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Comparative field performance over 3 years and two sites of transgenic wheat lines expressing HMW subunit transgenes

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Abstract A series of transgenic wheat lines expressing additional high molecular weight (HMW) subunit genes and the corresponding control lines were grown in replicate field trials at two UK sites (Rothamsted Research, approximately 50 km north of London and Long Ashton, near Bristol) over 3 years (1998, 1999, 2000), with successive generations of the transgenic lines (T_3, T_4, T_5) being planted. Four plots from each site were used to determine grain dry weight, grain nitrogen, dough strength (measured as peak resistance by Mixograph analysis) and the expression levels of the endogenous and "added" subunits. Detailed statistical analyses showed that the transgenic and non-transgenic lines did not differ in terms of stability of HMW subunit gene expression or in stability of grain nitrogen, dry weight or dough strength, either between the 3 years or between sites and plots. These results indicate that the transgenic and control lines can be regarded as substantially equivalent in terms of stability of gene expression between generations and environments.

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Introduction

Whereas conventional plant breeding involves the selection of novel combinations of many thousands of genes, transgenesis allows the production of lines which differ from the parental lines in the expression of only single or small numbers of genes. Consequently it should in principle be easier to predict the effects of transgenes than to unravel the multiple differences which exist between new, conventionally-produced cultivars and their parents. Nevertheless, there is considerable concern expressed by consumers and regulatory authorities that the insertion of transgenes may result in unpredictable effects on the expression of endogenous genes which could lead to the accumulation of allergens or toxins. This is because the sites of transgene insertion are not known and transgenic plants produced using biolistics systems may contain multiple and rearranged transgene copies (up to 15 in wheat) inserted at several loci which vary in location between lines (Barcelo et al. 2001; Rooke et al. 2003). Similarly, this apparently random insertion has led to the suggestion that the expression of transgenes may be less stable than that of endogenous genes between individual plants, between generations and between growth environments. Although there is evidence that the expression of transgenes introduced by biolistic

transformation is prone to silencing in a small proportion of wheat lines (e.g. Anand 2003; Howarth et al. 2005), many recent reviews including Kohli et al. (2003), Sahrawat et al. (2003), Jones (2005) and Altpeter et al. (2005) demonstrate the utility of biolistics transformation as a basis for stable genetic manipulation.

We have developed a series of transgenic wheat lines which express additional copies of wheat-derived genes encoding specific gluten proteins [called high molecular weight (HMW) subunits of glutenin], together with the marker genes used to facilitate the selection of transgenic plants (Barro et al. 1997; Rooke et al. 2003). These proteins are of particular interest because they are known to be the major determinant of dough strength, which in turn determines the suitability of flour for breadmaking or other end uses (Payne 1987; Shewry et al. 2003). Preliminary characterisation of the transgenic lines grown under glasshouse conditions showed both positive and negative impacts on dough strength (Barro et al. 1997; Rooke et al. 1999) and this has since been confirmed by large scale testing of grain grown in field trials (Darlington et al. 2003). These lines are therefore representative of the types of transgenic wheat which may be grown commercially in the future.

In order to assess the field performance of the transgenic lines and compare them with the corresponding control lines we have grown them in replicated plot trials on two UK sites for three seasons (1998, 1999, 2000). The material was then analysed to determine the relative expression levels of transgenic and endogenous HMW subunits as well as three other parameters relating to yield and quality. Grain dry weight and grain N content are major "point of sale" measures which are known to be affected by environmental and genetic factors. The peak resistance (PR) of dough determined using a Mixograph is a widely used measure of dough strength and was selected as an indicator of stability of functional properties. The results of these studies demonstrate that transgenes and transgenic plants are not intrinsically more or less stable over generations and between sites than are conventionally bred crops.

Materials and methods

Materials

The production and characterisation of the transgenic lines used in this study have been described by Barro et al. (1997) and Rooke et al. (2003) (Table 1). T_1 lines were grown in the glasshouse for three generations and progeny were selected for transgene expression by electrophoresis of half single seeds. The embryo halves of seeds expressing (for wheat lines coded B73-6-1, B102-1-1 and B102-1-2) or not expressing (for line B72-8-11a) the transgenes were then sown to provide the next generation. The harvesting of seed from resulting plants, grown in the glasshouse, allowed T_3 seeds to be planted in the field in 1998 and T_4 and T_5 seeds from the previous years' field trials in 1999 and 2000.

