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Identification of chromosome arms influencing expression of the HMW glutenins in wheat

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Abstract The high-molecular-weight (HMW) glutenin genes, located on the group 1L chromosome arms, are a major determinant for baking quality in wheat (*Triticum aestivum* L.). In addition, the HMW glutenin genes provide a valuable model system for studying the evolution and regulation of orthologous and paralogous genes in polyploid species. The goal of this study was to identify loci that modify the expression of the HMW glutenins, and to map them to specific chromosome arms. Comparisons were made between endosperms with zero versus three (or three versus six) doses for each of the 42 chromosome arms of wheat. SDS-PAGE and scanning densitometry were used to quantify the protein expression levels of the four HMW glutenin genes in cv. Chinese Spring, for each of the dosage comparisons. Fifteen chromosome arms were found to have significant effects on *Glu-B1-1*, excluding the structural gene dosage effect: eight positive effects on 1AL, 2AS, 2BL, 2DS, 5DS, 6AL, 6DL, and 7AL and seven negative effects on 1BS, 1DS, 1DL, 4DL, 6BS, 6DS, and 7AS. Nineteen chromosome arms had significant effects on *Glu-B1-2*, excluding the structural gene dosage effect: eight positive effects on 1AL, 2AS, 2BS, 3AL, 4BL, 6DS, 7BL and 7DS and 11 negative effects on 1AS, 1BS, 1DS, 1DL, 2AL, 2BL, 3DS, 4BS, 4DL, 5BL, and 6BS. Twenty chromosome arms had significant effects on *Glu-D1-1*, excluding the structural gene dosage effect: 11 positive effects on 1AL, 1BL, 2BS, 2DS, 5BS, 5DS, 6AL, 6DS, 6DL, 7AL, and 7BL and nine negative effects on 1AS, 1BS, 1DS, 2BL, 4DL, 5BL, 5DL, 6BL, and 7DS. Twenty-five chromosome arms had significant effects on *Glu-D1-2*, excluding the structural gene dosage effect: 17 positive effects on 1BL, 2AS, 2BS, 2DS, 2DL, 3AS, 3AL, 3BS, 5AS, 5BS, 5DL, 6AL, 6DL, 7AL, 7BS, 7BL, and 7DL and eight negative effects on 1DS, 4DL, 5AL, 5BL, 6BS, 6BL, 6DS and 7DS. Of the 164 gene-chromosome arm tests performed, about 52% (85/164) showed no significant effects, and 48% (79/164) showed significant effects, excluding the structural gene dosage effects. Of the significant effects, 56% (44/79) were positive effects, and 44% (35/79) were negative effects. Comparisons of dosage effects on orthologous loci (both x-type or both y-type HMW glutenins) showed that orthologous HMW glutenin genes are largely influenced by the same regulatory systems. Less correlation was found for comparisons between paralogous genes, although considerable conservation was observed at this level as well. These observations suggest that after polyploidization, many of the duplicated orthologous regulatory loci were inactivated by mutation, thus consolidating control over the HMW glutenin genes. Possible candidates for orthologous regulatory genes were identified in maize and barley. This study represents the first comprehensive search of the wheat genome for regulators of the HMW glutenins.

Keywords HMW glutenins · Dosage analysis · Regulatory genes

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Introduction

Baking quality is an important target for improvement in wheat (*Triticum aestivum* L.) breeding programs, and the

high-molecular-weight (HMW) glutenins play a key role in this trait (Dong et al. 1991; Rogers et al. 1991; Ahmad 2000). Gluten is a complex mixture of more than 50 proteins that largely determines the quality of wheat flour for baking (Shewry et al. 1995b). Most important are the HMW glutenins, which confer strength (elasticity) to dough (Shewry and Tatham 1997). Gliadins are also important, giving dough viscosity. The structural genes for these important loci have been genetically mapped and cloned. HMW glutenin loci are found on the long arm of the homoeologous group 1 chromosomes. The entire set of HMW glutenin genes has been cloned and sequenced from the wheat cultivar Cheyenne (Halford et al. 1987; Anderson and Greene 1989; Anderson et al. 1989) as well as several genes from other cultivars.

