 S	1.0 ± 0.12	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.08	–	0.6 ± 0.05	0.0 ± 0.0	0.6 ± 0.05
	27 T2 S	1.0 ± 0.17	0.2 ± 0.04	0.0 ± 0.0	0.6 ± 0.05	–	0.05 ± 0.03	0.05 ± 0.03	0.1 ± 0.05
F	97 T1 S	3.1 ± 0.09	1.2 ± 0.04	0.0 ± 0.0	1.9 ± 0.05	–	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.05
	1 T1 6b (†)	11.1 ± 0.2	1.3 ± 0.05	1.1 ± 0.04	2.2 ± 0.04	–	2.3 ± 0.10	1.7 ± 0.05	2.5 ± 0.05
	18 T2 6b (†)	9.8 ± 0.32	0.9 ± 0.07	1.0 ± 0.06	1.9 ± 0.05	–	2.2 ± 010	1.5 ± 0.06	2.4 ± 0.08
	24 T2 S	4.0 ± 0.11	1.3 ± 0.04	0.0 ± 0.0	2.6 ± 0.09	–	0.0 ± 0.0	0.6 ± 0.05	0.6 ± 0.05
	5 T2 (‡)	10.7 ± 0.5	1.0 ± 0.02	1.1 ± 0.04	1.5 ± 0.07	–	3.0 ± 0.17	1.5 ± 0.11	2.6 ± 0.18

Fig. 4 SDS-PAGE of endosperm proteins of a control wild-type seed (W.T.), a T_1 seed from event D (T_1) an homozygous T_2 seed (T_2) and its T_3 progeny (T_3, *lanes 1–7*)

Fig. 5 SDS-PAGE of endosperm proteins from control wild-type seeds (W.T.); two T_1 seeds from event E (*lanes 1, 2*); three T_2 seeds from the T_1 plant shown in *lane 2* (*lanes 3–5*) and two T_3 seeds from each of the T_2 plants shown in *lanes 3* (*lanes 6, 7*), *4* (*lanes 8, 9*) and *5* (*lanes 10,11*)

copy number and multiple insertion sites of the transgenes as shown by Southern blotting (Fig. 2 C). When the DNA from the T_1 transgenic plant (Fig. 2 C, lane 6) from event E was restricted with *Eco*RV at least nine extra hybridization bands were observed at 11.6, 10.2, 9, 7.3, 5.9, 4.9 (doublet), 2.95 and 2.1 kb. The strong band at 5.9 kb coincides with one of the endogenous bands as with other transgenes. Densitometric analysis of the autoradiograph suggests that the copy number of the transgenes is about 20.

Figure 6 A shows protein fractions from seeds from event F, where the expression of both transgenes was stronger and the silencing of the remaining subunits

