Theor Appl Genet (1999) 99:851–858

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Altered functional properties of tritordeum by transformation with HMW glutenin subunit genes

Received: 15 January 1999 / Accepted: 5 February 1999

Abstract The high-molecular-weight (HMW) subunits of wheat glutenin are the major determinants of the gluten visco-elasticity that allows wheat doughs to be used to make bread, pasta and other food products. In order to increase the proportions of the HMW subunits, and hence improve breadmaking performance, particle bombardment was used to transform tritordeum, a fertile amphiploid between wild barley and pasta wheat, with genes encoding two HMW glutenin subunits (1Ax1 and 1Dx5). Of the 13 independent transgenic lines recovered (a transformation frequency of 1.4%) six express the novel HMW subunits at levels similar to, or higher than, those of the endogenous subunits encoded on chromosome 1B. Small-scale mixograph analysis of T_2 seeds from a line expressing the transgene for 1Dx5 indicated that the addition of novel HMW subunits can result in significant improvements in dough strength and stability, thus demonstrating that transformation can be used to modify the functional properties of tritordeum for improved breadmaking.

Key words Tritordeum · Transformation · HMW glutenin subunits · Seed protein engineering · Dough functionality

Communicated by J.W. Snape

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Introduction

Tetraploid pasta wheat (*Triticum turgidum* conv. *durum* Dest. em.M.K., genome constitution AABB) is well adapted to the hot dry Mediterranean climate of southern Europe and North Africa, where it is traditionally used to make pasta, flat breads, bulgar and couscous (Matsuo 1996). It is not well suited for making leavened bread, lacking the D genome associated with high-gluten visco-elasticity present in hexaploid bread wheat (*Triticum aestivum* L., AABBDD), although significant amounts are used for this purpose in southern Italy and Greece (Matsuo 1996). Tritordeum (x *Tritordeum* Ascherson et Graebner) is an amphiploid derived from crossing the South American wild barley *Hordeum chilense* Roem et Schulz ($H^{ch}H^{ch}$) with either pasta wheat or bread wheat, resulting in hexaploid and octaploid forms, respectively (Martin and Chapman 1977; Martin and Sanchez-Monge Laguna 1982; Martin et al. 1996).

Tritordeums have been shown to have good agronomic performances and similar yields and protein contents to the parental wheat varieties (Ballesteros 1993; Alvarez et al. 1992). However, the grain quality is generally inferior to those of the wheat parents (Alvarez et al. 1994) and none of the hexaploid tritordeums so far produced are suitable for commercial bread production.

Genetic transformation of tritordeum has recently been achieved, by particle bombardment of immature inflorescences (Barcelo et al. 1994; Barcelo and Lazzeri 1995). This provides an opportunity to improve the breadmaking performance of tritordeum by the introduction of gluten protein genes that are known to be associated with breadmaking quality. One group of gluten proteins is of particular interest in this respect as they appear to be major determinants of gluten visco-elasticity and hence breadmaking quality. These are the high-molecular-weight (HMW) subunits of wheat glutenin. Two HMW subunit genes are present on each group-1 chromosome, encoding a high M_r x-type and a low M_r y-type subunit. However, variation in the expression of these genes results in differences in the number of proteins

present, with 1Dx, 1Dy and 1Bx subunits present in all cultivars of bread wheat and 1Ax and/or 1By subunits in some cultivars only. 1Ay subunits are not present in any widely grown cultivars (see Payne 1987). Differences in quality associated with this variation in subunit number may be due to a quantitative effect, each subunit accounting for about 2% of the total grain protein (Halford et al. 1992). However, it is also possible to demonstrate that allelic-expressed subunits differ in their quality, allowing them to be assigned "quality scores" (Payne et al. 1987). This is particularly notable with the subunits encoded by chromosome 1D, with the subunit pair 1Dx5+1Dy10 showing a higher quality than the allelic pair 1Dx2+1Dy12. These 1D-encoded subunits are, of course, absent from pasta wheats, with most cultivars expressing only 1Bx or 1Bx + 1By subunits (Branlard et al. 1989). The 1B-encoded subunits would also be expected to be present in derived tritordeum lines.

We have therefore used a genetic modification approach to improving the breadmaking quality of tritordeum, by transferring genes for quality-associated HMW subunits (Payne 1987) encoded by chromosomes 1A (1Ax1) and 1D (1Dx5) of bread wheat. This approach has previously been applied to bread wheat (Altpeter et al. 1996; Blechl and Anderson 1996) and improved mixing properties demonstrated (Barro et al. 1997). We demonstrate here that similar improvements can be achieved in tritordeum.

Materials and Methods

Plant material and plasmid constructs

Immature inflorescences from three tritordeum lines (HT28, HT31, HT174), derived from crosses between durum wheat and the wild barley *Hordeum chilense* (Martin and Chapman 1977), were used as target tissues for transformation by particle bombardment. Plants were grown in the greenhouse with supplementary lighting provided by sodium lamps with a day temperature of 18–20°C and a night temperature of 16–18°C.