Field trials

Lines were grown in field experiments over 3 years (1998, 1999 and 2000) on two sites: Rothamsted Research (RRes) (approximately 50 km north of London, UK) and Long Ashton Research Station (LARS) (Bristol, UK) using a replicated randomised block design. Each experiment comprised four blocks of 21 (7 × 3) 1 m^2 plots surrounded by a 2 m guard strip of a wheat cultivar which flowered at a different time from the trial wheat lines. Each plot was separated by a 0.5 m path. At each site, the whole experiment was netted during establishment and grain filling and was surrounded either by 20 m of a break crop or fallow land. The lines were randomly allocated to plots within each block, with one plot of each line per block. Additional plots were also grown of some lines to facilitate more detailed studies (see Darlington et al. 2003). Seeds were sown, harvested and threshed by hand. The plots were subjected to standard agronomic treatments for the two sites including 100 kg/Ha of N applied in the form of

Table 1 Characteristics of the control and transgenic lines used in this study

Line	Characteristics	Endogenous HMW subunit genes	HMW subunit transgenes	Transgene insertions
L88-6	Parental line, derived from Olympic x Gabo	1Ax1, 1Bx17, 1By18, 1Dx5, 1Dy10	None	–
L88-31	Parental line. Sister line of L88-6 derived from same cross	1A null, 1Bx17, 1By18, 1D null	None	–
B72-8-11a	Null transformant of L88-31	1A null, 1Bx17, 1By18, 1D null	None	–
B73-6-1	L88-6 transformed with 1Dx5 gene	1Ax1, 1Bx17, 1By18, 1Dx5, 1Dy10	1Dx5	Three loci, 10–15 copies
B102-1-1	L88-31 transformed with Ax1 gene	1A null, 1Bx17, 1By18, 1D null	1Ax1	One locus, 2–4 copies
B102-1-2	L88-31 transformed with 1Ax1 gene	1A null, 1Bx17, 1By18, 1D null	1Ax1	Two loci, 4–5 copies

The table is based on data in Lawrence et al. (1988), Barro et al. (1997) and Rooke et al. (2003)

Extran (Hydro Agri, Immingham, UK) within the first few weeks after sowing. Consent to release these transgenic lines (licence number 97/R8/3) was obtained from the UK Department of the Environment Advisory Committee on Releases to the Environment under Part VI of the Environmental Protection Act 1990 and the Genetically Modified Organisms (Deliberate Release) Regulations 1992 as amended (1995 and 1997).

Analysis of grain

The nitrogen content of milled whole grain was determined by Dumas analysis using a Leco FP428 combustion analyser. Grain samples were also milled using a Brabender Quadrumat Junior laboratory mill and the mixing properties (PR) of the white endosperm flour samples determined in duplicate using a 2 g Mixograph (Rath et al. 1990).

Protein expression levels in four lines (L88-6, B73-6-1, B102-1-1 and B102-1-2) were determined by SDS-PAGE using a Tris–borate gel system to separate total protein fractions extracted from white flour samples as described by Shewry et al.(1995). This method was selected because preliminary studies in our laboratory showed higher reproducibility than analysis by HPLC. Gels were stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in 40% (v/v) methanol, 10% (w/v) trichloroacetic acid and destained in 10% (w/v) trichloroacetic acid. Five replicate extracts from samples of lines in plots within blocks from the randomised block experiments were separated and in a randomised order, run on the gels. There were 30 lanes (samples) per gel but, due to distortion, data from the first and last lanes on gels were excluded from analysis. Scans of the gels were analysed using *phoretix*™ software (Nonlinear Dynamics, Newcastle, UK).

Statistical analysis

The method of REsidual Maximum Likelihood (REML) as developed by Patterson and Thompson (1971) and implemented in the GenStat™ Statistical system (GenStat release 8.2(c) 2005, Lawes Agricultural Trust, Rothamsted Research) was used to fit a mixed model to data on traits (1,000 grain weight, nitrogen content as a percentage of dry weight and peak resistance) and determined by gel scanning (percentages of lanes corresponding to endogenous and transgenic subunits 1Ax1, 1Dx5 and 1Bx17 + 1By18). A natural logarithmic transformation was used for peak resistance and the gel scanning data to account for heterogeneity of variance in these data sets. For the traits data, the variability due to the blocks within the experiments (site by year) was taken as a random term (source of design variation) in the model and the effects of line, site and year as fixed terms for assessment of statistical significance. For the gel scanning data, the variability due to the blocks as well as the variability due to the gels and lanes within gels was accounted for as random terms. Here, the analysis also included an assessment of the significance of systematic trend across the gels by including an extra model parameter with the *lane* term for autoregressive variation. There was no such significant variation for any of the variates analysed ($P >$ 0.05), so this extra parameter was not required and the model used for gel scanning data was

$$\begin{aligned} y_{ijklmn} = {} & \mu + site.year.block_{ijk} + gel_l + lane_m + gel.lane_{lm} \\ & + site_i + year_j + line_n + site.year_{ij} + site.line_{in} \\ & + year.line_{jn} + site.year.line_{ijn} + \varepsilon_{ijklmn}, \end{aligned} \quad (1)$$

for data $y_{ijklmn} = \log_e$(% of subunit in lane), where μ is a constant and the effects of each term are as given for $i=1,2$ (LARS, RRes); $j=1, 2, 3$ (1998, 1999, 2000); $k=1, 2, 3, 4$ for blocks; $l=1, 2, ..., 20$ for gels; $m=2, 3, ..., 29$ (omitting the first and 30th lanes) and $n=1, 2, 3, 4$ for lines (L88-6, B73-6-1, B102-1-1 and B102-1-2). A dot indicates the interaction between model terms, and the equation has the random terms of the mixed model on the first line and the fixed terms on the second and third lines. The ε_{ijklmn} term is the error for fitting each observation.

