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Changes in High Molecular Weight Glutenin Subunit Composition Can Be Genetically Engineered without Affecting Wheat Agronomic Performance

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ABSTRACT

The genomes of modern cultivars have been painstakingly selected for the presence of favorable alleles at multiple loci, which interact to produce superior phenotypes. Genetic transformation provides a tool to introduce new genes without altering the original gene combinations. However, the random genetic and epigenetic changes sometimes generated by the transformation process have been associated with losses in agronomic performance. The agronomic performance of 50 transgenic wheat (*Triticum aestivum* L.) lines containing additional copies of native or modified high molecular weight glutenin subunit (HMW-GS) genes and the selectable marker *bar*, their untransformed parent 'Bobwhite', four lines containing only *bar*, and 10 null segregant lines were assessed in small plot trials over 2 yr and three locations. Most of the transgenic lines did not show significant changes in performance relative to Bobwhite, although the transgenic lines as a group tended toward lower performance. Null-segregant and *bar*-only lines performed similarly to Bobwhite. No relationship could be established between performance and particular transgenes or their expression levels. Despite the overall lower performance of the transgenic lines, many with agronomic performance equivalent to Bobwhite were identified. These findings suggest that extant techniques for genetic engineering of wheat are capable of producing agronomically competitive lines for use as cultivars or parents in breeding programs.

MODERN WHEAT PRODUCTION systems rely on cultivars that produce high yields of grain that meets specific quality parameters. Decades of hybridization and selection for incremental improvements have created allelic packages that interact to produce the particular phenotype desired for a given use, for example, high yielding hard wheat for the production of reasonably priced, high quality breads. Genetic manipulations that utilize in vitro techniques may introduce undesirable genetic and epigenetic variability for agronomic and quality traits. The expression of introduced gene(s) may also cause undesirable pleiotropic interactions. Ultimately, these unintended sources of variability will affect the ease with which in vitro-based asexual

manipulations can be used for the introduction of specific characteristics.

Commonly used techniques for asexual manipulation of cereal crops, such as in vitro selection or DNA delivery via particle bombardment (Weeks et al., 1993; Wan and Lemaux, 1994; Kumar et al., 2004) or *Agrobacterium tumefaciens*-mediated transfer (Cheng et al., 1997; Tingay et al., 1997), require manipulation of cells grown in vitro. The in vitro environment often induces multiple heritable epigenetic and genetic changes, that is, somaclonal variation (SCV) (Larkin and Scowcroft, 1981; Karp, 1994; Olhoft and Phillips, 1999; Kaeppler et al., 2000).

Field studies of transgenic and nontransgenic tissue culture-derived cereal plants have documented reductions in agronomic performance that would significantly impact their use in breeding programs. These studies have also identified tissue culture-derived lines with performance similar to their wild-type parent. Examples include studies of regenerated barley (see Bregitzer et al. [2002] and other studies cited therein) and wheat (Hanson et al., 1994; Barro et al., 2002; Zhou et al., 2003). Furthermore, studies of barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), and rice (*Oryza sativa* L.) have shown that the transformation process generates variability over and above that produced by the in vitro processes alone (Schuh et al., 1993; Bregitzer et al., 1998; Choi et al., 2000a, 2000b, 2001).

Several studies have examined the extent to which transgene expression, and not SCV, is responsible for observed phenotypic variability. Reported effects of transgene expression range from severe stunting and phenotypic abnormalities in nuclear tobacco transformants expressing trehalose (Lee et al., 2003), to slight but detectable performance reductions both from SCV and the expression of a heat stable β -glucanase in barley (Horvath et al., 2001), to undetectable effects either of transgene expression or SCV in transgenic wheat lines encoding 5-enolpyruvylshikimate-3-phosphate synthase (Zhou et al., 2003) or high molecular weight (HMW) glutenin transgenes (Barro et al., 2002). In the latter study, no differences were detected between wheat lines constitutively expressing *uidA* and *bar* and an endosperm-expressed HMW glutenin transgene, segregants containing only the HMW glutenin transgene, two nontransformed regenerants, and the two original parents from which these lines were derived. Thus, the literature has shown that transgenic plant performance can be, but is not necessarily, affected both by SCV and/or the presence of a transgene.