Three plasmids containing scorable and/or selectable markers were used: plasmid pAHC25 (Christensen and Quail 1996) contains the scorable *uid*A gene, encoding for β-glucuronidase (GUS), and the *bar* gene which confers resistance to the herbicide BASTA, both under the control of the maize ubiquitin promoter; plasmids pAct1-DGus (McElroy et al. 1990) and pAct1-Dneo (constructed by E. Mueller, University of Hamburg), contain the scorable *uid*A and selectable neomycin phosphotransferase (*neo*) genes, respectively, both under the control of the actin1-D promoter from rice. Two plasmids containing HMW subunit genes were used: plasmid pHMW1Dx5 (denoted p1Dx5), containing a 8.7-kb *Eco*RI genomic fragment including the coding sequence of the *Glu-DI-1b* (1Dx5) gene (Anderson et al. 1989) flanked by approximately 3.9-kb and 2.0-kb of 5′ and 3′ sequences, respectively (Halford et al. 1989), and plasmid pHMW1Ax1 (denoted p1Ax1) containing a 7.0-kb *Eco*RI fragment including the complete coding sequence of the *Glu-A1–1a* (1Ax1) gene flanked by approximately 2.2 kb and 2.1 kb of 5′ and 3′ sequences, respectively (Halford et al. 1992). Thus, in both cases, the HMW subunit genes were driven by their own promoters. The 1Dx5 promoter has been shown to be endosperm-specific in transgenic tobacco (Halford et al. 1989) and wheat (Lamacchia et al., unpublished results). In bombardments either the plasmid pAHC25 or the plasmids pAct1-DGus and pAct1-Dneo were delivered in combination with the HMW-subunit plasmids. In all co-transformation experiments 1:1 molar ratios of the respective plasmids were used.

Production of transgenic plants

Immature tillers containing inflorescences were harvested, surface-sterilised (0.5% sodium hypochlorite) for 30 min, and washed with sterile distilled water. Immature inflorescences were isolated and prepared as described by Barcelo and Lazzeri (1995). For each bombardment 25 to 30 approximately 1-mm pieces of inflorescences were placed in the centre of a 10-cm-diameter Petri dish containing L7P4 induction medium (Rasco-Gaunt and Barcelo 1998). Explants were cultured in darkness at 24°C for 1 day prior to bombardment.

Plasmid DNA was precipitated onto gold particles (Heraeus, diameter 0.4–1.2 mm) following the protocol of Barcelo and Lazzeri (1995). Bombardments were carried out using a PDS 1000/He gun (Bio-Rad) with a distance of 5 cm from the stopping plate and a helium pressure of 1100 psi. After bombardment, explants were spread over the surface of the medium in the original dish and cultured at 24°C in darkness for 3 weeks for the induction of embryogenesis. For selection, calluses bearing somatic embryos were transferred to RZ regeneration medium (Rasco-Gaunt and Barcelo 1998) supplemented with either 2 mg l^{-1} L-phosphinothricin (L-PPT, the active ingredient of BASTA) or 50 mg l^{-1} G418 (geneticin disulphate), and cultured for a further 3 weeks before transfer to R medium (Rasco-Gaunt and Barcelo 1998) containing 2 mg l^{-1} L-PPT or 50 mg l^{-1} G418 respectively. Successive 3-week rounds of selection were applied until all the control plants had been killed. Surviving plantlets were then transferred to soil and grown to maturity in the greenhouse under the same conditions as donor plants.

Analysis of transgenic plants

GUS activity was examined histochemically using 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) as described by Barcelo and Lazzeri (1995). Leaf and root tissues were cut into small pieces, immersed in X-Gluc buffer and incubated overnight at 37°C. Chlorophyll was then extracted from leaf pieces by incubation in 70% ethanol for 1 h and then in several changes of 100% ethanol until the material had cleared.

For PCR and Southern-blot analyses, total genomic DNA was isolated from leaf tissue of primary transformants and their progeny, using a CTAB method (Stacey and Isaac 1994). PCR was carried out, with 50–200 ng of genomic DNA, in a reaction mixture containing 50 mM KC1, 10 mM Tris-HC1 (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP, 0.3 μM of each primer, and 0.66 units of Dynazme DNA polymerase (Flowgen, Lichfield, UK). PCR analysis was carried out for the *uid*A gene (5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′, 5′-ATCGCC-GCTTTGGACATACCATCCGTA-3′, annealing temperature 62°C, amplified product 1.05-kb in size), the *bar* gene (5′-GTCTGCACCATCGTCAACC-3′, 5′-GAAGTCCAGCTGCCA-GAAAC-3′, annealing temperature 57°C, amplified product 443 bp in size), the *neo* gene (5′-GAGGCTATTCGGCTATGACTG-3′ 5′-ATCGGGAGCGGCGATACCGTA-3′, annealing temperature 57°C, amplified product 700-bp in size) and the 1Dx5 gene (D'Ovidio and Anderson 1994). PCR-amplification was carried out using 1 cycle of denaturation at 94°C for 5 min, primer annealing for 30 s and a 2-min extension at 72°C, followed by 30 cycles of 94°C for 1 min, annealing for 30 s and extension at 72°C for 2 min with a 3-s increment per cycle and a final extension cycle of 72°C for 10 min. Products of PCR-amplification were analysed by electrophoresis in 1% agarose gels. Integration of the HMW subunit genes was subsequently confirmed by Southern analysis of digested genomic DNA. Restriction digests were carried out with either *Eco*RV, which cuts once within the p1Ax1 construct and releases a 5.8-kb fragment from p1Dx5 or *Sca*I, which cuts once within the p1Dx5 construct. Digested DNA was separated by elec-