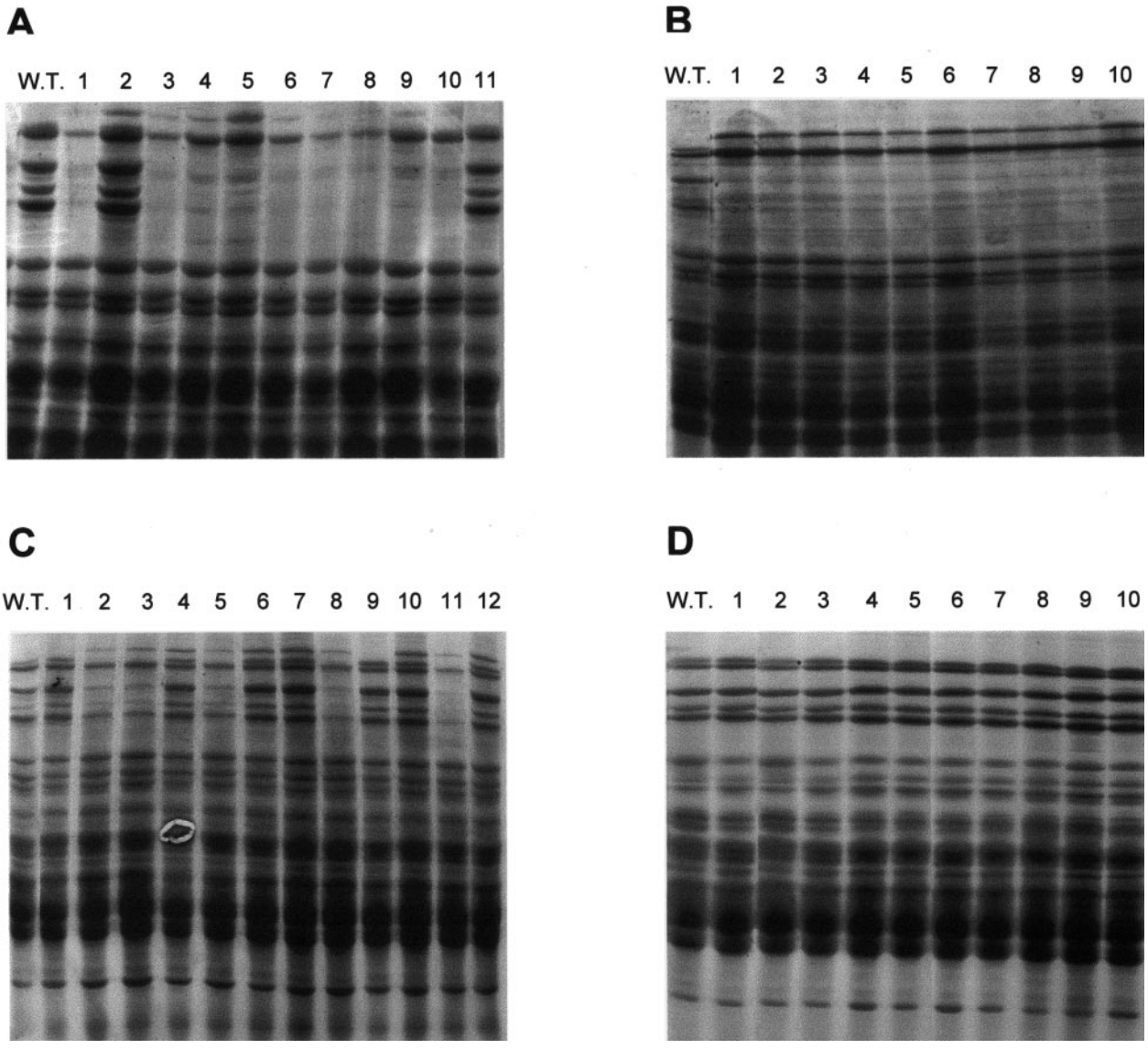

Fig. 6 A—D SDS-PAGE of endosperm proteins of 11 T_1 seeds from event F (**panel A**); ten T_2 seeds from an homozygous T_1 plant (**panel B**); 12 T_2 seeds from an heterozygous T_1 plant (**panel C**) and 10 negative T_2 seeds (**panel D**). W.T., wild-type

(1Ax2*, Bx7, By9 and Dy10) nearly complete. This transgenic event gave 102 T_1 seeds of which 97 show silencing (nine of these are shown in Fig. 6 A, lanes 1, 3–10), five were negative (one is shown in Fig. 6 A, lane 11) and one expressed six subunits including the 1Ax1 transgene protein (shown in Fig. 6 A, lane 2). In contrast with event E, the pattern of silencing was not stable in the T_2 progenies of event F. T_2 progeny from 12 of the 97 T_1 seeds from event F which showed silencing were analysed. Seven out of these retained the silencing pattern with the expression of subunits 1Ax1 and 1Dx5. The progeny of one such plant are shown in Fig. 6 B. In this case 24 seeds were analysed and all showed strong expression of both the 1Ax1 and 1Dx5 transgenes and near complete silencing of the other subunits. Segregation occurred in the other five T_2 plants, with silenced and non-silenced seeds. Two of these families have some T_2 seeds showing a pattern of six expressed subunits, the five endogenous HMW glutenin subunits plus the transgene 1Ax1. This is illustrated in Fig. 6 C, where 5 of the 12 seeds (lanes 4, 6, 7, 10, 12) show such an expression pattern. In this case subunit 1Ax2* is expressed together with the 1Ax1 transgene and the pattern of expression is identical to that of a single T_1 seed shown in Fig. 6 A, lane 3. Another five seeds of the T_2 family in Fig. 6 C (lanes 2, 3, 5, 8, 11) showed weaker silencing of all HMW glutenin subunits than in the T_1 plant, while two seeds expressed only the five endogenous subunits and not the transgene. Figure 6 D shows a family of non-silenced seeds.