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Abbreviations: SCV, somaclonal variation; HMW, high molecular weight; HMW-GS, high molecular weight glutenin subunits.

Designing reasonable breeding objectives and efficient breeding schemes for the use of transgenic parents requires knowledge of their characteristics. The probability of recovering lines with altered performance for unselected traits will affect decisions on the number of individual transgenic lines that should be screened to identify suitable parents, the nature of the breeding process that should be used, and the potential utility of transgenic cultivars in the marketplace. If the effects of SCV or transgene expression are small or occur in only a small portion of the population, identifying a suitable line may be merely a matter of screening a sufficiently large number of transgenic lines. On the other hand, widespread existence of SCV in a transgenic population would require hybridization and selection to separate variant alleles from the transgene, a process that might require multiple cycles of hybridization and selection within large populations. Horvath et al. (2001) showed both the potential and the limitations of the latter approach. They were able to improve the yield and 1000-kernel weight of transgenic barley lines expressing a heat stable β -glucanase by hybridization and selection for agronomic performance. However, null segregants from these crosses had higher 1000-kernel weight than transgenic segregants, suggesting that some of the performance reductions were attributable to the expression of the transgene and not SCV. If performance reductions are caused by the expression of the transgene itself and if such reductions are severe, then the goal of producing a cultivar for standard agricultural markets—in the absence of premium payments based on the value of the transgene-encoded trait—may not be feasible.

The study reported here was conducted as part of a two-phase evaluation of the agronomic performance and grain quality of transgenic wheat lines containing

bar only or *bar* plus transgenes encoding native or recombinant HMW-GS. The selectable marker *bar* encodes phosphinothricin acetyl transferase, enabling herbicide-based identification of transgenic cells and plants using phosphinothricin (glufosinate) (De Block et al., 1987). HMW-GS are seed storage proteins found only in endosperm tissue of wheat and they typically comprise 5 to 10% of flour protein (Shewry et al., 1992). This paper reports the agronomic performance of 54 such transgenic lines—as measured in replicated small-plot trials grown in California and Idaho—relative to the performance of the wild-type parental control, and relative to 10 transgene-null plant populations that segregated from the first and second generation of nine of the transgenic events.

MATERIALS AND METHODS

Transformation Plasmids

The six different plasmids diagrammed in Fig. 1 and carrying HMW-GS coding regions under control of their native promoter and transcription termination sequences were used in transformation experiments. For simplicity, the plasmids are referenced in other sections and in the figures with a short designation of the HMW-GS they encode: Dx5 for pK+Dx5B; Dy10 for pK-Dy10A; Ax2* for pK-Ax2E; Hybrid for pGlu10H5; LongDx5 for pGlu10/5-Dx5-853; and ShortDx5 for pGlu10/5-Dx5-441 (Fig. 1).

The complete sequences of plasmids pK+Dx5B (accession number X12928), pK-Dy10A (accession number X12929), and pK-Ax2E (accession number M22208) have been published (Anderson et al., 2002). They are pBluescript (Stratagene, La Jolla, CA) clones of the native genomic DNA *EcoRI* fragments derived from the hard red winter wheat 'Cheyenne' that contain the *GluD1-1*, *GluD1-2*, and *GluA1-1* genes, respectively, encoding HMW-GS Dx5, Dy10, and Ax2*, respectively.