trophoresis in 0.6% (w/v) agarose gels and the DNA transferred, by capillary blotting, to a positively charged Boehringer nylon membrane according to the manufacturer's instructions (Boehringer Mannheim). Southern filters were hybridised with PCR-generated digoxigenin-labelled probes produced using primers for the 1Ax1 (5′-GTGTGAGCGCGAGCTCCAGGAA-3′, 5′-CGGAGAAGTTGGGTAGTACCCTGC-3′, annealing temperature 60°C, amplified product 421-bp in size) and 1Dx5 subunit genes (D'Ovidio and Anderson 1994), with p1Ax1 and p1Dx5 as the DNA templates, respectively. Hybridised probe DNA was detected using a chemiluminescent detection system (Boehringer Mannheim).

Total proteins were extracted from single half-grains or flour samples in 25 µl mg^{-1} of 62.5 mM Tris-HC1 buffer, pH 6.8, containing 2% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.002% (v/v) bromophenol blue, and separated by SDS-PAGE using a Tris-borate buffer system and 10% (w/v) acrylamide gels (Shewry et al. 1995).

Samples of T_2 grain for mixograph analysis were initially milled to obtain white flour and the mixing properties determined using a 2-g mixograph as described by Békés and Gras (1992). Mixing parameters were determined by a previously reported computer programme modified to excise the portions of the recording, during which mixing was halted (Gras et al. 1990; Gras and Békés 1996).

Results

Production of transgenic plants

Three hexaploid tritordeum lines (HT28, HT31, HT174) were used for transformation. As hexaploid tritordeum lacks the D genome, and thus does not contain *Glu-D1*-encoded subunits, and none of these lines express subunit 1Ax1, they provide a good background in which to explore the consequences of transformation with the 1Ax1 and 1Dx5 glutenin subunit genes.

Bombardment of 777 inflorescence explants with three of the plasmid combinations (pAHC25 + p1Ax1, pAHC25 + p1Dx5, and pAHC25 + both HMW plasmids) gave rise to 97 PPT-resistant plants of which 15 were PCR-positive for the *bar* gene. Therefore, the frequency of escapes under PPT selection was 85%. Of the 15 transformants, nine were shown by Southern analysis to be independent lines; giving a transformation frequency for tritordeum cultures selected on PPT of 1.2%. In contrast, G418 selection was more efficient giving a lower frequency of escapes (38%) and a higher frequency for the production of independent transgenic lines (2.7%). Of 146 explants bombarded with pAct1-DGus + pAct1-Dneo + p1Dx5, eight G418-resistant lines were recovered of which five plants were transgenic for the *neo* gene and a minimum of four were independent lines.

A total of 13 independent transgenic plants were recovered from the four different plasmid combinations (see Table 1). Of these, nine were produced under PPT selection following bombardment with the plasmid pAHC25, one in combination with p1Ax1, two with p1Dx5, and six with both HMW subunit plasmids. The remaining four lines were produced under G418 selection following bombardment with pAct1-DGus + pAct1-Dneo + p1Dx5. From these experiments, the co-integration frequency in tritordeum for one or more marker genes and one or more HMW subunit transgenes was 92%. Of the four lines produced under G418 selection, one contained the *neo* marker gene only, the remaining three lines contained both the *neo* and 1Dx5 subunit transgenes but only one contained copies of the *uid*A coding region. Of the nine lines identified as PCR-positive for the *uid*A coding region, two were shown to have GUS activity in leaf and/or root tissues. Three of the 13 transgenic lines were sterile while the remaining ten were fertile and set seed.

Analysis of integrated HMW glutenin subunit transgenes

Integration of HMW subunit genes into the tritordeum lines was confirmed by PCR and Southern analysis. PCR primers were used to confirm the presence of the 1Dx5

Table 1 Transgenic tritordeum lines: estimation of transgene insertion number, and expression of HMW subunits in T_1 endosperm.

