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Analysis of HMW glutenin subunits encoded by chromosome 1A of bread wheat (*Triticum aestivum* L.) indicates quantitative effects on grain quality

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Summary. A gene encoding the high-molecular-weight (HMW) subunit of glutenin 1Ax1 was isolated from bread wheat cv Hope. Comparison of the deduced amino acid sequence with that previously reported for an allelic subunit, 1Ax2*, showed only minor differences, which were consistent with both subunits being associated with good bread-making quality. Quantitative analyses of total protein extracts from 22 cultivars of bread wheat showed that the presence of either subunit 1Ax1 or 1Ax2*, when compared with a null allele, resulted in an increase in the proportion of HMW subunit protein from ca. 8 to 10% of the total. It is suggested that this quantitative increase in HMW subunit protein may account for the association of 1Ax subunits with good quality.

Key words: Wheat – HMW glutenin subunits – Bread-making quality – Gene – Protein sequence

Introduction

The high-molecular-weight (HMW) subunits of wheat glutenin have been studied in detail because of their role in determining bread-making quality. They are encoded by loci (*Glu-1*) on the long arms of the group 1 chromosomes (Payne et al. 1980; Lawrence and Shepherd 1981), each of which consists of two genes (Harberd et al. 1986). These encode different types of subunit (low M_r x-types and high M_r y-types), but not all the genes are expressed in bread wheat. Thus, cultivars always contain 1Dx, 1Dy and 1Bx subunits, sometimes 1By and 1Ax subunits, but never 1Ay subunits (Payne et al. 1981 b).

The HMW subunits also occur in allelic forms which differ in their mobility on SDS-PAGE, and Payne and coworkers have shown that bread-making quality is particularly associated with variation at the *Glu-D1* and *Glu-A1* loci (Payne et al. 1979, 1981 a; Payne 1987). In the case of the *Glu-D1* locus, good quality is specifically associated with a pair of subunits (1Dx5+1Dy10) (Payne et al. 1981 a), and comparison of their amino acid sequences with those of allelic poor quality subunits (1Dx2+1Dy12) has indicated differences that could affect the structure and physical properties of gluten (Flavell et al. 1989; Shewry et al. 1989).

Two allelic subunits encoded by the *GluA1* locus occur in bread wheats, called 1Ax1 and 1Ax2*. Payne et al. (1979, 1981 a) initially showed that subunit 1Ax1 was correlated with good quality when compared with the null (silent) allele, using SDS-sedimentation as an indirect measurement of bread-making quality. Moonen et al. (1983) subsequently showed that both 1Ax1 and 1Ax2* were superior to the null allele in baking tests, although 1Ax2* appeared to be better than 1Ax1. The difference between 1Ax1 and 1Ax2* has not been substained by other workers, and Payne et al. (1987) have given both subunits the same "quality score" (3 out of a maximum of 4).

In the present paper we compare the nucleotide and deduced amino acid sequences of a gene encoding subunit 1Ax1 with those reported previously for subunit 1Ax2* (Anderson and Greene 1989). We also demonstrate that the presence of either of these subunits results