In addition to being important agriculturally, the HMW glutenin genes represent a valuable model system for studying the evolution and regulation of orthologous and paralogous genes. Different cultivars of wheat contain between three and five HMW glutenin subunits: none or one encoded by the locus on 1AL (*Glu-A1*), one or two encoded by the locus on 1BL (*Glu-B1*), and two encoded by the locus on 1DL (*Glu-D1*) (Alvarez et al. 2000). Each HMW glutenin locus on the group 1 long arms consists of two tightly-linked paralogous genes, called x-type (specified by *-1*) and y-type (*-2*), believed to have originated from an ancient duplication event with subsequent divergence (Anderson et al. 1998). There is a complete orthologous set of x-type genes at the A, B, and D genome *Glu-1* loci; as well as a complete orthologous set of y-type genes at the A, B, and D genome *Glu-2* loci. However, no cultivars produce all six possible protein subunits due to various gene inactivations. The x- and y-type protein subunits can be distinguished based on electrophoretic mobility and isoelectric focusing. X-types show slower migration on SDS-PAGE and also have only about half the cysteine content of y-types. At *Glu-A1*, the x-type subunit gene is sometimes active; the y-type subunit gene is silent. Most of the variation in quality can be attributed to the y-type subunits, but allelic variation at both of these genes results in differences in bread quality (Payne 1987). For example, for *Glu-D1-1* alleles (x-type), subunit Dx5 gives better quality than subunit Dx2. For *Glu-D1-2* alleles (y-type), subunit Dy10 gives better quality than subunit Dy12 (Flavell et al. 1989). The cultivar Chinese Spring contains only four active HMW glutenin genes. Both the x-type and y-type genes at *Glu-A1* are silent. *Glu-B1-1* contains the x-type subunit Bx7 and *Glu-B1-2* contains the y-type subunit By8. *Glu-D1-1* contains the x-type subunit Dx2, and *Glu-D1-2* contains the y-type subunit Dy12 (Harberd et al. 1986).

The expression of genes is controlled by networks of regulatory proteins that recognize specific DNA elements in enhancers or the promoter and then interact with transcription factors at the promoter to modulate transcription level (Wyrick and Young 2002). Genes regulating seed storage protein (SSP) expression have been found for a number of cereals. The *Lys3* gene in barley, *Hordeum vulgare* L., controls expression of the hordein SSPs, probably at transcription or early transcript processing (Kreis et al. 1984). In maize (*Zea mays* L.) a number of genes have been discovered that regulate the expression of the zein SSPs – for example, the *Opaque* and *Floury* genes. Studies on the interactions of these genes indicate that there are multiple regulatory pathways controlling zein expression in maize (Motto et al. 1989; Schmidt 1992). For example, *Opaque-2* and *Opaque-7* appear to belong to different regulatory pathways, regulating the 22-kDa and 20-kDa α-zein proteins, respectively. *Opaque-6* seems to act at a higher level regulatory point, affecting transcription of both the 22-kDa and 20-kDa α-zeins. The two genes *Defective endosperm-B30* and *Mucronate* affect yet a different regulatory pathway than *Opaque-2* (Motto et al. 1989). *Opaque-2* encodes a regulatory protein with a leucine-zipper DNA binding domain. The Opaque-2 protein is required for transcription of the 22-kDa α-zein genes (Thompson and Larkins 1994). At least two other genes that improve the seed characteristics of the opaque phenotype, called *opaque-2* modifiers, act to increase transcription of the 27-kDa γ-zein genes and increase stability of the transcripts (Burnett and Larkins 1999).

Studying two different developmental stages in wheat – 10-day-old first leaf seedlings and 7-day-old etiolated seedlings – Colas des Francs and Thiellement (1985) and Thiellement et al. (1986) found numerous dosage-sensitive regulatory genes. They analyzed proteins using two-dimensional SDS-PAGE and altered the dosage of chromosome arms using ditelosomic lines, a strategy similar to the one used in this study. They found that: (1) regulatory loci were frequently found, and many structural genes had regulatory loci on more than one chromosome arm; (2) homoeologous chromosome arms usually did not cause similar effects on the expression of a particular protein; (3) different regulators were found for the different developmental stages.

Guo and Birchler (1994) used dosage analysis in maize to clarify our understanding of the genetic basis of aneuploid syndromes. Chromosome arm dosage in the embryo and endosperm was varied at three levels for chromosome arms covering about half of the maize genome. Using Northern analysis of endosperm RNA, expression of model genes was examined. They found many positive (direct) and negative (inverse) effects on transcription of the genes by varying the dosage of unlinked chromosome arms, i.e., arms that did not carry the structural genes. This showed that these unlinked arms contained dosage-sensitive regulatory genes that modified expression of the model genes. Their findings were similar to those found earlier in the wheat study: (1) regulatory loci were frequently found, and many structural genes had regulatory loci on more than one chromosome arm; (2) different regulators were found for the different tissue types (embryo versus endosperm). They examined ten gene/tissue combinations, varying the dosage of chromosome arms over approximately half of the maize genome, and found that each gene had an average of 6.6 chromosome arms with dosage-sensitive effects. Taking into account that only half of the genome was tested, one