Line	Genotype	Plasmids	PCR			Estimated transgene insertion number		Subunit expression in T_1 endosperm	
			uidA	*bar*	*neo*	1Ax1	1Dx5	1Ax1	1Dx5
1 (B39-8-3a)	HT31	p1Dx5+pAHC25	+	+	NA	NA	≥2	NA	ND[a]
2 (B42-1-1)	HT28	p1Dx5+pAHC25	+	+	NA	NA	4–6	NA	None
3 (B48-2-1)	HT28	p1Dx5+pAct1-Dgus+pAct1-Dneo	–	NA	+	NA	≥1	NA	High
4 (B48-2-2)	HT28	p1Dx5+pAct1-Dgus+pAct1-Dneo	+	NA	+	NA	≤8	NA	None
5 (B48-3-1)	HT28	p1Dx5+pAct1-Dgus+pAct1-Dneo	–	NA	+	NA	0[b]	NA	NA
6 (B48-6-3)	HT31	p1Dx5+pAct1-Dgus+pAct1-Dneo	–	NA	+	NA	3–4	NA	Medium
7 (B52-8-2)	HT174	p1Ax1+pAHC25	+	+	NA	>15	NA	None	NA
8 (B76-3-2)	HT28	p1Ax1+p1Dx5+pAHC25	+	+	NA	>5	>5	Medium	High
9 (B76-3-5)	HT28	p1Ax1+p1Dx5+pAHC25	+	+	NA	0–1	1–2	ND	ND[a]
10 (B76-3-8)	HT28	p1Ax1+p1Dx5+pAHC25	–	+	NA	4	0[b]	Medium	None
11 (B76-7-2)	HT31	p1Ax1+p1Dx5+pAHC25	+	+	NA	2	0[b]	Medium	None
12 (B79-2-3)	HT31	p1Ax1+p1Dx5+pAHC25	+	+	NA	≤4	≥1	Medium	Medium
13 (B79-3-1)	HT31	p1Ax1+p1Dx5+pAHC25	+	+	NA	0–1	1–2	ND	ND[a]

[a] Line sterile
[b] PCR negative for 1Dx5
NA not applicable; ND not determined

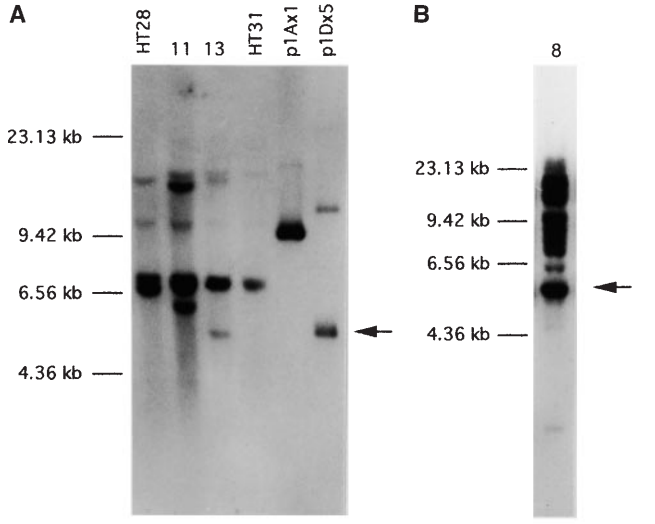

Fig. 1A, B Southern analysis of transgenic T_0 tritordeum lines for the identification of HMW subunit transgenes. Southern filters were hybridised with a probe to the 1Ax1 subunit transgene; however, the probe cross-hybridises with both transgenes and the endogenous HMW subunit genes (see Results section) identifying four and three bands in HT28 and HT31, respectively (see panel **B**). **A** Southern analysis of transgenic lines 11 and 13 (genotype HT31). Two extra hybridised bands are present in both lines compared to the donor HT31. The smallest of which in line 13 is identical in size to the 5.8-kb fragment released from p1Dx5 (indicated by the *arrow*). **B** Southern analysis of line 8 (genotype HT28) containing multiple integrated HMW transgene copies. The *arrow* indicates the 5.8-kb fragment released from p1Dx5