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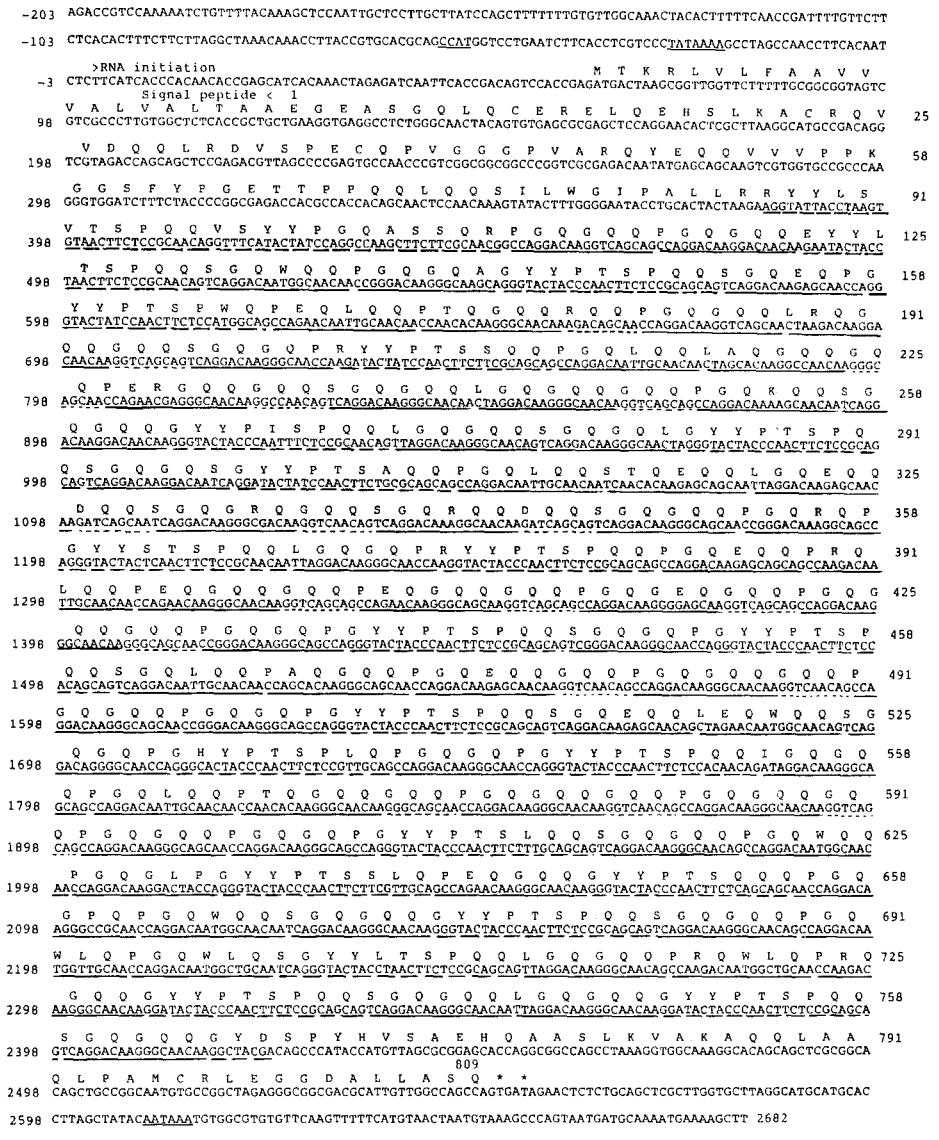

Fig. 1. The nucleotide and deduced amino acid sequences of the *Glu-1A-1* gene encoding HMW subunit 1Ax1. The putative TATA box and CCAT sequence in the 5′ region and polyadenylation signal in the 3′ region are *underlined.* Repetitive hexapeptides, nonapeptides and tripeptides in the encoded protein are *underlined* with *unbroken*, *broken* and *dotted lines*, respectively

in a significant increase in the proportion of HMW subunits present in gluten, and speculate that their effect on bread-making quality is mainly quantitative.

Materials and methods

Isolation and characterisation of a gene encoding subunit 1Ax1

The hexaploid wheat cultivar 'Hope' was obtained from the wheat collection maintained at Plant Breeding International, Cambridge. Its identity was supported by SDS-PAGE, which showed the expected presence of HMW subunits 1, 6+8, 5+10.

A genomic library was constructed as described by Flavell et al. (1989), using the vector NM1130 kindly provided by Dr. N. Murray (Edinburgh). NM1130 is an insertion vector with an *Eco*RI cloning site in the *imm* 434cI, which causes a shift from turbid to clear plaques. The vector was digested with *Eco*RI and ligated with the size-fractionated wheat DNA and packaged in vitro, as described by Maniatis et al. (1982). The packaged mixture was plated on *E. coli* strain K803 (*Sup* E *mei*$^-$ *hod* S$^-$ r_k^- m_k^-) which is *Rec*A$^+$.

The phage library containing wheat DNA was plated and screened by plaque hybridisation using, as a probe, the HMW glutenin cDNA pTAG 1290 (Thompson et al. 1983) labelled with ^{32}P. Hybridising plaques were picked and purified by several rounds of plaque purification on *E. coli* strain Ww 265 (*gyz* A96, *Rec* A1, *end* A1, *thi-1 hod* R1–7 R_k^- M_k^+ *Sup* E44), a *rec* A$^-$ strain also called Dh-1. Cloned DNAs were prepared following growth of phage on plates (Maniatis et al. 1982). Cloned DNA *Eco*RI inserts were subcloned in pUC19 plasmid vector grown in *E. coli* strain ED 8800 (r_k^- m_k^- *Sup* E *Sup* F *lac*2 M13 *met Rec* A56). An insert believed to correspond to the wheat *Eco*RI DNA fragment containing the gene coding for HMW glutenin subunit 1 was restricted with *Hin*dIII and *Eco*RI, run on an agarose gel, blotted onto nitrocellulose and hybridized with ^{32}P-labelled pTAG 1290 cDNA. Hybridising fragments of 2.5 kb and 7.0 kb were observed, respectively, corresponding to the hybridising pattern expected for the Hope DNA restriction fragments containing the HMW glutenin subunit 1 gene, as previously determined by Harberd et al. (1986) using nullisomic, tetrasomic and intervarietal chromosome substitution lines of wheat.