Table 1 Effects[a] of chromosome arms on expression of the HMW glutenin genes

Arm[b]	*Glu-B1-1* (Bx7)			*Glu-B1-2* (By8)			*Glu-D1-1* (Dx2)			*Glu-D1-2* (Dy12)		
	A[c]	B	D	A	B	D	A	B	D	A	B	D
1S	NS	–* 0.83	–** 0.74	–** 0.86	–** 0.76	–** 0.74	–** 0.84	–** 0.71	–** 0.79	NS	NS	–** 0.80
1L	+** 1.17	+** 24.42	–** 0.58	+** 1.57	+** 24.44	–** 0.61	+** 1.20	+* 1.29	+** 30.35	NS	+** 1.49	+** 5.56
2S	+	NS	+* 1.19	+	+* 1.14	NS	NS	+** 1.22	+** 1.38	+	+** 1.31	+** 1.31
2L	NS	+	NS	–* 0.85	–	NS	NS	–	NS	NS	NS	+** 1.14
3S	NS	NS	NS	NS	NS	–* 0.90	NS	NS	NS	+** 1.20	+** 1.27	NS
3L	NS	NS	NS	+* 1.19	NS	NS	NS	NS	NS	+** 1.42	NS	NS
4S	NS	NS	NS	NS	–	NS	NS	NS	NS	NS	NS	NS
4L	NS	NS	–** 0.81	NS	+** 1.32	–* 0.88	NS	NS	–* 0.87	NS	NS	–* 0.89
5S	NS	NS	+** 1.29	NS	NS	NS	NS	+** 1.33	+** 1.24	+* 1.17	+** 1.23	NS
5L	NS	NS	NS	NS	–	NS	NS	–	–	–	–	+
6S	NS	–** 0.73	–** 0.86	NS	–* 0.81	+** 1.32	NS	NS	+** 1.23	NS	–** 0.85	–** 0.83
6L	+** 1.21	NS	+* 1.11	NS	NS	NS	+** 1.43	–* 0.91	+** 1.37	+** 1.58	–* 0.88	+** 1.34
7S	–* 0.90	NS	NS	NS	NS	+	NS	NS	–	NS	+** 1.33	–
7L	+** 1.18	NS	NS	NS	+* 1.37	NS	+** 1.28	+** 1.30	NS	+** 1.38	+* 1.19	+* 1.21

*$P<0.05$; **$P<0.01$; NS, not significant

[a] Direction of effect: +, increase in protein with increasing dosage (from 0 to 3 doses); –, decrease in protein with increasing dosage (from 0 to 3 doses). The numbers indicate the ratio of CS (3 doses)/the ditelosomic (0 doses). Ditelosomic stocks known to have impure backgrounds which may affect the results are: 2AL, 2DS, 4AS, 6AS. Ditelosomic stocks known to have additional partial terminal deletions are: Dt 1BS, a deletion in 7AL; Dt 2AS, a deletion in 3BS; Dt 2BL, a deletion in 4AL; Dt 5BL, a deletion in 2DS; Dt 6BS, a deletion in 2BS (Devos 2000; Devos et al. 1999). Where the ditelosomic stock was not available, the effect of the arm was inferred by comparing the effects of the whole chromosome and the other arm. The ratio used to measure the effect of the whole chromosome was tetrasomic (6 doses)/CS (3 doses). Where these comparisons gave a significant effect, they are indicated by a sign but not a significance level or ratio. These arms include: 2AS, 2BL, 4AL, 4BS, 5AL, 5BL, 5DL, and 7DS

[b] Homoeologous chromosome arm tested

[c] A, B, and D refer to the A, B, and D genomes, respectively

would expect about 13.2 dosage-sensitive regulators per gene on average. This may still be an underestimate as one chromosome arm may actually have several regulatory loci whose overall effect is averaged when varying the dosage of the whole arm. About 55% of the loci had a negative (inverse) effect, 39% had a positive (direct) effect, and 6% showed a zig-zag effect with increasing dosage (first up then down, or first down then up).

There are several examples where chromosomes not carrying the structural genes have been observed to affect the expression of SSPs in wheat. Bittel et al. (1991) found that chromosome 1D contains sequences that induce and suppress expression of different gliadin genes located on chromosome 6A. Brown and Flavell (1981) found that additional copies of chromosome 2A silenced the expression of gliadin genes encoded on chromosome 6D.