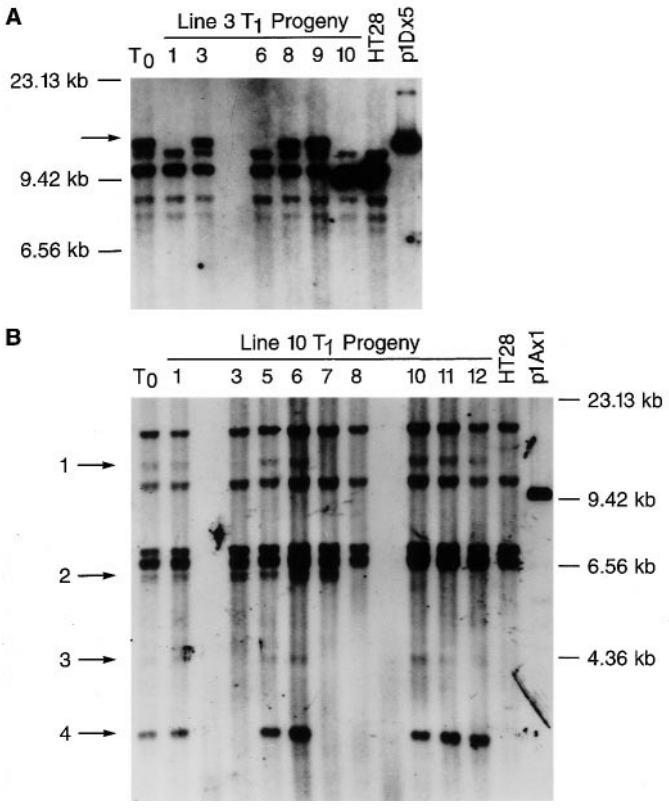

Fig. 2A, B Southern analysis of HMW subunit transgene inheritance and segregation in T_1 progeny of tritordeum lines 3 and 10. **A** Southern analysis of line 3 (genotype HT28). DNA was hybridised with a probe for the 1Dx5 subunit gene. A single extra hybridised band, compared to the HT28 donor, is present in T_0 DNA of line 3 (indicated by *arrow*). Segregation of this transgenic band is observed in the T_1 progeny of line 3 (the band is present in progeny 3, 8 and 9). **B** Southern analysis of line 10 (genotype HT28). DNA was digested with the restriction enzyme *Eco*RV, which cuts once within p1Ax1, and hybridised with a probe to the 1Ax1 subunit gene. Four extra hybridised bands, compared to donor HT28, are present in T_0 DNA of line 10 (indicated by the *numbered arrows*). Segregation of these transgenic bands is observed in the T_1 progeny of line 10; all four bands are present in progeny 1, 5 and 6; bands 1, 3 and 4 are present in progeny 10, 11 and 12; band 2 is only present in progeny 3 and 7, and progeny 8 is a null segregant

subunit gene. However, due to the high level of sequence homology between the HMW subunit genes, the primers designed for the 1Ax1 subunit gene could not distinguish this gene from the other subunit genes present in the tritordeum lines. Therefore, lines transformed with this gene were confirmed by Southern analysis.

The high level of sequence similarity between all the HMW subunit genes results in cross-hybridisation in Southern analysis and, therefore, probes designed for either the 1Ax1 or 1Dx5 transgene recognise and anneal to both the endogenous and transgenic HMW subunit genes. For example, the hybridisation patterns of the tritordeums HT28 and HT31 are shown in Fig. 1A; however, additional hybridised DNA fragments are evident in DNA from lines 11 and 13 and confirm the integration of copies of the HMW subunit transgenes. For Southern analysis, genomic DNA was digested with an enzyme that cuts once within the plasmid sequence. Following hybridisation with a probe for the transgene, and depending on the position of the next enzyme site within the plant DNA, different-sized transgenic bands will be expected each representing a plasmid DNA/plant DNA junction. Counting the number of extra hybridising bands, compared to the parental line, allows an estimation of the transgene insertion number. However, the identification of transgenes in lines bombarded with both HMW subunit genes is complicated because the probes recognise both transgenes equally and it may not be possible to specifically associate hybridised DNA fragments with the different subunit transgenes.

Southern analyses of T_0 lines 11, 13 and 8 are presented in Figs. 1A and 1B. DNA was digested with *Eco*RV which restricts once within the plasmid p1Ax1 and releases a 5.8-kb fragment, containing the 1Dx5 gene, from p1Dx5. Line 11 (genotype HT31) was determined by PCR analysis to be negative for the 1Dx5 gene and therefore, the 2 extra hybridising bands observed by Southern analysis (Fig. 1A) suggest that 2 copies of the 1Ax1 subunit gene have been integrated into the genome of this line. Of the two extra hybridising bands observed for line 13 (Fig. 1A; genotype HT31), the smallest was the size expected for the fragment released from p1Dx5 (arrowed) and indicates the integration of a complete copy of the 1Dx5 gene coding region. The low signal in-

tensity of this band does not suggest the integration of more than one or two 1Dx5 gene copies. The second transgenic band observed for line 13 probably represents the insertion of a single copy of the 1Ax1 subunit transgene, although truncations or rearrangements of p1Dx5 may lead to bands varying in size from that expected. Line 8 (genotype HT28) appears to contain multiple copies of both the 1Ax1 and 1Dx5 subunit transgenes (Fig. 1B). The strong signal strength of the 5.8-kb band indicates the integration of multiple copies of the 1Dx5 coding region, while the presence of multiple extra hybridising bands of sizes ranging from 3 to >23 kb suggests the integration of multiple copies of the 1Ax1 subunit transgene (although they may also represent copies of 1Dx5, as discussed for line 13).