For the 1,000 grain weight and nitrogen as a percentage of dry weight data, a similar model was used

$$\begin{aligned} y_{ijkn} = {} & \mu + site.year.block_{ijk} \\ & + site_i + year_j + line_n + site.year_{ij} + site.line_{in} \\ & + year.line_{jn} + site.year.line_{ijn} + \varepsilon_{ijkn}, \end{aligned} \quad (2)$$

with y_{ijkn} being the 1,000 grain weight, or N as percentage of dry weight data and the model terms as denoted in Eq. 1, except with $n=1,..., 6$ (L88-31, L88-6, B73-6-1, B102-1-1, B102-1-2 and B72-8-11a).

For the $\log_e$ (peak resistance) data, there were duplicate samples per block, so the model was

$$\begin{aligned} y_{ijknm} = {} & \mu + site.year.block.sample_{ijkm} \\ & + site_i + year_j + line_n + site.year_{ij} + site.line_{in} \\ & + year.line_{jn} + site.year.line_{ijn} + \varepsilon_{ijknm}, \end{aligned} \quad (3)$$

with y_{ijknm} being $\log_e$(peak resistance), $n=1, ..., 6$ again and $m=1,2$ for the two samples.

The significance of the fixed model terms was assessed using the Wald test (incorporated within the REML procedure (Welham and Thompson 1997)). This provided *P*-values assuming an asymptotic chi-squared distribution for a model term's variation on the relevant degrees of freedom for the term. This was followed by inspection of means tables and calculation of mean ratios of standard errors of means, or standard deviations of contributions to interactions for corresponding year by site combinations across lines, to consider stability of progeny lines to parental lines within and across environments.

For the gel scanning data (for subunits 1Ax1, 1Dx5 and 1Bx17 + 1By18) from the set of four lines, a linear

discriminant analysis (LDA) (see for example, Mardia et al. 1979) was used to consider the differences between lines, sites and years as a whole.

The purpose of LDA here is to locate vectors through the multi-dimensional data (i.e. three dimensions) which maximises the difference between the line by site by year groups (24), so that it is possible to allocate each data point or "unit" to its particular group. The vectors are formed as linear combinations of the three original variates (1Ax1, 1Dx5 and 1Bx17 with 1By18 data), and as in principal components analysis (PCA), the first vector accounts for the most variability with subsequent vectors accounting for less. Saving the first two discriminant vectors, the original data points may then be transformed to this new co-ordinate scale to give unit scores. It is then possible to visualise the differences between the groups by plotting the discriminant scores for the group means.

Results and discussion

The high molecular weight (HMW) subunits of wheat glutenin have been studied in detail because of their role in determining dough strength and grain processing quality (Payne 1987; Shewry et al. 2003). They are encoded by a small family of six genes, of which three, four, or five are expressed in hexaploid bread wheat. There is a clear relationship between the number of genes expressed, the amount of HMW subunit protein and dough strength, and this has led to a number of studies in which transgenesis has been used to increase HMW subunit copy number (Blechl and Anderson 1996; Altpeter et al. 1996; Barro et al. 1997; Alvarez et al. 2000). We have generated a series of transgenic lines in which additional HMW subunit genes are expressed in two related lines. These were produced from the F_2 generation of a cross between the cultivars Olympic and Gabo and hence each has 50% background from each parent. They were selected to differ in the number of expressed HMW subunit genes, with two (1Bx17 + 1By18) being expressed in L88-31 and five (1Ax1, 1Bx17 + 1By18, 1Dx5 + 1Dy10) in L88-6.

The characteristics of the lines used for this study are summarised in Table 1 while SDS-PAGE separations of the total grain proteins are shown in Fig. 1. These comprise the two parental lines, L88-6 and L88-31, L88-6 transformed to express the 1Dx5 transgene (B73-6-1) and L88-31 transformed to express the 1Ax1 transgene (B102-1-1, B102-1-2). A further control line was also used, B72-8-11a, which is a null line selected by segregation from L88-31 transformed with the 1Dx5 gene. It should be noted that line B102-1-2 contains two bands corresponding to the products of 1Ax1 transgenes, the lower of which (see arrow in Fig. 1, track d) probably results from a gene rearrangement during transformation. These bands were combined for gel scanning. Such rearrangements probably occur due to recombination within the sequences encoding the repetitive domains of the proteins and have been discussed previously (Barro et al. 1997). Similarly, an additional minor band of slower mobility in the transgenic line B73-6-1 (see arrow in Fig. 1, track b) may correspond to an extended form of subunit 1Dx5 resulting from recombination. In order to determine the performance of these lines under field conditions, they were grown in replicate field trials on two sites in the UK (Rothamsted, near London and Long Ashton Research Station, near Bristol) over 3 years (1998–2000).