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                                *
1Ax1  EGEASGQLQCERELQEHSLKACRQVVDQQLRDVSPECQPVGGGPVARQYEQQVVVPPKGGSFYPGETTPPQQLQQ
1Ax2* EGEASGQLQCERELQEHSLKACQQVVDQQLRDVSPECQPVGGGPVARQYEQQVVVPPKGGSFYPGETTPPQQLQQ

                                                                          *
      SILWGIPALLRRYYLSVTSPQQVSYYPGQASSQRPGQGQQPGQGQQEYYLTSPQQSGQWQQPGQGQAGYYPTSPQ
      SILWGIPALLRRYYLSVTSPQQVSYYPGQASSQR......PGQGQQEYYLTSPQQSGQWQQPGQGQSGYYPTSPQ

          *
      QSGQEQPGYYPTSPWQPEQLQQPTQGQQRQQPGQGQQLRQGQQGQQSGQGQPRYYPTSSQQPGQLQQLAQGQQGQ
      QSGQKQPGYYPTSPWQPEQLQQPTQGQQRQQPGQGQQLRQGQQGQQSGQGQPRYYPTSSQQPGQLQQLAQGQQGQ


      QPERGQQGQQSGQGQQLGQGQQGQQPGQKQQSGQGQQGYYPISPQQLGQGQQSGQGQLGYYPTSPQQSGQGQSGY
      QPERGQQGQQSGQGQQLGQGQQGQQPGQKQQSGQGQQGYYPISPQQLGQGQQSGQGQLGYYPTSPQQSGQGQSGY


      YPTSAQQPGQLQQSTQEQQLGQEQQDQQSGQGRQGQQSGQRQQDQQSGQGQQPGQRQPGYYSTSPQQLGQGQPRY
      YPTSAQQPGQLQQSTQEQQLGQEQQDQQSGQGRQGQQSGQRQQDQQSGQGQQPGQRQPGYYSTSPQQLGQGQPRY

                                      **
      YPTSPQQPGQEQQPRQLQQPEQGQQGQQPEQGQQGQQPGQGEQGQQPGQGQQGQQPGQGQPGYYPTSPQQSGQGQ
      YPTSPQQPGQEQQPRQLQQPEQGQQGQQPEQGQQGQQQRQGEQGQQPGQGQQGQQPGQGQPGYYPTSPQQSGQGQ


      PGYYPTSPQQSGQLQQPAQGQQPGQEQQGQQPGQGQQGQQPGQGQQPGQGQPGYYPTSPQQSGQEQQLEQWQQSG
      PGYYPTSPQQSGQLQQPAQGQQPGQEQQGQQ.........PGQGQQPGQGQPGYYPTSPQQSGQEQQLEQWQQSG

                                                          *
      QGQPGHYPTSPLQPGQGQPGYYPTSPQQIGQGQQPGQLQQPTQGQQGQQPGQGQQGQQPGQGQQGQQPGQGQQPG
      QGQPGHYPTSPLQPGQGQPGYYPTSPQQIGQGQQPGQLQQPTQGQQGQQPGQGQQGQQPGEGQQGQQPGQGQQPG

                                  *
      QGQPGYYPTSLQQSGQGQQPGQWQQPGQGLPGYYPTSSLQPEQGQQGYYPTSQQQPGQGPQPGQWQQSGQGQQGY
      QGQPGYYPTSLQQSGQGQQPGQWQQPGQGQPGYYPTSSLQPEQGQQGYYPTSQQQPGQGPQPGQWQQSGQGQQGY


      YPTSPQQSGQGQQPGQWLQPGQWLQSGYYLTSPQQLGQGQQPRQWLQPRQGQQGYYPTSPQQSGQGQQLGQGQQG
      YPTSPQQSGQGQQPGQWLQPGQWLQSGYYLTSPQQLGQGQQPRQWLQPRQGQQGYYPTSPQQSGQGQQLGQGQQG