Fig. 1. Diagrammatic representation of the high molecular weight glutenin subunit (HMW-GS) DNA sequences introduced by genetic transformation. Thickest boxes are coding regions, medium boxes are 5' flanking regions including promoters, and thin boxes are 3' flanking regions including transcription termination and polyA addition sites. Sequences from the native wheat gene *GluD1-2* (Dy10) are in black, native gene *GluD1-1* (Dx5) are in white, and native gene *GluA1-1* (Ax2*) are in diagonal stripes. The coding sequences are divided into three regions corresponding to HMW-GS protein domains: the short N-terminal nonrepetitive region, the long repetitive region, and the short C-terminal nonrepetitive region shared by all HMW-GS. Shorthand names used in this report for the HMW-GS are to the left. (The pBluescript plasmid backbones are not shown.)

The other three plasmids contain recombinant HMW-GS coding sequences flanked, on the 5' side, by 2.8 kb of DNA from the region upstream of the native Dy10 start codon, including the Dy10 promoter, and, on the 3' side, by 2 kb of DNA from the 3' region of the native Dx5 gene including its transcription terminator. pGlu10H5 has been described previously (Blechl and Anderson, 1996) and encodes a hybrid subunit consisting of the N-terminal nonrepetitive region from Dy10 fused to the repetitive and C-terminal regions of Dx5. Plasmids pGlu10/5-Dx5-441 and pGlu10/5-Dx5-853 have not been described previously; they contain the coding sequences constructed by D'Ovidio et al. (1997) for length variants of Dx5 with repetitive regions 441 amino acids (short Dx5) and 853 amino acids (long Dx5), compared to the native Dx5 subunit, which has a repetitive domain of 696 amino acids. All plasmids have the pBluescript backbone (Stratagene).

To enable herbicide-based selection of transformants, plasmids pAHC20 (Christensen and Quail, 1996) or pUBI:BAR (Cornejo et al., 1993), were introduced in conjunction with one or two of the HMW-GS plasmids described above. Both of the former plasmids contain the herbicide resistance gene *bar* (De Block et al., 1987) under control of the maize *Ubi1* promoter and first intron. Transgenic wheat lines containing only the pUBI:BAR plasmid (with no changes in HMW-GS content) are called BAR in this report.

Generation and Identification of Primary Transformants

The wheat lines that are the subject of this paper resulted from 15 different bombardment experiments. Transformation was achieved by particle bombardment of immature embryos of the hard white spring wheat Bobwhite using methods described previously (Weeks et al., 1993; Okubara et al., 2002). One or two HMW-GS encoding plasmids in two- or three-fold molar excess were co-bombarded with one of the selection plasmids, pAHC20 or pUBI:BAR. Stable transformants were selected by regeneration in the presence of 3 mg L⁻¹ bialaphos {4-[hydroxy(methyl)phosphinoyl]-L-homoalanyl-L-alanyl-L-alanine} (Okubara et al., 2002). Seeds of resistant plants were screened for expression of the HMW-GS-encoding plasmids by SDS-PAGE of endosperm proteins as described previously (Blechl and Anderson, 1996) or by using Nu-PAGE Novex Bis-Tris 4 to 12% gradient gels and MES SDS running buffer, as described by the manufacturer (Invitrogen, Carlsbad, CA).

Establishment of Transgenic Wheat Lines

Each line selected for study originated from a unique transformation event, except for LongDx5-E tall and short, two lines derived from a unique event by phenotypic selection in the field (see Results). SDS-PAGE of endosperm proteins was used to identify homozygous progeny of plants that showed altered expression of HMW-GS as a result of the HMW-GS transgene(s), based on the observation of stable and uniform expression alterations among individual plants within a line for two consecutive generations. In early generations, lines were advanced by single seed descent. The final derivation of lines was made by bulking seed from the 20 to 25 progeny of homozygous plants expressing the transgene. Nine lines were derived in the T₂ generation, 26 in the T₃, 10 in the T₄, one in the T₅, two in the T₆, and one in the T₁₀ generation. Further generation advances were made via bulks as necessary to produce sufficient seed for testing. In a similar manner, four lines containing only *bar* were established as marker gene-only controls; these lines were derived in the T₂, T₃, and T₄ generations. The presence of *bar* was inferred from the ability of 3-

to 4-wk-old embryos to precociously germinate on media containing 3 mg L⁻¹ bialaphos (Weeks et al., 1993) and confirmed by PCR, as described in the next section.