Some progress has already been made toward understanding the regulatory system controlling transcription of the HMW glutenins in wheat. A 40-bp enhancer element has been discovered 170 bp upstream of the HMW glutenin transcription start site (Thomas and Flavell 1990). Anderson et al. (1998) examined the promoter sequences of several HMW glutenin genes and found conservation of sequence about 1,200 bp 5′ to the start codon and 200–400 bp 3′ to the stop codon. Presumably, the sequences important for regulation of the HMW genes are contained within this range. The objective of this project was to identify and map genes that regulate the expression of the HMW glutenin genes in bread wheat. This study represents the first comprehensive search of the wheat genome for genes regulating the expression of the HMW glutenin genes.

Materials and methods

Creation of endosperms with zero and three copies of chromosome arms

For the chromosome arms for which they are available, ditelosomic (Dt) lines with the Chinese Spring (CS) background (Sears 1954) were used to create wheat seeds with endosperms possessing zero doses of the arm which is missing in the Dt line. For example, to obtain zero doses of 1AS, the Dt 1AL line was used. CS was used to create endosperms possessing the normal three doses of the chromosome arm. Where Dt lines were not available for a chromosome arm, tetrasomic lines were used to compare the effects of three copies (normal CS) versus six copies (tetrasomic) of the chromosomes in the endosperm. For all chromosomes, at least one arm could be analyzed using Dt lines, so the effect of the other arm was deduced by comparing the first arm and the whole chromosome. Plants were grown in the greenhouse, and endosperm proteins were extracted from seeds using the protocol of Galili et al. (1986).

SDS-PAGE, scanning densitometry, and statistical analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of endosperm proteins was performed on Protean II SDS-PAGE systems following the manufacturer's recommended protocols (Bio-Rad, Hercules, Calif.). The gels were 37.5 (acrylamide): 1 (bisacrylamide) (AmresCo, Solon, Ohio); resolving gels were 10% acrylamide. Seven replications of each comparison were carried out on a single gel, with each replicate containing protein from a different seed. Gels were stained with GelCode Blue (Pierce, Rockford, Ill.), destained, and dried between sheets of clear cellulose film (Shewry et al. 1995c). Dried gels were scanned on a Bio-Rad GS-710 imaging densitometer and images were analyzed with Quantity One image analysis software (Bio-Rad). Optical density of specific bands, expressed as a percentage of the total protein in the lane, was used to quantify expression of specific HMW glutenin proteins. *t*-tests were performed to find significant differences in expression for particular HMW glutenin genes between the two dosage levels. Quantity One software indicated that the images were within the linear response range.

Results

The dosage effects of each chromosome arm on expression of the HMW glutenin genes are shown in Table 1. The structural gene dosage effects for *Glu-B1-1* and *Glu-B1-2*, located on chromosome arm 1BL, can be seen in the 1BL row of Table 1. The structural gene dosage effects for *Glu-D1-1* and *Glu-D1-2*, located on chromosome arm 1DL, can be seen in the 1DL row of Table 1.

Fifteen chromosome arms were found to have significant effects on *Glu-B1-1*, excluding the structural gene dosage effect: eight positive effects on 1AL, 2AS, 2BL, 2DS, 5DS, 6AL, 6DL, and 7AL and seven negative effects on 1BS, 1DS, 1DL, 4DL, 6BS, 6DS, and 7AS. Nineteen chromosome arms had significant effects on *Glu-B1-2*, excluding the structural gene dosage effect: eight positive effects on 1AL, 2AS, 2BS, 3AL, 4BL, 6DS, 7BL and 7DS and 11 negative effects on 1AS, 1BS, 1DS, 1DL, 2AL, 2BL, 3DS, 4BS, 4DL, 5BL, and 6BS. Twenty chromosome arms had significant effects on *Glu-D1-1*, excluding the structural gene dosage effect: 11 positive effects on 1AL, 1BL, 2BS, 2DS, 5BS, 5DS, 6AL, 6DS, 6DL, 7AL, and 7BL and nine negative effects on 1AS, 1BS, 1DS, 2BL, 4DL, 5BL, 5DL, 6BL, and 7DS. Twenty-five chromosome arms had significant effects on *Glu-D1-2*, excluding the structural gene dosage effect: 17 positive effects on 1BL, 2AS, 2BS, 2DS, 2DL, 3AS, 3AL, 3BS, 5AS, 5BS, 5DL, 6AL, 6DL, 7AL, 7BS, 7BL, and 7DL and eight negative effects on 1DS, 4DL, 5AL, 5BL, 6BS, 6BL, 6DS and 7DS.