Inheritance of the HMW subunit transgenes was confirmed by Southern analysis of T_1 progeny from lines 3 and 10 (Fig. 2A and 2B). Compared to the tritordeum control (HT28) a single extra hybridising band was observed in Southern analysis of T_0 DNA from line 3 (Fig. 2A). The genomic DNA had been digested with *Sca*I, which cuts once in the p1Dx5 plasmid sequence, and the transgenic band was observed to be identical in size to the linearised p1Dx5 plasmid. This may indicate the presence of plasmid copies integrated into the plant genome in head-to-tail arrays or concatamers; plasmid concatamers have been previously observed in soybean and tritordeum lines produced by direct gene transfer (Christou et al. 1989; Barcelo et al. 1994). The transgenic 1Dx5 band segregates in the T_1 progeny of line 3 (Fig. 2A; progeny 3, 8 and 9 contain the band) and, moreover, the segregation of the 1Dx5 subunit transgene correlates with the segregation of the selectable *neo* marker gene (data not shown) indicating that the transgenes have been integrated at the same genetic locus.

As line 10 was determined to be PCR-negative for the 1Dx5 subunit transgene the four extra hybridising bands observed in Southern analysis (Fig. 2B) probably indicate the integration into this line of four copies of the 1Ax1 subunit transgene. Segregation of the transgenes has occurred in the T_1 progeny and it can be observed (Fig. 2B) that the transgenes are not all transmitted together. Three of the transgene bands (bands 1, 3 and 4) appear to have been integrated into the same locus and segregate together, while the fourth band (band 2) of approximately 6.3-kb in size is inherited independently. Inheritance of HMW subunit bands 1, 3 and 4 is correlated with the segregation of the selectable *bar* transgene (data not shown) and the presence in the endosperm of the 1Ax1 subunit (data not shown). Subunit 1Ax1 was not present in the endosperm of progeny containing only the 6.3-kb hybridising band 2 (progeny 3 and 7; protein data not shown) and therefore the HMW transgene is either incomplete or inactive.

Estimations of HMW subunit transgene insertion number, determined by counting the number of extra hybridised bands in Southern analyses, for the 13 tritordeum lines are presented in Table 1. The gene-insertion number ranges from a minimum of one (line 3) to greater than 15 (line 7). However, the majority of lines have six or fewer HMW subunit transgene insertions. This is in agreement with previous reports on transgenic wheat lines (Blechl and Anderson 1996; Barro et al. 1997, 1998).

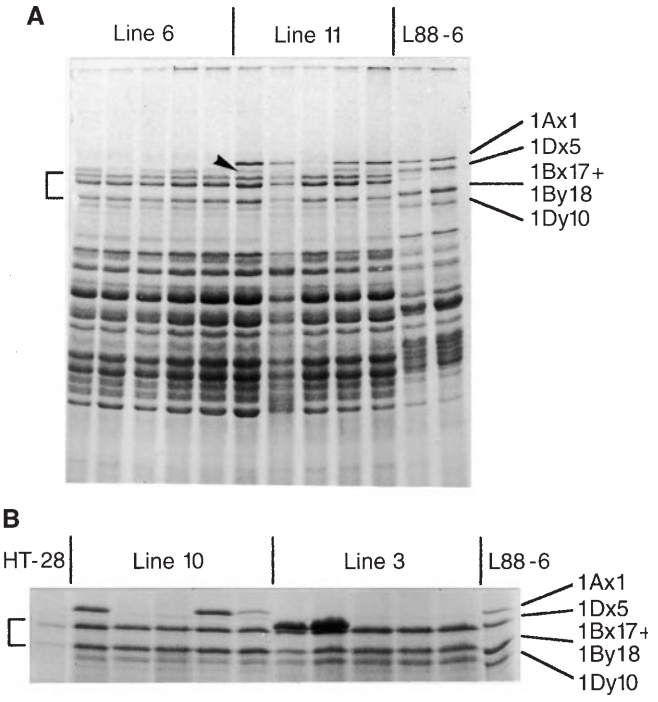

Fig. 3A, B HMW glutenin subunit expression in transgenic lines. **A** SDS-PAGE of individual T_1 seeds of lines 6 (genotype HT31) and 11 (genotype HT31). Line 6 shows expression of subunit 1Dx5, see comparison with the standard wheat line L88–6, while line 11 shows expression of subunit 1Ax1. Line 11 also contains an additional band of faster mobility than the transgene product (see *arrowhead*). The *bracket* indicates the endogenous HMW and D hordein subunits present in HT31-genotype lines. **B** SDS-PAGE of individual T_1 seeds of lines 10 (genotype HT28) and 3 (genotype HT28). Only the top portion of the gel containing the HMW glutenin and D hordein subunits is shown and the bracket indicates the endogenous subunits present in genotype HT28. Line 10 shows expression of subunit 1Ax1, see comparison with the standard wheat line L88–6, while line 3 shows high-level expression of subunit 1Dx5

Expression of HMW subunits in transgenic plants

SDS-PAGE of single seeds of the three donor tritordeum lines showed that each contained HMW subunits and D hordein, the barley homologue of the HMW subunits, derived from both the wheat and *H. chilense* parents (indicated in Fig. 3).