Seed for the 2002 field trials was hand harvested from single row plots grown at the Aberdeen location in 2000 and 2001. At this point, the transgenic HMW-GS and *bar* lines planted in the 2002 field trial had undergone between 3 and 12 sexual self-pollination generations after regeneration; most were in the T₅ to T₉ generations. The same seed was also used to plant single-row increase plots in 2002, which were hand harvested and used to plant the 2003 trials.

Establishment of Null (Nontransgenic) Segregant Control Lines

For nine different transgenic events, DNA was isolated from T₁ or T₂ plants as described by Dellaporta et al. (1983) and screened by polymerase chain reaction (PCR) for the absence of *bar* and HMW-GS transgenes, as described by Okubara et al. (2002). To distinguish between endogenous and transgenic HMW-GS sequences, the following primers were designed to amplify a 375-bp fragment that spans the junction between the wheat genomic sequences downstream of the *Glu-D1-1* gene and the pBluescript plasmid backbone in the HMW-GS plasmids: forward primer 5'CGACGCTGATTGGTAC3' and reverse primer 5'CCCTATCTCGGTCTATTC3'. Amplification by Taq polymerase (PerkinElmer, Foster City, California or Promega Corporation, Madison, WI) was conducted in an MJ Research PTC-200 (Waltham, MA) Peltier thermal cycler for 36 cycles with an annealing temperature of 52°C. Primers used for the detection of *bar* amplified a 446-bp fragment that spans the junction of the *Ubi1* promoter and the *bar* coding region: forward primer 5'CCTGCCTTCATACGCTATTTA-TTTC3' and reverse primer 5'CTTCAGCAGGTGGGTGTAGAGCGTG3'. Amplification was conducted as above except the annealing temperature was 62°C. Seeds from 10 to 12 transgene-negative plants segregating from each initial transformation event were harvested, pooled, and planted in the greenhouse for increase. Greenhouse seedling assays for sensitivity to glufosinate-ammonium applied as a spray were conducted as additional confirmation of the absence of *bar*. The progeny of these plants were grown at the Aberdeen field location in 2001 for increase, and tested in 2002 replicated trials as T₀-derived T₃ or T₁-derived T₄ populations. This seed was also used to plant single-row increase plots in 2002, which were hand harvested and used to plant the 2003 trials. The lines are named by the event from which they were derived followed by "null."

Agronomic Evaluations

All transgenic and null segregant lines and the wild-type Bobwhite control were grown under irrigation using prevailing agronomic practices for small plot evaluations of spring wheat at Aberdeen, ID, and Davis, CA (2002 and 2003), and at El Centro, CA (2002). Planting dates for Aberdeen were 8 Apr. 2002 and 1 Apr. 2003; for Davis, 24 Jan. 2002 and 7 Feb. 2003; and for El Centro, 15 Jan. 2002. All locations utilized a randomized complete block experimental design with four replicates. Data was collected for all replicates, except for protein contents at Aberdeen in 2003 where two composite samples (reps 1 and 2, 3, and 4) were analyzed per line. Grain was milled to pass through a 1.0-mm screen and protein determinations were made using a Dickey John Instalab 600 NIR analyzer (Auburn, IL) as specified by the manufacturer.