Of the 164 gene-chromosome arm tests performed, about 52% (85/164) showed no significant effects, and 48% (79/164) showed significant effects, excluding the structural gene dosage effects. Of the significant effects, 56% (44/79) were positive effects, and 44% (35/79) were negative effects. The average number of regulatory effects found per HMW glutenin gene was 19.8. As noted above, the genome-adjusted average estimate for several maize genes was 13.2 (Guo and Birchler 1994). Taking into account that wheat is hexaploid and maize probably has a tetraploid history, it is interesting that both species have an average of 6.6 regulators per gene on a per genome basis.

Figure 1 shows three examples of the zero to three dosage comparisons for chromosome arms 1BL, 1DL, and 6AL. Deletion of the structural genes for the HMW glutenins abolishes their expression. There is no expression of *Glu-B1-1* and *-2* when 1BL is deleted, and no expression of *Glu-D1-1* and *-2* when 1DL is deleted. The largest positive and negative regulatory effects found are also shown. *Glu-B1-1* expression shows a 42% decrease with increasing dosage of 1DL from zero to three. *Glu-D1-2* expression shows a 58% increase with increasing dosage of 6AL from zero to three.

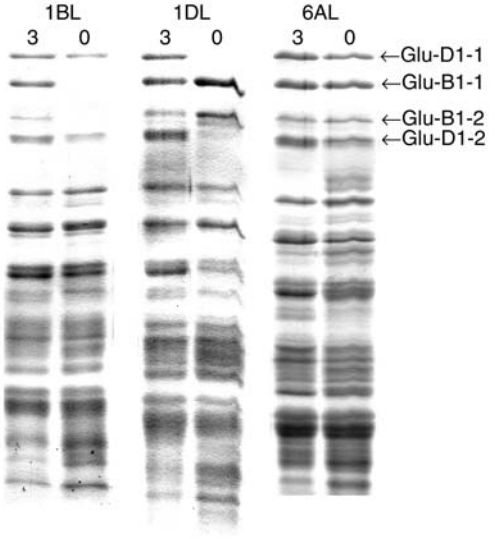

Fig. 1 SDS-PAGE of endosperm proteins comparing the three (3) and zero (*O*) dose levels for chromosome arms 1BL, 1DL, and 6AL. The effect of deleting the structural genes for the HMW glutenins can be seen for the 1BL and 1DL lanes

Discussion

Eukaryotes employ several layers of regulatory control over gene expression. These include transcriptional control (mediated by activator and repressor regulatory proteins), transcript processing control, control of transcript transport to the cytoplasm, transcript degradation control, transcript translational control, and protein degradation control. Cereal SSP genes are thought to be primarily regulated at the transcriptional level (Thomas and Flavell 1990). Final HMW glutenin protein content in the seed integrates over all of these levels. Regulatory genes found to control the expression of HMW glutenins at the protein level could be acting at any one (or some combination) of the above control levels. An issue with measuring final protein quantity is that protein level may be influenced by factors which are not specific for a particular protein. For example, in this study, altering the dosage of a chromosome arm could have a general effect on all proteins by perturbing normal seed development or interfering with amino acid availability for protein synthesis. The data collected in this experiment are the amount of specific protein bands as a percentage of the total endosperm protein in the gel lane. Thus, factors that affect general protein synthesis (total protein in the lane) should not disproportionately affect a particular protein band. The great majority of chromosome arm dosage effects in this study do not affect all of the HMW glutenins equally. Of the 40 chromosome arm or whole chromosome dosage effects (excluding chromosomes with structural genes) shown in Table 1, 37/40 have at least one HMW glutenin gene that is not significantly affected by dosage modulation. One chromosome arm caused significant effects in opposite directions for different HMW glutenin genes (6DS). Only two chromosome arms caused significant effects in the same direction for all four genes (1DS and 4DL). Thus, it does not appear that this experiment detected general factors affecting protein synthesis, such as availability of amino acids. A significant contribution of Guo and Birchler (1994) was to demonstrate that aneuploid syndromes, which can have large phenotypic effects, are caused by multiple *trans*-acting dosage effects on individual genes. These data support this conclusion.

A negative regulator gene decreases the expression of the HMW glutenin gene as its dosage increases. So, with a negative regulator, the aneuploid condition of zero doses of the regulator in the ditelosomic causes an increase in the expression of the HMW gene relative to normal (three doses). A positive-effect regulator gene increases the expression of the HMW glutenin gene as its dosage increases. So, with a positive-effect regulator, the aneuploid condition of zero doses of the regulator in the ditelosomic causes a decrease in the expression of the HMW gene relative to normal (three doses). All of the dosage effects found were less than a 100% increase or 50% decrease relative to the normal dosage (except for structural gene dosage effects). This is not surprising because in hexaploid wheat, any modifier gene would be expected to have orthologs on at least some of the homoeologous chromosomes which would compensate to some degree for the loss of regulatory loci on one chromosome. The largest positive effect was found for 6AL on *Glu-D1-2*, a 58% increase over zero doses. The largest negative effect was found for 1DL on *Glu-B1-1*, a 42% decrease below zero doses. In maize, many of the regulatory genes of the zein SSPs are linked with the structural genes. If linked regulatory loci were present on the chromosome arms holding the structural genes in this study, it was not possible to detect them due to the overwhelming structural gene dosage effects. It is also possible that for the chromosome arms that showed significant effects, more than one regulatory locus was present.