Densitometric analysis of events E and F (Table 2) showed that the percentage of the HMW subunits in the gel decreased from 11% in the wild-type grain to 4.1% and 3.1% for events E (Fig. 5) and F (Fig. 6 A), respec-

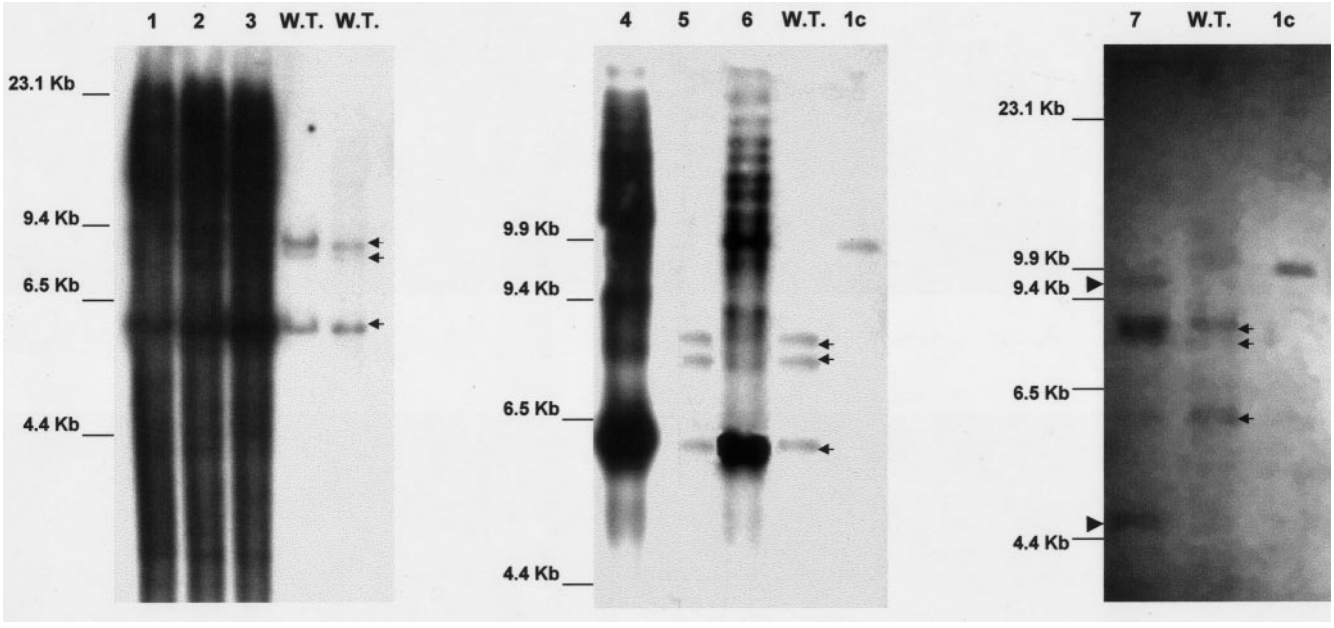

Fig. 7 Southern blot of transgenic plants of seven T_1 plants (*1–7*) from event F. Genomic DNA was digested with *Eco*RV. *Large arrow-heads* extra transgenic bands; *small arrowheads* endogenous bands; W.T., wild-type

tively. Subunit 1Ax1, which is absent from the background, accounted for up to 1.3% of the total protein in the transgenic seeds (Table 2, bottom line), being equivalent to the level of expression of the endogenous 1Ax1 subunit present in other lines. HMW subunit 1Dx5, which migrates close to or together with subunit 1Ax2* on SDS-PAGE (Figs. 5 and 6, W.T.), makes a large contribution to the HMW subunits present in the silenced plants (Table 2 and Fig. 6 A), especially in event F, suggesting that it results from expression of the 1Dx5 transgene rather than the endogenous gene. However, in the T_1 seed expressing six subunits (Fig. 6 A, lane 3), its T_2 progeny (18 seeds), and the five T_2 seeds shown in Fig. 6 C, the amounts of the 1Dx5 subunit expressed did not exceed that in the controls (Table 2, event F).

Figure 7 shows Southern hybridizations of DNA from seven T_1 wheat plants of event F using *Eco*RV and the 1Ax1 gene as a probe. At least two patterns of hybridization with high copy numbers and different insertion sites are apparent when comparing lanes 1–3 with lanes 4 and 6. The DNA in lane 5 is from a negative T_1 plant in which only the endogenous HMW subunits are present (Fig. 7) which agrees with the SDS-PAGE pattern of the seed (data not shown). At least 20 extra bands are seen in lanes 1–3 when compared to the wild type (W.T.) lanes; the strongest coinciding with one of the endogenous bands at 5.9 kb. Other strong bands are at 17.9, 15.8, 11.1, 8.9, and 3 kb. The copy number of the transgenes was estimated by densitometry to be about 50. On the other hand, although only about 15 bands are seen in lanes 4 and 6, the major band is again at 5.9 kb and the total transgene copy number is about 40 (lanes 4 and 6 gave similar results in spite of the fact that lane 4 was loaded with more DNA). A completely different pattern of transgene integration is apparent in Fig. 7, lane 7, in which the DNA comes from the transgenic T_1 plant shown in Fig. 6 A, lane 3. This Southern blot shows only three bands, the strongest being at 7.6 kb which corresponds to one of the endogenous bands. In addition, only two extra bands are seen at 9.5 and 4. 5 kb. In this case the copy number is about four, i.e., 10-times lower than in the other plants. Thus, these results suggest that segregation, rearrangements and elimination of insertion sites have taken place in event F. The latter may be associated with the reversion of silencing described above.