At Aberdeen, plots consisted of seven rows 2.4 m in length on 17.8-cm centers. Plots were arranged in multiple ranges of

side-by-side plots, with approximately 36 cm between adjacent plots and with 1.2-m alleyways separating each range. Similar field designs were employed at Davis and El Centro, except that plot lengths varied from approximately 1.6 to 2.4 m at Davis, and the alleyways at El Centro were 0.9 m. Soil tests at all three locations showed sufficient levels of P and K. At Aberdeen preplant soil N levels were adequate, so no additional application of fertilizer was made. At Davis, N was applied in the form of ammonium nitrate at, respectively for 2002 and 2003, 112 kg ha⁻¹ preplant + 112 kg ha⁻¹ at tillering, and 45 kg ha⁻¹ preplant + 112 kg ha⁻¹ at tillering. At El Centro, urea (30 kg ha⁻¹) and P₂O₅ (30 kg ha⁻¹) were applied preplant, and anhydrous ammonia (50 kg ha⁻¹) was applied just before heading. Herbicide applications were made as follows: Aberdeen, Bronate (3,5-dibromo-4-hydroxybenzonitrile plus 2-methyl-4-chlorophenoxyacetic acid) at 0.3 L ha⁻¹; Davis, MCPA (2-methyl-4-chlorophenoxyacetic acid) at 0.2 L ha⁻¹; El Centro, Butril (3,5-dibromo-4-hydroxybenzonitrile) 0.3 L ha⁻¹; and Carbyne (M-chloro, 4-chloro-2-butyryl ester) at 0.3 L ha⁻¹. Sprinkler irrigation was provided as needed based on crop usage and weather conditions. Plots were harvested using small-plot combines.

Statistical Analyses

All data were analyzed using SAS v. 8.0 Proc GLM (SAS Institute Inc., Cary, NC). For the Aberdeen and Davis locations, the model used to examine line performance data included year, rep(year), line, and line × year as sources of variance. Rep(year), year, and line × year were considered random. Line × year was used as the error term for all line comparisons. For the El Centro location, where there was only 1 yr of data, the model included rep and line as sources of variance. To identify lines with differences from the Bobwhite wild-type control, comparisons of least-squared means of line performance were examined via the Dunnett's test. Specific comparisons of individual lines were made via single degree of freedom contrasts constructed using the lsmeans/pdiff option.

RESULTS

Fifty-four independent transformation events propagated as homozygous, advanced generation lines were included in these agronomic evaluations. In addition, 10 null segregant lines, derived from nine of the transgenic events, were evaluated. For the purposes of this paper, the lines are named by an abbreviation for the added HMW-GS-encoding plasmid, or BAR, (Fig. 1, Materials and Methods) followed by a unique event letter. The four transgenic lines containing only *bar* exhibited no changes in HMW-GS gene or protein composition (e.g., lane BAR of Fig. 2C) and expressed *bar*, as determined by their ability to germinate in the presence of 3 mg L⁻¹ bialaphos (Weeks et al., 1993) and by greenhouse assays for seedling resistance to glufosinate-ammonium applied as a 0.05% spray (data not shown).

The 49 lines containing added native or recombinant HMW-GS transgenes exhibited a wide range of glutenin subunit compositions as assessed by SDS-PAGE (Fig. 2). The nontransformed parental cultivar, Bobwhite (C lanes in Fig. 2), contains HMW-GS Ax2*, Bx7, By9, Dx5, and Dy10. The HMW-GS gene transformants comprise one line transformed with the native gene encoding Ax2* (Lane 1, Fig. 2A); 12 lines transformed

with the native gene encoding Dx5 (e.g., Lanes 4 and 5, Fig. 2A); six lines transformed with the native gene encoding Dy10 (e.g., Lanes 2 and 3, Fig. 2A); seven lines cotransformed with two separate plasmids containing native genes coding for Dx5 and Dy10 (e.g., Lanes 6, 7, and 8, Fig. 2A); nine lines transformed with a recombinant gene encoding a hybrid subunit between Dy10 and Dx5 (e.g., Lanes 1 and 2, Fig. 2B) (Blechl and Anderson, 1996); eight lines transformed with a recombinant gene encoding a Dx5 variant subunit with a longer repeat region (e.g., Lanes 3 and 4, Fig. 2B) (D'Ovidio et al., 1997); and seven lines transformed with a recombinant gene encoding a Dx5 variant subunit with a shorter repeat region (e.g., Lanes 5 and 6, Fig. 2B) (D'Ovidio et al., 1997).