Are orthologous genes regulated by loci on the same chromosome arms?

Two comparisons can be made for orthologous genes: the x-type HMW glutenin genes and the y-type genes. When comparing the regulation of the x-type genes, *Glu-B1-1* and *Glu-D1-1*, 40 comparisons were made, excluding the structural gene dosage effects: 15% (6/40) of the chromosome arms showed positive effects on both genes; 7.5% (3/40) showed negative effects on both genes; 45% (18/40) showed non-significant effects on both genes; 5% (2/40) showed opposite effects on the two genes; 27.5% (11/40) showed a significant effect on one gene and no significant effect on the other gene. Of the 11 comparisons that showed a significant effect on both genes, about 82% (9/11) caused effects in the same direction. Only 8% (2/11) of the chromosome arms with significant effects on both genes gave effects in opposite directions. These exceptional cases were found for chromosome arms 2BL and 6DS.

For the y-type genes, *Glu-B1-2* and *Glu-D1-2*, 40 comparisons were made, excluding the structural gene dosage effects: 10% (4/40) of the chromosome arms showed positive effects on both genes; 10% (4/40) showed negative effects on both genes; 17.5% (7/40) showed non-significant effects on both genes; 5% (2/40) showed opposite effects on the two genes; 57.5% (23/40) showed a significant effect on one of the genes and no significant effect on the other gene. Of the ten comparisons that showed a significant effect for both genes, 80% (8/10) had effects in the same direction. Only 20% (2/10) of the chromosome arms had effects in opposite directions. These exceptional cases were found for chromosome arms 6DS and 7DS.

These observations for orthologous gene pairs indicate that orthologous HMW glutenin genes are largely controlled by the same regulatory systems. Taking into account the proportions of positive, negative, and non-significant effects on each gene, we performed a chi-square test to evaluate the results obtained for both orthologous pairs combined (Table 2). The test showed that the observed pattern was significantly different than what would be expected due to chance ($P < 0.05$). Thus, orthologous genes were influenced in the same direction more often than would occur by chance.

One interesting result found was that chromosome arms that carried orthologous structural genes did not al-

Table 2 Chi-square test comparing expected effects versus observed effects for both orthologous gene pairs combined (*NS* not significant)

Effect type	Expected (e)	Observed (o)	$(o–e)^2/e$
Both +	5.5	10	3.68
Both –	3.6	7	3.21
Both NS	22.0	25	0.41
Opposite	8.9	4	2.70
One significant one NS	39.9	34	0.87
$\sum$			10.87

ways have effects in the same direction. Chromosome arm 1BL, which carries the *Glu-B1-1* and *Glu-B1-2* structural genes, had a significant positive effect on expression of both *Glu-D1-1* and *Glu-D1-2*, which reside on chromosome arm 1DL. However, chromosome arm 1DL, which carries the *Glu-D1-1* and *Glu-D1-2* structural genes, had a significant negative effect on expression of both *Glu-B1-1* and *Glu-B1-2*, which reside on chromosome arm 1BL. The negative dosage effect of 1DL on *Glu-B1-1* and *Glu-B1-2* may be attributable to competition between these orthologous genes for shared transcription factors, or amino acids for protein synthesis. Thus, there is not necessarily a regulatory locus on 1DL that exerts control over the HMW glutenin genes on 1BL. However, the positive effect of 1BL on the 1DL HMW glutenin loci seems to point to a positive regulatory locus there.

The short arms of homoeologous group six chromosomes contain gliadin seed storage protein genes. The short arms of the homoeologous group one chromosomes also contain gliadin and LMW glutenin genes. Several of the short arms of the homoeologous group one chromosomes do cause negative dosage effects on expression of the HMW glutenins, and some have no significant effect. However, the situation is more complex with the short arms of the homoeologous group six chromosomes. Some of these arms have positive effects on the expression of the HMW glutenins, some have negative effects, and some have no significant effect. For the arms with negative effects, competition for amino acids in protein synthesis may be occurring.

Are paralogous genes regulated by loci on the same chromosome arms?