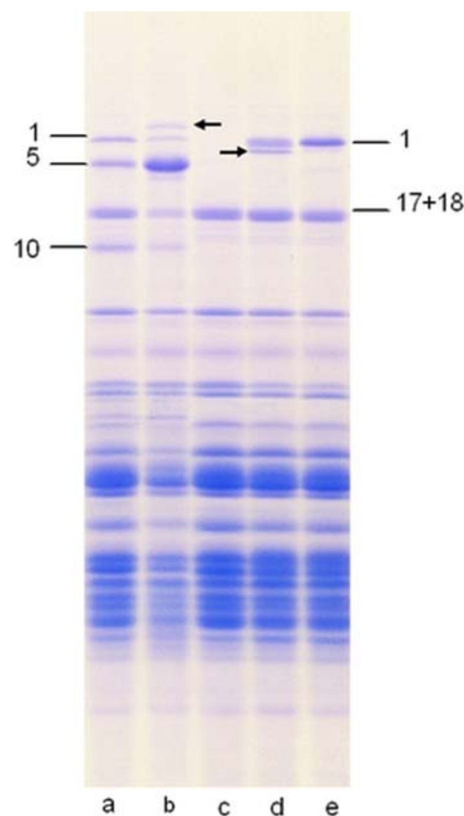

Fig. 1 SDS-PAGE analyses of total protein samples from flour of the control and transgenic lines. *a* L88-6 (control), *b* B73-6-1 (transgenic), *c* L88-31 (control), *d* B102-1-1 (transgenic), *e* B102-1-2 (transgenic). Subunits 1Ax, 1Dx5, 1Bx17 + 1By18 and 1Dy10 are indicated. *Arrows* in tracks b and d indicate additional HMW subunit bands which may be encoded by rearranged transgenes. Line B72-8-11a is a null segregant with the same patterns as L88-31 and is therefore not shown

Two series of analyses were carried out to compare the stability of the transgenic and control lines. Firstly the stability of expression of the transgenes was determined, in comparison with the endogenous HMW subunit genes in the same lines and with the control line L88-6 expressing five endogenous HMW subunit genes. Secondly, the 1,000 grain weight, seed nitrogen content and mixing properties were compared for the three transgenic and three control lines. In both cases these analyses were carried out on four randomly selected plots from each site/year combination.

Table 2 Stability of HMW subunit expression in the lines L88-6, B73-6-1, B102-1-1 and B102-1-2

Model term	1Ax1	1Dx5	1Bx17 + 1By18
Site	< 0.001	0.060	0.019
Year	< 0.001	0.191	< 0.001
Line	< 0.001	< 0.001	< 0.001
Site·year	0.449	0.744	0.162
Site·line	< 0.001	0.702	< 0.001
Year·line	< 0.001	< *0.001*	0.002
Site·year·line	< *0.001*	0.271	< *0.001*

The statistical significance of the effects in the model of $\log_e$ percentage of lanes on gels for the three subunits, given by Eq. 1. Most important significant effects are given in italics. There was no significant variability between sites for subunit 1Dx5

Stability of HMW subunit expression

Quantitative gel scanning was used to determine the proportions of HMW subunits (expressed as % total protein in gel lane) in the three transgenic lines (B73-6-1, B102-1-1, B102-1-2) relative to the L88-6 control line which expresses five endogenous HMW subunit genes. Statistical analysis was then used to calculate whether the subunits encoded by the transgenes differed in stability of expression from those encoded by the corresponding endogenous genes in other lines and by the homoeologous genes in the same lines. It should be noted that subunit 1Dx5 in B73-6-1 is the product of both endogenous and transgenes and that the data for amount and stability apply to the combined products of these genes. Similarly, it should be noted that subunits 1Bx17 and 1By18 co-migrate on SDS-PAGE and that combined values for these two proteins were therefore determined.

The data obtained for subunit amount (determined as a percentage of the total protein in the lane) were $\log_e$ transformed and analysed by fitting the linear mixed model (Eq. 1), in order to assess the significance of year, site and line effects. There were significant interactions between the three factors for 1Ax1 ($P < 0.001$) and 1Bx17 + 1By18 ($P < 0.001$) but not for 1Dx5 which showed a significant interaction ($P < 0.001$) between lines and years but no significant variability between sites (Table 2). Comparison of the mean values (Table 3) confirmed the differences in the proportions of subunits in the different lines which were apparent by visual inspection. For example, there were higher proportions of 1Dx5 in B73-6-1 compared to L88-6 and of 1Ax1 in B102-1-1 and B102-1-2 compared with L88-6 and B73-6-1. Gel scanning also demonstrated that greater variability existed in the proportion of subunit 1Ax1 in material grown in 1998, with B102-1-1 and B102-1-2 differing significantly from each other and from the other five site/year combinations in the material from Long Ashton and the proportion of the same subunit in B73-6-1 grown at both sites differing from the other four site/year combinations. Subunit 1Dx5 differed significantly between L88-6 and B73-6-1, but within lines occured significant differences at the 5% level occurred across years for L88-6 only. Subunits 1Bx17 + 1By18 also showed significant differences between pairs of lines grown on the Long Ashton and Rothamsted sites in 1998. There were no significant differences for individual lines within or across sites for 1999 and 2000.