      YYPTSPQQSGQGQQGYDSPYHVSAEHQAASLKVAKAQQLAAQLPAMCRLEGGDALLASQ-809
      YYPTSPQQSGQGQQGYDSPYHVSAEHQAASLKVAKAQQLAAQLPAMCRLEGGDALLASQ-794
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Fig. 2. Comparison of the deduced amino acid sequences of *Glu-1A-1* genes encoding subunit 1Ax1 and subunit 1Ax2* (Anderson and Green 1989)

The HMW subunit gene was located on 2 *Hin*dIII fragments, the 2.5-kb fragment (above) and a smaller fragment of ~800 bp. The latter did not hybridise to pTAG 1290. These, and an overlapping *Sph*I fragment of ~2,400 bp, were cloned in M13 mp18 and mp19 for sequencing and in pUC18 and pUC19 for the production of sequential overlapping deletions. The deletions were generated by digestion of linearised plasmid clones with exonuclease III, followed by blunting with nuclease S1 and religation. The shortened inserts were then excised and ligated into M13 mp18 or mp19 for sequencing. Analyses of the derived sequences were carried out using the University of Wisconsin sequencing programmes (Devereux et al. 1984).

Quantitative analysis of HMW subunits

Milled samples were extracted with 10 vol. of 3.33% (w/v) SDS, 100 m*M* dithiotheitol and 10% (v/v) glycerol in 0.067 *M TRIS*-HCl (pH 6.8), by suspension in a sonic bath for 30 min, followed by standing at 20 °C overnight and finally boiling for 2 min. The supernatant after centrifugation was separated on a 10% acrylamide gel using a *TRIS*/borate buffer system (see Bunce et al. 1985) at three different loads. Four separations at each 100 cl, taken from separate gels, were scanned using an LKB ultroscan XL laser scanner. The combined areas of the HMW subunit peaks were expressed as a percentage of the total areas of all protein peaks. For the Avalon samples the protein extracts were adjusted to 10 mg/ml before separation.

The mean effects of different subunit compositions on the combined proportions of HMW subunits in cultivars with four and five subunits were estimated using the restricted maximum likelihood algorithm (REML) of the statistical package GENSTAT (Genstat 5 Committee 1990). This method of analysis can account for the unbalanced distribution of subunit composition over the experiment, which was designed as four gels run per tank, with two tanks run simultaneously at each of three times.

Results

The amino acid sequence of subunit 1Ax1

The nucleotide and derived amino acid sequences of a gene encoding subunit 1Ax1 from the cultivar Hope are given in Fig. 1. Comparison of the N-terminal amino acid sequence of the encoded protein with those determined for a number of purified HMW subunits (but not 1Ax1, which was N-terminally blocked) (Shewry et al. 1984) shows the presence of a 21-residue signal peptide, the mature protein consisting of 809 residues with an M_r of 87,680. It resembles other HMW subunits (see Shewry et al. 1989) in that it has a clear domain structure, with nonrepetitive domains of 86 and 42 residues, respectively, flanking a repetitive domain of 681 residues. The latter consists of tandem and interspersed repeats based on tripeptide (consensus GQQ), hexapeptide (consensus PGQGQQ) and nonapeptide (consensus GYYPTSPQQ) motifs, which are underlined in Fig. 1. Assuming that the transcription start site is 61 bp upstream of the ATG initiation codon, as determined for the gene encoding subunit 1Dx2 (Sugiyama et al. 1985), there is a TATA box beginning at −30 and a CCAT sequence at −56. It

also has two stop codons (TGA.TAG) and a putative polyadenylation signal (AATAAA) ca. 50 bp downstream from the first of these, but differs from other HMW subunit genes in that it lacks a second putative polyadenylation signal (AATAAT) further downstream.

The amino acid sequence of the mature subunit 1Ax1 is very similar to that of subunit 1Ax2* (Fig. 2). It differs from subunit 2* in seven single amino acid substitutions (all except one in the repetitive domain), and in the insertion of a single hexapeptide and adjacent hexa- and tripeptides. The net effects of these changes are increases in the numbers of glycine, glutamine and proline residues (derived from the inserted repeat peptides), with minor changes in other residues. These include a decrease in the lysine content from six to five residues (Table 1). Although there is no change in the net content of lysine, this residue is involved in two substitution events. In particular, subunit 1Ax1 has arginine instead of glutamine adjacent to a cysteine residue.