To compare the responses of the x-type genes to the y-type genes, all chromosome arms where both orthologs (for the x-type or y-type genes) had significant effects in the same direction were checked for the other gene type (paralog), excluding the structural gene dosage effects. Fifteen comparisons showed both orthologs with significant effects in the same direction, for either the x-type or y-type genes. About 13% (2/15) of the chromosome arms showed significant effects in the same direction as the original orthologs for both of the paralogs; about 73% (11/15) showed one paralog with a significant effect in the same direction and the other paralog with a non-significant effect; about 13% (2/15) showed both paralogs with non-significant effects. No comparison showed paralogs with significant effects in the opposite direction of the first orthologous pair. In these comparisons, the majority (73%) of the orthologous pairs had one gene that retained sensitivity to the chromosome arm that affected its paralogs and one gene that was no longer influenced by the chromosome arm that affected its paralogs. Thus, a striking pattern of conservation was also found between the regulatory systems of the paralogous HMW glutenin genes, although the correlation between the paralogous genes was less than that found for the orthologous genes. This seems to indicate that after divergence of the paralogs, these genes lost sensitivity over time to regulatory loci that once exerted some control over their expression. The difference in correlation between orthologs and paralogs is consistent with the belief that the divergence between the x-type and y-type genes is older than the divergence between the B and D genomes.

Do homoeologous chromosome arms contain orthologous regulatory loci controlling the same genes?

Excluding structural gene dosage effects there were 52 tests where all three homoeologous chromosome arms were checked for dosage effects on the genes *Glu-B1-1*, *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*. About 8% (4/52) showed all three arms with significant effects in the same direction; 25% (13/52) showed two arms with effects in the same direction, with one arm non-significant; about 37% (19/52) showed two arms with no significant effects; about 19% (10/52) showed all three arms with non-significant effects; about 6% (3/52) showed two arms with significant effects in the same direction and one arm with a significant effect in the opposite direction. This pattern was seen for the *Glu-D1-1*/6L, *Glu-D1-2*/5L, and *Glu-D1-2*/6L gene/homoeologous chromosome arm combinations. About 6% (3/52) showed two arms with opposite effects, and one arm with no significant effect. This pattern was seen for the *Glu-B1-2*/4L, *Glu-B1-2*/6S, and *Glu-D1-2*/7S gene/homoeologous chromosome arm combinations. Thus, only in about 8% of the cases did all three of the homoeologous arms maintain the same regulatory effect over a particular HMW glutenin gene. In the other cases, one or two of the homoeologous arms diverged to have no effect, or in a few cases, opposite effects. These observations are similar to those of Colas des Francs and Thiellement (1985) and Thiellement et al. (1986), reviewed above.

Orthologous candidate regulatory genes from other cereal species

The prolamin SSPs are present only in the Poaceae, and include the gliadins and LMW and HMW glutenins of wheat, the zeins of maize, and the hordeins of barley

(Shewry et al. 1995a). Regulators of these genes in maize and barley are possible candidate orthologs of the wheat regulatory effects on the HMW glutenins found in this study. In fact, Holdsworth et al. (1995) found that the maize Opaque-2 transcription factor activates transcription of the wheat LMW glutenin gene in transient assays in plant and yeast cells. The zeins, hordeins, gliadins, and LMW and HMW glutenins also share a conserved upstream sequence, the "-300 element", believed to be involved in transcription regulation of these genes (Thompson and Larkins 1989).

The barley *lys3a* mutation, which reduces B and C hordein mRNA expression in barley endosperm (Kreis et al. 1984), is located on chromosome 5HL (Lundqvist et al. 1997). Thus, the *Lys3* gene from barley is a possible orthologous candidate for the 5L effects found for the HMW glutenins. These interactions included the 5AL effect on *Glu-D1-2*; the 5BL effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 5DL effects on *Glu-D1-1* and *Glu-D1-2*.

Using comparative mapping (Ahn et al. 1993; Moore et al. 1995) and the bin map coordinates of maize genes (MDB 2001), a number of orthologous candidates were found from maize. Two possible candidates were found which map to the wheat group 1L chromosome arms: *Opaque-14* (on maize chromosome arm 6L) and *Floury-3* (maize 8L). Excluding the structural gene dosage effects, 1AL affected expression of *Glu-B1-1*, *Glu-B1-2*, and *Glu-D1-1*; 1BL affected *Glu-D1-1* and *Glu-D1-2*. 1DL affected *Glu-B1-1* and *Glu-B1-2*.