In order to compare the relative stability of expression of the subunits, the ratios relative to the control line (L88-6) were calculated in terms of the back-transformed means of percentage of lanes and standard

Table 3 Mean proportions of HMW subunits for line by year by site combinations

Line	Site	Year	1Ax1	1Dx5	1Bx17 + 1By18
L88-6	LARS	1998	1.077 (2.935)	1.328 (3.773)	1.616 (5.032)
		1999	1.053 (2.865)	1.421 (4.141)	1.635 (5.132)
		2000	1.201 (3.322)	1.545 (4.688)	1.725 (5.614)
	RRes	1998	1.019 (2.769)	1.239 (3.452)	1.604 (4.974)
		1999	1.147 (3.150)	1.437 (4.208)	1.682 (5.377)
		2000	1.219 (3.385)	1.477 (4.380)	1.713 (5.546)
B73-6-1	LARS	1998	0.350 (1.420)	*2.206 (9.079)*	1.172 (3.227)
		1999	0.406 (1.501)	*2.310 (10.074)*	1.235 (3.438)
		2000	0.476 (1.610)	*2.303 (10.004)*	1.286 (3.620)
	RRes	1998	0.266 (1.305)	*2.094 (8.117)*	1.109 (3.031)
		1999	0.414 (1.513)	*2.230 (9.300)*	1.217 (3.377)
		2000	0.563 (1.756)	*2.297 (9.944)*	1.307 (3.695)
B102-1-1	LARS	1998	**1.521 (4.579)**		1.553 (4.727)
		1999	**1.771 (5.877)**		1.712 (5.542)
		2000	**1.855 (6.390)**		1.771 (5.876)
	RRes	1998	**1.777 (5.910)**		1.703 (5.490)
		1999	**1.861 (6.433)**		1.785 (5.957)
		2000	**1.870 (6.490)**		1.756 (5.790)
B102-1-2	LARS	1998	**1.246 (3.477)**		1.393 (4.027)
		1999	**1.646 (5.189)**		1.690 (5.421)
		2000	**1.738 (5.684)**		1.766 (5.848)
	RRes	1998	**1.604 (4.971)**		1.673 (5.326)
		1999	**1.725 (5.614)**		1.745 (5.723)
		2000	**1.767 (5.853)**		1.755 (5.781)
SED (df)			0.05697 (452)	0.1115 (452)	0.04277 (452)

Values are percentage of lanes on gels, $\log_e$ transformed, with back-transformed means in parenthesis. Bold indicates that subunit 1Ax1 is transgenic for B102-1-1 and B102-1-2. Italics indicates that subunit 1Dx5 is endogenous and transgenic in B73-6-1. Sites are Rothamsted Research (RRes) and Long Ashton Research Station (LARS)

Table 4 Relative stability of subunit expression in the lines

Line	1Ax1	1Dx5	1Bx17 + 1By18
L88-6	1.000	1.000	1.000
B73-6-1			
Within site			
LARS	1.086 (0.183)		1.057 (0.047)
RRes	1.207 (0.192)		1.298 (0.251)
Overall	1.147(0.122)	0.839 (0.179)	1.177 (0.126)
B102-1-1			
Within site			
LARS	1.479 (0.142)		1.559 (0.357)
RRes	0.941 (0.231)		1.194 (0.123)
Overall	1.210 (0.171)		1.377 (0.188)
B102-1-2			
Within site			
LARS	1.211 (0.128)		1.083 (0.127)
RRes	1.115 (0.164)		1.203 (0.120)
Overall	1.163 (0.095)		1.143 (0.083)

Means of the ratios of standard errors of corresponding site–year values given in Table 3 (with standard errors in parenthesis) are used to compare the overall stabilities of subunit expression in the transgenic and (L88-6) lines with the standard errors on the transformed scale. L88-6 is set with a ratio as unity. There was no statistically significant site difference for the subunit 1Dx5