The absence of 1Ax1 and 1Dx5 subunits from the three donor tritordeum lines allowed the transgene products to be clearly identified by SDS-PAGE. Examples of four lines are shown in Fig. 3, lines 6 and 11 (both in the HT31 background) in Fig. 3A, and lines 10 and 3 (both HT28) in Fig. 3B. The transgene products (1Dx5 in lines 3 and 6, 1Ax1 in lines 10 and 11) can be clearly identi-

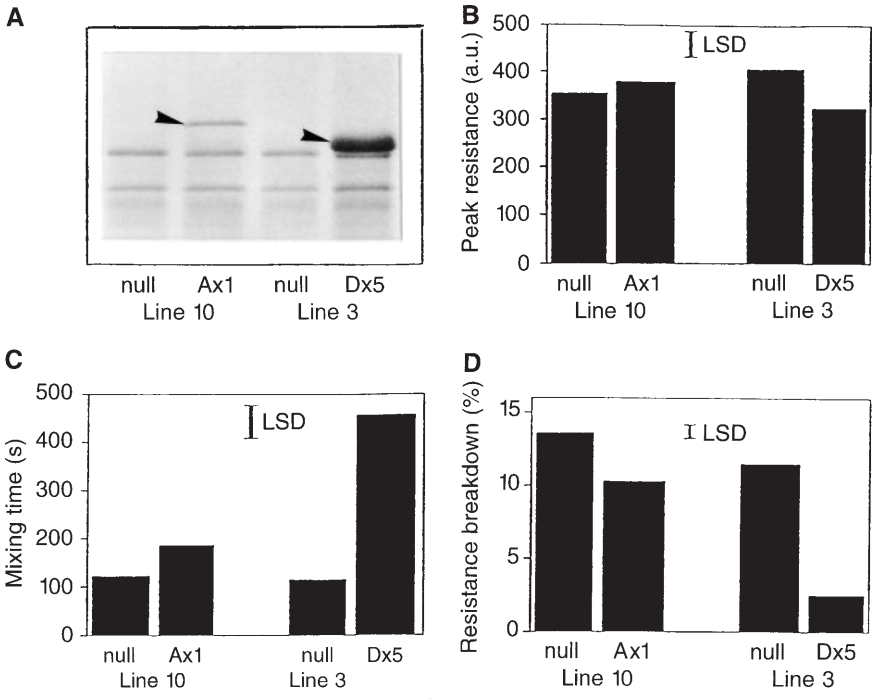

Fig. 4A–D Analysis of the mixing properties of dough from tritordeum line 10 (1Ax1) and line 3 (1Dx5). **A** HMW subunit composition of the flours determined by SDS-PAGE. T_2 seeds from positive and null segregants of lines 10 and 3 were milled to give white flour. SDS-PAGE of the flour proteins confirms the presence, in the positive segregants, of the 1Ax1 subunit in line 10 and high levels of 1Dx5 in line 3. **B** The peak dough resistances, given in arbitary units, for doughs made from null and positive T_2 seeds of lines 10 and 3. **C** Mixing time, given in seconds, for doughs made from the null and positive T_2 seeds of lines 10 and 3. **D** Resistance breakdown, given as %, for doughs made from the null and positive T_2 seeds of lines 10 and 3

fied by comparison with the standard wheat line L88–6 (containing subunits 1Ax1, 1Dx5, 1Dy10, 1B17, 1By18). Although expression levels varied between T_1 seed in most lines the levels of the subunits encoded by the transgenes were similar to, or higher than, those of the endogenous HMW subunits (Table 1, Fig. 3). However, three lines (2, 4 and 7) did not express the transgenes. For line 7 the lack of expression appeared to be a result of poor transgene transmission as PCR analysis revealed that T_1 progeny had not inherited either marker or the HMW subunit transgenes. Poor transmission may result from the loss of unstably integrated transgenes or from insertional mutagenesis and a disruption of essential gene function, which thereby results in a failure to produce progeny plants carrying the transgenic locus.

Of the six lines (lines 8–13) regenerated after co-bombardment with both 1Ax1 and 1Dx5 genes, two were found to be negative for the 1Dx5 transgene by PCR and contained only copies of the subunit 1Ax1 gene (see previous), which were expressed at a medium level (lines 10 and 11) as shown in Figs. 3A and B, respectively. A further two lines (9 and 13) were sterile and could not, therefore, be assayed for transgene expression. The remaining two lines, 8 and 12, expressed both HMW subunits at medium or high levels.

Closer examination of the SDS-PAGE patterns of the transgenic lines reveals that some appear to contain HMW subunit bands which correspond neither to the transgene products nor to the endogenous HMW subunits. Such a band is present in line 11, migrating between the 1Ax1 transgene product and an endogenous subunit (see arrowhead in Fig. 3A). This may correspond to a rearranged form of subunit 1Ax1, arising from unusual crossing over as discussed by D'Ovidio et al. (1996). Similar additional bands were observed previously in wheats transformed with the same HMW subunit genes (Barro et al. 1997) and most were recognised, in Western blotting, by a monoclonal antibody specific for the 1Ax and 1Dx HMW subunits.