Both 1Ax1 and 2* have four cysteine residues, three in the N-terminal domain and one in the C-terminal domain. All of these can be expected to form disulphide bonds, as free cysteine residues have not been detected in wheat gluten proteins. Although the precise disulphide patterns are not known, at least one interchain disulphide bond must be formed, as subunit 1Ax1 is only present in glutenin polymers that are stabilised by disulphide bonds. The arginine residue at position 23 in 1Ax1 could possibly affect the disulphide bonding of the adjacent cysteine due to a charge effect, but none of the other substitutions are likely to affect disulphide bond formation.

The absence of major differences between the amino acid sequences of subunits 1Ax1 and 1Ax2* is consistent with their similar effects on grain quality. Recent studies by Autran (1990) have indicated differences in the total amounts of HMW subunit proteins present in cultivars with three, four and five expressed subunit genes, indicating that the improved quality associated with the presence of a 1Ax subunit could result from an increase in the total amount of HMW subunit protein. We therefore compared the amounts of HMW subunit protein present in varieties with and without a 1Ax subunit.

Quantitative determination of HMW subunits

A sample of 22 wheat lines was selected to study the effects of the presence of 1Ax HMW subunits on total subunit amount. All had subunits 1Dx5+1Dy10, and either 1Bx7+1By9 or 1Bx7+1By8. In addition, 8 lines also had subunit 1Ax1 and 4 had subunit 1Ax2*. This gave a total of six classes (Table 2). Although the lines were multiplied in the glasshouse at Rothamsted, they did show considerable variation in total grain nitrogen (from 1.47 to 2.75% at 14% H_2O). We therefore also

Table 1. Comparison of the amino acid compositions and general characteristics of subunits 1Ax1 and 1Ax2*

Residue	Residues per mole		Mole %	
	1Ax1	1Ax2*	1Ax1	1Ax2*
Ala	19	18	2.349	2.267
Cys	4	4	0.494	0.504
Asp	6	6	0.742	0.756
Glu	24	24	2.967	3.023
Phe	1	1	0.124	0.126
Gly	146	140	18.047	17.632
His	4	4	0.494	0.504
Ile	4	4	0.494	0.504
Lys	5	6	0.618	0.756
Leu	40	39	4.944	4.912
Met	1	1	0.124	0.126
Asn	0	0	0.000	0.000
Pro	98	95	12.114	11.965
Gln	282	276	34.858	34.761
Arg	19	19	2.349	2.393
Ser	60	61	7.417	7.683
Thr	26	26	3.214	3.275
Val	12	12	1.483	1.511
Trp	9	9	1.112	1.134
Tyr	49	49	6.057	6.171

Characteristics	1Ax1	1Ax2*
Mol.wt	87,680.13	86,308.38
No. of residues	809	794
No. of charged residues	6	5
Calculated isoelectric point	5.30	5.52

Data for 1Ax2* are from Anderson and Greene (1989)

Table 2.

Subunit composition			No. of cultivars	HMW subunits (% total)
1A	1B	1D		
A. Variation in the proportions of HMW subunits in cultivars with four and five subunits				
–	7+8	5+10	6	7.609
–	7+9	5+10	4	8.564
1	7+8	5+10	3	10.316
1	7+9	5+10	5	10.625
2*	7+8	5+10	1	9.631
2*	7+9	5+10	3	9.835
Maximum SE of differences = 0.3499				
B. Proportions of HMW subunits in cultivars with four and five subunits				
Four subunits			10	8.015
Five subunits			12	10.211
SE of differences = 0.1411				

The varieties analysed were Tonic, Adam, Markus, Masterpiece, Fournil, Hira, Koga-2, Magdalena, Vuka, Broom, Opal, Bonanza, Jerico, Sicco, Monopol, Musket, Red Fife, Holdfast, Kavkaz, Bezostoja, Marquis, Era

studied a series of 19 field-grown samples of cv Avalon, with nitrogen contents ranging from 1.58 to 2.48% (at 14% H_2O). A simple linear regression of the total grain nitrogen of these samples against the combined proportions of HMW subunits showed that the slope of the line was not significantly different from zero ($P<0.122$), implying no evidence of association.