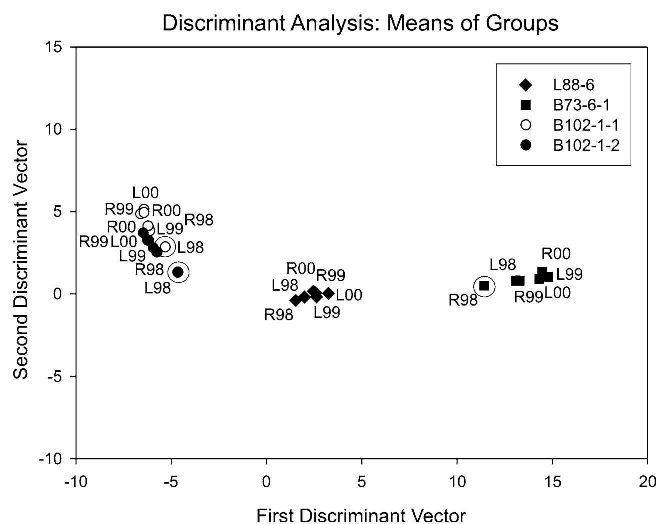

Fig. 2 Linear discriminant analysis (LDA) of HMW subunit expression levels for L88-6 (control), B73-6-1, B102-1-1 and B102-1-2 (transgenic) grown on two sites over 3 years. The points are labelled by site, with *R* for RRes and *L* for LARS, and by year, 1998, 1999 or 2000

errors of means on the transformed scale. Data for the 3 years were then combined to give means of the ratios and standard errors of these means within site and overall (Table 4). In Table 4 the relative stability of expression of the endogenous subunits is expressed as 1.000 and subunits which show back-transformed mean ratios greater than this are less stable in their expression. Conversely, those that show values lower than unity are more stable. These results show that the subunits encoded by the transgenes do not differ significantly in their stability of expression from the subunits encoded by endogenous genes. For example, subunit 1Dx5 in B73-6-1 (which is encoded by the endogenous gene and transgenes) shows a value of 0.839 ± 0.179 compared with L88-6 set as 1.000. Similarly, subunit 1Ax1 shows values of 1.210 ± 0.171 in B102-1-1 (transgenic) and 1.163 ± 0.095 in B102-1-2 (transgenic) compared with 1.147 ± 0.122 in B73-6-1 and compared with 1.000 in L88-6 (endogenous). Similar conclusions can be drawn when comparing subunits encoded by transgenes and endogenous genes in the same lines, such as 1Ax1 in B102-1-1 (1.210 ± 0.171) and B102-1-2 (1.163 ± 0.095) and 1Bx17 + 1By18 in the same lines (1.377 ± 0.188 and 1.143 ± 0.083, for B102-1-1 and B102-1-2, respectively).

The relative stability of subunit expression in the lines can also be represented visually using LDA (Fig. 2). This figure is derived from the analysis of proportions of subunits 1Ax1, 1Dx5, 1Bx17 + 1By18 from the four lines (L88-6, B73-6-1, B102-1-1, B102-1-2) over all years and sites and shows the scores of the group (line by site by year) means. In this analysis, the first two discriminant vectors (represented as the *x*- and *y*-axes in Fig. 2) account for 58.9 and 19.4% of the total variation, respectively, with subunit 1Dx5 being of most importance in the discrimination of data to the groups for the first vector and subunits 1Ax1/1Bx17 + 1By18 for the second. This is apparent in Fig. 2, in which the clusters of points (discriminant scores on the new axes given by the two vectors) comprising B102-1-1/B102-1-2, L88-6 and B73-6-1 are clearly separated by the first vector and those comprising B102-1-1/B102-1-2 and L88-6/B73-6-1 by the second. The drift in values within lines for 1998 noted above can be seen for B102-1-1, B102-1-2 and B73-6-1. However, the clusters formed by the points pertaining to lines are of similar area in all four lines and hence, most importantly, the points within the clusters (site by year combinations) remain in fairly close proximity given that the LDA aims to separate the line by site by year data as much as possible. This finding is therefore consistent with the component HMW subunits of the lines showing similar levels of stability of expression across sites and years.

Stability of seed weight, seed N and processing quality

Three parameters were measured on all six lines in order to compare their stability of agronomic performance and processing quality: 1,000 grain weight, grain nitrogen (expressed as % dry weight) and PR as a measure of dough strength during mixing. Dough strength is particularly relevant as the HMW subunits are major determinants of this aspect of dough functionality (Payne 1987). Hence, the optimisation of the amount and composition of the HMW subunits is an important target for wheat improvement whether by plant breeding or plant biotechnology. In order to measure dough strength we milled grain and mixed the white endosperm flour in a 2 g Mixograph. This machine records the increase in stress as dough is mixed to its maximum resistance and the subsequent decrease in stress on

Table 5 Statistical significance of the effects in the model of 1,000 grain weight, nitrogen as a percentage of dry weight and peak resistance, given Eqs. 2 and 3

Model term	1,000 grain weight	*N* as % dry weight	Peak resistance
Site	0.122	< 0.001	< 0.001
Year	< 0.001	< 0.001	< 0.001
Line	< 0.001	< 0.001	< 0.001
Site·year	0.977	< 0.001	0.037
Site·line	< *0.001*	0.086	< 0.001
Year·line	*0.002*	< 0.001	< 0.001
Site·year·line	0.704	< *0.001*	< *0.001*

Most important significant effects are given in italics. Significant site by line and year by line interactions occurred for 1,000 grain weight

over-mixing (Walker and Hazelton 1996). A number of measurements are taken and we selected PR (measured in arbitrary units) to compare the properties of flours from the transgenic and control lines.