Effects of the HMW subunits on grain functionality

Segregation of the transgene products was observed in the T_1 seed of all the expressing lines, either for the amounts or for absence/presence of the transgene products. In order to determine the effects of the transgene products on the processing properties of the grain, the endosperm-halves of single T_1 seeds from line 10 and line 3 were analysed to identify progeny which expressed the transgenic subunits or lacked expression (null segregants). The embryo-halves of the grain were then grown to give T_2 seeds that were again screened to eliminate plants that were still segregating for expression of the transgene product. T_2 seeds from positive segregants (taken to be homozygous) and null segregants were then milled to give white flour. About 5 g of flour was recovered from each plant, allowing duplicate analysis to be made using a 2-g mixograph.

The mixograph measures changes in dough resistance during mixing, giving information on dough strength (i.e. visco-elasticity) and stability. A range of parameters are measured, three of which are most important. These are the maximum resistance achieved (peak resistance, PR) which is measured in arbitrary units, the time taken to achieve maximum resistance (mixing time, MT) measured in seconds, and the rate of resistance loss after the maximum is achieved (resistance breakdown, RBD) ex-

pressed as %. Strong wheats suitable for breadmaking have greater peak resistances, longer mixing times, and lower resistance breakdown than wheats used for other purposes.

Parameters determined from the mixographs obtained for lines 10 and 3 are shown in Fig. 4, together with SDS-PAGE separations of the HMW subunits present in total proteins extracted from the flours. The results were obtained from three null segregants for each line and either three 1Ax1-expressing progeny of line 10 or two 1Dx5-expressing progeny of line 3. It is clear that the expression of subunit 1Dx5 in line 3 is associated with a highly significant improvement in dough strength, measured as MT, but only a small effect on PR. The reasons for these different effects on MT and PR, both measures of dough strength, are not known. There was also an increase in dough stability measured as RBD. In contrast, the expression of subunit 1Ax1 in line 10 gave only a small increase in MT with little or no effect on PR and RBD.

Discussion

These experiments extend the previous reports of tritordeum transformation (Barcelo et al. 1994; Barro et al. 1998) by co-introducing copies of HMW glutenin subunit genes along with marker genes and demonstrating modified gluten functionality.

Two selection systems were used in the transformation procedure, the *bar* and the *neo* genes with PPT and G418 as the selective agents, respectively. G418 selection resulted in a lower frequency of escapes and a higher frequency of production of transgenic lines; however, the mean transformation efficiency of 1.4% is comparable with previous reports (Barcelo et al. 1994; Barro et al. 1998). Co-bombardment of marker and HMW subunit genes on separate plasmids was found to be an efficient delivery system as 92% of the lines contained both marker and HMW subunit transgenes, a similar frequency was observed following bombardment of similar constructs into bread wheat (Barro et al. 1997).

Integration of the HMW subunit genes was examined by Southern blotting. Transgene insertion numbers were relatively low (≤6) for the majority of lines, although lines 7 and 8 contained complex multi-copy integration patterns. In at least two of the lines (lines 10 and 12; data not shown for line 12) transgenes had been inserted into multiple loci which, although it results in segregation of the transgenes in the successive generation, has the potential to separate the marker genes from the gene of interest.

Expression levels of the novel HMW subunits were found to vary, with some being as high as or higher than the levels of the endogenous subunit genes. Similar results were observed with transgenic bread wheat (Barro et al. 1997). There appeared to be no correlation between expression level and transgene insertion number; the two highest levels of subunit expression were shown by a line containing multiple transgene insertions (line 8) and a line with a low copy number (line 3). Two lines appeared not to express the HMW transgenes (lines 2 and 4), this may be due to the presence of incomplete copies of the transgenes or as a result of transgene silencing, although co-suppression of endogenous HMW subunits was not observed.

The small-scale mixograph results demonstrate that transformation of tritordeum with additional genes for HMW subunits can result in a significant improvement in dough strength. Of the two lines studied, this was more marked with line 3 which expressed subunit 1Dx5, than in line 10 which expressed subunit 1Ax1. This may relate partly to expression levels, the 1Dx5 protein being expressed at a higher level. However, it may also be related to the intrinsic properties of this protein which forms part of a pair of subunits (1Dx5 and 1Dy10) which are associated with good breadmaking quality when compared with allelic subunits encoded by the same locus (1Dx2 and 1Dy12). The production of lines expressing high levels of additional HMW subunits, such as line 3, may lead to tritordeums of sufficiently good quality for commercial breadmaking.

Acknowledgements The IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK. L.R. was supported by the BBSRC CWIS Programme and the MAFF Crop Molecular Genetics Initiative. F.B. acknowledges the Spanish Ministry of Agriculture, Food and Fisheries (INIA) for the award of a fellowship. Part of the work was supported by the EU FAIR project CT170 “Improving the quality of EU wheats for the use in the food industry, EUROWHEAT”. Support from the British Council, “Acciones Integradas” Programme, is also acknowledged. The authors acknowledge Fiona Gilzean, Ian Pearman and Julian Franklin for their care of the plants. The experiments comply with the current laws of the countries in which they were performed.

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