Discussion

In this paper transformation of a commercial spring bread-wheat cultivar with the HMW glutenin subunit genes 1Ax1 and 1Dx5 is described. Two transgenic events overexpressed the 1Dx5 subunit without changes in the other endosperm proteins. As a result, the contribution of HMW glutenin subunits to total protein increased by 10 and 22%, respectively (Table 2). Barro et al. (1997) have previously shown expression of the 1Dx5 transgene both in a background with five subunits including 1Dx5 and in a line which expresses only subunits 1Bx17 and By18. They were also able to introduce the 1Ax1 transgene alone, or with 1Dx5, and to analyze the mixing properties of the resulting dough, showing that expression of the transgenes correlated with increasing dough strength.

Altpeter et al. (1996) and Blechl and Anderson (1996) transformed the cultivar “Bobwhite” with a chimaeric gene comprising mostly the subunit 1Ax1 and the 1Dx5 genes, respectively. Neither demonstrated gene suppres-

sion but Blechl and Anderson suggested that it could have occurred in one line (Blechl and Anderson 1996; Blechl et al. 1998) while Altpeter et al. (1996) speculated that integration of the 1Ax1 transgene could result in silencing, particularly of the 1Ax2* gene. Blechl et al. (1997) have since confirmed that suppression does indeed occur in some lines.

Of the six transgenic events described here, four show silencing of one or all the HMW glutenin subunits. Two types of phenomena were observed. Firstly, the expression of the 1Ax1 transgene was associated with inactivation of the allelic gene 1Ax2* as shown in Figs. 3 and 4 for events C and D, with inactivation of the 1Ax2* gene being complete when the transgene was homozygous and in a low copy number. However, in spite of this silencing, the contribution of the HMW glutenin subunits to the total endosperm protein increased by over 20% (Table 2, event C). Secondly, in two cases the silencing of all HMW glutenin subunits was observed (events E and F, Figs. 5 and 6). In these events silencing was associated with a high copy number of the transgenes and multiple insertion sites (Fig. 2 B and 7).

These silencing phenomena presumably involve homology dependent gene silencing (Meyer and Saedler 1996) and resemble co-suppression in which mutual inactivation of transgenes and homologous genes occurs. However, in the four cases (events C, D, E and F) the transgenes remain fully expressed. Events E and F have high copy numbers of the transgenes and multiple insertion sites and it is possible that co-suppression occurs at some of the sites while the transgenes at other sites remain active.

Only partial homology may be required for inactivation, limited, for example, to the promoter or even to a short segment of at least 90 bp (Vaucheret 1993). However, in the cases reported here the 1Dx5 transgene shows complete homology with the endogenous 1Dx5 gene and high homology with the other HMW subunit gene, while the 1Ax1 transgene shows high homology with all the endogenous HMW subunit genes but particularly with the allelic 1Ax2* gene that was silenced in events C and D. Although inactivation has been observed even with a single copy of the transgene (Meyer and Saedler 1996), increasing numbers of copies and unlinked sites of insertion increase inactivation. The complete silencing of HMW glutenin observed in events E and F (Figs. 5 and 6) was associated with transgene copy numbers of about 20 to 50 and multiple insertion sites (Figs. 2 C and 7). Moreover, reversion of the inactivation was observed in event F where it was associated with a loss of transgene copies and a reduction in insertion sites.

Transgene-induced silencing may take place at the transcriptional or post-transcriptional level. The former occurs mainly when multiple copies of the transgene are present (Vaucheret et al. 1998) while the latter was discovered in petunia and is referred to as co-suppression (Napoli et al. 1990). However, co-suppression has since been described at both levels of inactivation (Meyer and Saedler 1996). Transgene silencing is an increasingly important and interesting phenomenon because it is involved normally in plant defense mechanisms against invasive DNA and may also be an unwanted consequence of the genetic engineering of plants (Matzke et al. 1996; Kumpatla et al. 1998). However, in the present case it could result in the generation of material with novel functional properties. It is noteworthy that in four of the transgenic events reported in this paper the contribution of the HMW glutenin subunits to total endosperm protein was increased by 10–20%, which may result in higher dough visco-elasticity.

The wheat transformation experiments described here with the HMW subunits clearly provide an interesting system to explore the transgene-induced silencing phenomenon in a small and well-characterized multigene family, the products of which can be readily separated and quantitized.

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