The linear mixed models given in Eqs. 2 and 3 were used to obtain the significance of the line, site and year factors together with their interactions (Table 5). For 1,000 grain weight, although there was not an overall significant effect of site, there were significant two-way interactions between lines and sites ($P < 0.001$) and between years and lines ($P = 0.002$). For nitrogen as a percentage of dry weight, and for the peak resistance data, there was a significant interaction between the three factors ($P < 0.001$) (Table 5).

The means for the combinations of the factors are given in Table 6. The greatest 1,000 grain weights were obtained for L88-6 at LARS in 1998 and 1999, and the greatest difference across the two sites was also for L88-6. The differences in 1,000 grain weight for lines across sites, taking overall LARS—RRes means, were 1.41 (B102-1-1), −0.75 (B102-1-1), −0.57 (B72-8-11a), 2.35 (B73-6-1), 2.6 (L88-6) and 0.47 (L88-31), so significant ($P < 0.05$) site differences only occurred for L88-6 and B73-6-1. Given the years by lines interaction, year 2000 gave generally low 1,000 grain weights for all lines compared to the other years. For nitrogen as a percentage of dry weight, the highest percentages were seen consistently at RRes compared to LARS for all line by year combinations. Although all maximum differences within sites (comparing years) for lines were significant,

Table 6 Mean values of 1000 grain weight, nitrogen as a percentage of dry weight and peak resistance for line by year by site combinations

Line	Site	Year	1,000 grain weight (g)	*N* as % dry weight	Peak resistance (arbitrary units)
L88-6	LARS	1998	41.05	2.485	6.092 (442.3)
		1999	41.36	2.063	5.110 (165.7)
		2000	36.34	2.559	5.998 (402.6)
	RRes	1998	38.20	2.559	6.037 (418.6)
		1999	39.57	2.422	5.102 (164.4)
		2000	33.17	2.736	6.052 (425.0)
B73-6-1	LARS	1998	33.67	2.593	5.080 (160.8)
		1999	35.67	2.255	4.224 (68.30)
		2000	31.38	2.591	4.932 (138.7)
	RRes	1998	31.15	2.659	4.968 (143.7)
		1999	33.35	2.653	4.184 (65.60)
		2000	29.16	2.881	5.050 (156.0)
B102-1-1	LARS	1998	32.08	2.489	5.947 (382.6)
		1999	34.33	2.428	5.059 (157.4)
		2000	28.59	2.733	5.953 (384.9)
	RRes	1998	30.35	2.785	6.024 (413.2)
		1999	31.97	2.725	5.167 (175.4)
		2000	28.47	2.892	5.965 (389.6)
B102-1-2	LARS	1998	30.06	2.380	5.894 (362.9)
		1999	33.96	2.203	4.985 (146.2)
		2000	30.63	2.567	5.869 (353.9)
	RRes	1998	31.58	2.688	6.028 (414.9)
		1999	33.94	2.543	5.115 (166.5)
		2000	31.37	2.677	5.886 (360.0)
L88-31	LARS	1998	33.22	2.333	5.698 (298.3)
		1999	33.89	2.069	4.805 (122.1)
		2000	32.30	2.480	5.682 (293.5)
	RRes	1998	33.73	2.464	5.729 (307.7)
		1999	34.02	2.371	4.809 (122.6)
		2000	30.23	2.611	5.654 (285.4)
B72-8-11a	LARS	1998	31.59	2.265	5.671 (290.3)
		1999	33.98	2.154	4.766 (111.4)
		2000	30.25	2.437	5.660 (287.1)
	RRes	1998	31.78	2.459	5.702 (299.5)
		1999	34.14	2.372	4.949 (141.0)
		2000	31.60	2.588	5.622 (276.4)
SED (df)			1.428 (102)	0.04525 (102)	0.03620 (231)

Values are given with back-transformed means in parenthesis where applicable. Sites are Rothamsted Research (RRes) and Long Ashton Research Station (LARS)

the greatest differences within sites were for LARS in the case of L88-6 (0.496) and L88-31 (0.411). In comparison, for the transgenic wheat lines, the maximum within site differences for B102-1-1 were 0.305 (LARS) and 0.167 (RRes), whilst for B102-1-2 they were 0.364 (LARS) and 0.145 (RRes) and for B73-6-1 were 0.338 (LARS) and 0.228 (RRes). For B72-8-11a, the maximum differences were 0.283 (LARS) and 0.216 (RRes). This indicates that no greater spread of values was obtained for the transgenic lines than for the control lines (Table 6).