The proportions of HMW subunits in total protein extracts were determined by laser densitometry of SDS-PAGE separations. Because the individual HMW subunits are not always completely resolved, it was decided to determine their combined rather than individual proportions. The means and standard errors of differences of the different samples and separations were calculated using residual maximum likelihood estimation (REML) to allow for imbalance in the data.

Comparison of the combined proportions of HMW subunits in the cultivars with four (i.e. no 1Ax subunits) and five subunits showed a clear difference (8.015 compared with 10.211), which was statistically significant (Table 2B). Comparison of the six classes showed further differences associated with subunit composition, the varieties with subunits 1Bx7+1By9 having a higher combined proportion of HMW subunits than those with 1Bx7+1By8, and varieties with 1Ax1 more than those with 1Ax2* (Table 2A). However, the number of cultivars present in the classes was lower (one to six) and the maximum SE of differences higher (0.3499), and these results should be treated with caution. There was no relationship between the total proportion of HMW subunit protein and grain nitrogen.

Discussion

Previous studies have demonstrated that the repetitive sequences present in the HMW subunits form regular β-turns, which could contribute to the elastic mechanism of gluten (Shewry et al. 1989). Flavell et al. (1989) have further suggested that the β-turns are more regularly distributed in subunit 1Dy10 than in 1Dy12, and that this could be responsible for the association of subunits 1Dx5+1Dy10 with good quality for bread-making. Similar comparisons of subunits 1Ax1 and 1Ax2* were made using the method of reverse turn prediction of Chou and Fasman (1978). These showed that one of the differences between the sequences, the substitution of Gln.Arg. for Pro.Gly. in a hexapeptide repeat, resulted in loss of a predicted reverse turn in the 1Ax2* subunit. The other single amino acid differences affected the probabilities, but not the overall predicted occurrence of reverse turns. The two deletions present in subunit 1Ax2* relative to subunit 1Ax1 would result in a slightly lower total content of turns in the subunit, but there is currently no evidence that subunit size is related to quality. We consider, therefore, that none of the differences between the repetitive domains of the two subunits would have sufficient effects on the conformations of the proteins to affect quality. The two subunits also have the same number of cysteine residues, which are potential cross-linking sites for the formation of disulphide-linked polymers. Only one substitution event could be predicted to affect the local environment of a cysteine residue, an arginine for glutamine adjacent to the first cysteine in subunit 1Ax1. However, there is no evidence that this substitution would affect the pattern of disulphide bond formation, and the structures of the two subunits are essentially consistent with similar roles in gluten structure and quality.

The results of the quantitative determinations of HMW subunits indicate that the increase in bread-making quality associated with the presence of a 1Ax subunit may result from an increase in the total proportion of HMW subunit proteins. This in turn may give a higher amount of high M_r gluten polymers (see Field et al. 1983). These quantitative differences agree with the data of Autran (1990), but disagree with Marchylo et al. (1989), who found no significant differences between the combined proportions of HMW subunits in five lines (two parents and three backcross progeny) with three, four and five subunits. This may be due to the larger number of lines analysed in our study. Our results are also consistent with a recent study by Seilmeier et al. (1991) who determined total protein, total gliadins, HMW subunits and LMW subunits in 24 lines of wheat. They showed that the amount of HMW subunits had the greatest effects on the sedimentation volume and dough resistance, both measurements of quality. They also quantitised individual subunits by HPLC, and their data clearly show that the presence of a 1Ax subunit results in increased total subunit protein (an average of 1.57% seed weight for 12 varieties with five subunits, compared with an average of 1.29% for seven varieties with four subunits).

The results reported here demonstrate that clear differences in the amount of HMW subunit protein are associated with gene expression, with ca. 8% HMW subunit protein present in lines with four expressed genes and 10% in lines with five. This indicates that individual subunit genes account on average for about 2% of the total grain proteins, although variation is clearly apparent on SDS-PAGE separations and is also indicated by the small differences that we observed between lines with 1By8 and 1By9, and lines with 1Ax1 and 1Ax2*. This suggests that improvements on quality could be effected by engineering wheat to increase the HMW subunit gene number by only one or two copies. Recent advances in the transformation of maize (Fromm et al. 1990; Gordon-Kamm et al. 1990) and rice (Rhodes et al. 1988; Toriyama et al. 1988; Shimamoto et al. 1989; Datta et al. 1990) and in the regeneration of wheat (Vasil et al. 1990) indi-

cate that it may soon be possible to test this hypothesis experimentally.

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