Three possible candidates were found which map to the wheat group 2S chromosome arms: *Opaque-2* (maize 7S), *Floury-1* (maize 2S), and *Opaque-15* (maize 7L). *Opaque-2* is known to encode a transcription factor which regulates the expression of the 22-kDa and 19-kDa α-zeins (Thompson and Larkins 1989). *Opaque-15* encodes a transcription factor which regulates expression of the 27-kDa γ-zeins and is believed to be an *o2* modifier, a gene that improves the soft texture of the *o2* mutant (Dannenhoffer et al. 1995). *Gzr1*, *gamma zein modifier 1*, also on maize 7L, is probably the same as *Opaque-15*. 2AS affected expression of *Glu-B1-1*, *Glu-B1-2*, and *Glu-D1-2*; 2BS affected expression of *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; 2DS affected expression of *Glu-B1-1*, *Glu-D1-1*, and *Glu-D1-2*.

Three possible candidates were found which map to the wheat group 5L chromosome arms: *Dzr1* (*zein-protein regulator 1*, on maize 4S), *Ohp1* (*opaque-2 heterodimerizing protein 1*, on maize 1L), and *Opaque-5* (maize 7L). *Dzr1* is a post-transcriptional regulator of the 10-kDa δ-zein gene (Chaudhuri and Messing 1994). *Ohp1* encodes a bZIP transcription factor which is thought to dimerize with the O2 transcription factor and bind to the 22-kDa α-zein promoter to stimulate transcription (Pysh et al. 1993). 5AL showed effects on *Glu-D1-2*; 5BL on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and 5DL on *Glu-D1-1* and *Glu-D1-2*.

De-B30* (*Defective endosperm-B30*) is linked to *Opaque-2* on maize chromosome 7S (5 cM away) and is a candidate gene that would be present on the wheat group 2S or 5L chromosome arms. 2S effects included the 2AS effects on *Glu-B1-1*, *Glu-B1-2*, and *Glu-D1-2*; the 2BS effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 2DS effects on *Glu-B1-1*, *Glu-D1-1*, and *Glu-D1-2*. 5L effects included the 5AL effect on *Glu-D1-2*; the 5BL effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 5DL effects on *Glu-D1-1* and *Glu-D1-2*.

Opaque-7 (maize 10L) is a candidate gene that would be present on the wheat group 2L or 5L chromosome arms. 2L effects included the 2AL effect on *Glu-B1-2*; the 2BL effects on *Glu-B1-1*, *Glu-B1-2*, and *Glu-D1-1*; and the 2DL effect on *Glu-D1-2*. 5L effects included the 5AL effect on *Glu-D1-2*; the 5BL effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 5DL effects on *Glu-D1-1* and *Glu-D1-2*.

Ohp2 (*opaque-2 heterodimerizing protein 2*, maize 5S) encodes a transcription factor which dimerizes with O2, similar to *Ohp1*, above. If there is an *Ohp2* wheat ortholog, it would be expected to be on the group 4L or 5L chromosome arms. 4L effects included the 4BL effect on *Glu-B1-2*, and the 4DL effects on all four HMW glutenin genes. 5L effects included the 5AL effect on *Glu-D1-2*; the 5BL effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 5DL effects on *Glu-D1-1* and *Glu-D1-2*.

Opaque-1 (maize 4L) is a candidate gene that would be present on the wheat group 5L or 6L chromosome arms. 5L effects included the 5AL effect on *Glu-D1-2*; the 5BL effects on *Glu-B1-2*, *Glu-D1-1*, and *Glu-D1-2*; and the 5DL effects on *Glu-D1-1* and *Glu-D1-2*. 6L effects included the 6AL effects on *Glu-B1-1*, *Glu-D1-1*, and *Glu-D1-2*; the 6BL effects on *Glu-D1-1* and *Glu-D1-2*; and the 6DL effects *on Glu-B1-1*, *Glu-D1-1*, and *Glu-D1-2*.

Taking into account the observations of chromosome arm dosage effects on orthologous and paralogous HMW glutenin genes, and comparisons of the effects of the three homoeologous chromosome arms, our hypothesis is that after polyploidization many of the duplicate orthologous regulatory loci were inactivated by mutation or deletion and that the system regulating the expression of the HMW glutenins is in a process of consolidation. This model is consistent with several studies showing that polyploidization leads to genome-wide deletions and epigenetic silencing of orthologous genes encoding transcription factors (Comai et al. 2000; Lee and Chen 2001; Ozkan et al. 2001; Pikaard 2001; Shaked et al. 2001). Future experiments will focus on measuring the effects of the dosage-sensitive regulatory loci on the gene expression of the HMW glutenins at the mRNA level. This will allow a determination of the level of regulatory control, and will eliminate any possible effects due to perturbations in amino acid metabolism caused by varying levels of chromosome dosage.

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