The values for PR were consistently lower in 1999 than for the other years regardless of site, which is related to the relatively high *P*-value (0.037) for the sites by years interaction. The values across sites for any given line were also reasonably consistent by year, with the 1999 data generally contributing most to the variability. Only B73-6-1 showed some inconsistency across years compared to the patterns of PR values exhibited over years by the other lines, and relatively high values of PR were recorded for the B102-1-1 and B102-1-2 samples from RRes in 1998 and also for L88-6 in the LARS samples from 1998 (Table 6). Mean ratio values, calculated using the corresponding year by site combinations and with standard errors and ranges of the ratios, are given in Table 7, comparing B102-1-1, B102-1-2 and B72-8-11a to L88-31, B73-6-1 to L88-6 and for L88-6 to L88-31. For PR, the ratios were calculated using the back-transformed means.

The differences between the mean values for 1,000 grain weight, grain nitrogen and PR (summarised in Table 6) are consistent with previous analyses of these lines. For example, Rooke et al. (1999) reported that B73-6-1 had a lower grain weight and lower PR than its parental line L88-6 while Popineau et al. (2001) reported similar Mixograph results for B73-6-1 and B102-1-2. However, the major point to be noted in the present study is that the means (Table 6), mean ratio values and ranges of ratios (Table 7) gave no indication that the transgenic lines differed in stability from the corresponding control lines.

The stabilities of the three traits across [in terms of contributions to the line by environment (two sites by 3 years) interaction] and within (in terms of contributions to the overall residual variation given the models) the six environments are compared in Table 8, with the values for L88-6 and L88-31 again being expressed as 1.000. In addition, the relative stabilities of L88-6 and L88-31 were expressed as the ratio L88-6:L88-31. The lines clearly show some differences in stability but these are not consistently related to the presence or absence of the transgenes. For example, L88-6 showed the greatest instability for 1,000 grain weight, nitrogen as a percentage of dry weight and PR when measured across all environments, but was more stable than most of the other lines within environments.

Table 7 Mean ratio values with standard errors (in parenthesis) and ranges of ratios for comparison of 1,000 grain weight, nitrogen as a percentage of dry weight and peak resistance (in arbitrary units)

Line	1,000 grain weight	*N* as % dry weight	Peak resistance
B102-1-1	0.941 (0.019)	1.122 (0.015)	1.337 (0.023)
	0.885–1.013	1.070–1.174	1.285–1.431
B102-1-2	0.971 (0.020)	1.052 (0.012)	1.265 (0.029)
	0.905–1.038	1.021–1.091	1.197–1.358
B72-8-11a	0.980 (0.018)	0.997 (0.010)	1.001 (0.030)
	0.937–1.045	0.971–1.041	0.962–1.151
L88-31	1.000	1.000	1.000
B73-6-1	0.849 (0.011)	1.056 (0.013)	0.372 (0.012)
	0.815–0.879	1.013–1.095	0.342–0.412
L88-6	1.000	1.000	1.000
L88-6:L88-31	1.162 (0.023)	1.034 (0.010)	1.400 (0.027)
	1.097–1.236	0.997–1.066	1.340–1.489

Values are calculated from those in Table 6, for B102-1-1, B102-1-2 and B72-8-11a to line L88-31, for B73-6-1 to line L88-6 and for L88-6 to L88-31. For peak resistance, ratios have been calculated using the back-transformed means

Table 8 Line stability across and within environments

Line	1,000 grain weight	*N* as % dry weight	Peak resistance
Across environments			
B102-1-1	0.718	2.252	0.912
B102-1-2	1.482	2.042	1.706
B72-8-11a	1.112	1.717	1.891
L88-31	1.000	1.000	1.000
B73-6-1	0.500	0.831	1.291
L88-6	1.000	1.000	1.000
L88-6:L88-31	1.709	2.799	1.926
Within environments			
B102-1-1	0.666	1.541	1.525
B102-1-2	0.972	1.247	0.968
B72-8-11a	0.840	1.062	1.996
L88-31	1.000	1.000	1.000
B73-6-1	0.954	0.931	1.501
L88-6	1.000	1.000	1.000
L88-6:L88-31	0.835	0.866	1.101

Across environments: Using the contributions to the line by environment (two sites by three years) interaction (variation about the environment means), the ratios of the standard deviation of these values for each line, to L88-31 (for B102-1-1, B102-1-2 and B72-8-11a) and L88-6 (for B73-6-1), are shown as well as the ratio of L88-6 to L88-31
Within environments: Using the contributions to the overall residual variation (variation about the line–site–year means), the ratios of the standard deviation of these values for each line to L88-31 (for B102-1-1, B102-1-2 and B72-8-11a) and L88-6 (for B73-6-1), are shown as well as the ratio of L88-6 to L88-31.

Conclusions

We have carried out replicate field trials on two sites over 3 years to compare the performance of transgenic and control wheats, measuring four parameters: subunit expression levels, grain dry weight, grain nitrogen and peak resistance on mixing. This has shown that the expression of the transgenes is similar to that of the corresponding genes in terms of stability from generation to generation, site to site and plot to plot. Furthermore, the control and transgenic lines showed

similar stability in terms of grain dry weight, grain total nitrogen and dough strength on mixing. It is concluded therefore that the transgenic wheat lines are not intrinsically more or less stable than those produced by conventional breeding.

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