In most of the lines, accumulation of the transgene-encoded HMW-GS was additive with that of the native HMW-GS (Fig. 2A, 2B). Ten of the transgenic lines exhibited some degree of transgene-mediated cosuppression as evidenced by decreased accumulation of all the native HMW-GS (examples in Fig. 2C). Clear differences in the expression levels were evident among lines transformed with the same construct. The examples (Fig. 2A, 2B) illustrate typical ranges from low to high expressers for various transgene-encoded HMW-GSs. In general, the larger subunits showed lower accumulation than the smaller subunits. For example, the HMW-GS levels were similar for the highest expressing LongDx5 line (Lane 4 of Fig. 2B) and the lowest expressing ShortDx5 line (Lane 5 of Fig. 2B).

Visual observations during field growth revealed that the majority of the transgenic wheat lines were similar to Bobwhite, although often their appearance was less uniform. The nonuniformity was generally difficult to quantify and not always noticeable in all plots of a given line. Notable exceptions included the lines LongDx5-E, BAR-D, and Dx5-L. Line BAR-D had a distinctly yellow-green appearance relative to the darker green of Bobwhite; the difference was particularly noticeable before anthesis. The leaves of line Dx5-L were relatively narrow and often twisted. In addition, during the seed increases before the tests reported here, line LongDx5-E was clearly segregating for short and tall plants, and selections of multiple short and tall variants of this line were made. These selections were bulked and tested as separate entries in the trials in Davis and Aberdeen, where they were designated "LongDx5-E short" and "LongDx5-E tall." There was insufficient seed to evaluate these lines at the El Centro location. Several of the lines were significantly different from Bobwhite for heading date or plant height at one or more locations (see below).

Initial examinations of the data suggested that there were significant line by location interactions. Preliminary analyses of variance also showed line by location interactions to be a significant ($P < 0.05$) source of variability. Therefore, the data from each location were analyzed separately as described in the Materials and Methods. Line × year interactions were not significant at either the Aberdeen or Davis locations; the line means for each location are presented in Tables 1–3.

Fig. 2. SDS-PAGE of seed proteins in transgenic wheat lines (numbered lanes) and their nontransgenic parent Bobwhite (C). Arrowheads to the left of the individual lanes indicate expected locations of bands corresponding to transgene-encoded high molecular weight glutenin subunit (HMW-GS). The gel positions and names of the HMW-GS native to Bobwhite are indicated to the right. (A) Numbered lanes contain protein extracts from greenhouse-grown seeds from homozygous transgenic wheat lines expressing transgene-encoded native HMW-GS: T₃ Ax2*-A (1), T₃ Dy10-A (2), T₆ Dy10-E (3), T₅ Dx5-G (4), T₅ Dx5-J (5), T₇ Dx5+Dy10-A (6), T₇ Dx5+Dy10-D (7), and T₇ Dx5+Dy10-G (8). (B) Numbered lanes contain protein extracts from greenhouse-grown seeds from homozygous transgenic wheat lines expressing transgene-encoded recombinant HMW-GS: T₁₁ Hybrid-C (1), T₁₂ Hybrid-G (2), T₆ LongDx5-C (3), T₅ LongDx5-G (4), T₄ ShortDx5-A (5), and T₈ ShortDx5-D (6). (C) Numbered lanes contain protein extracts from greenhouse-grown—except Hybrid-B, which was field-grown—seeds from homozygous transgenic wheat lines exhibiting transgene-mediated cosuppression of HMW-GS: T₉ Hybrid-A (1), T₅ LongDx5-A (2), T₄ Dx5-D (3), T₅ Dx5-A (4), and T₇ Hybrid-B (5). Lane BAR contains T₁₁ seed extract from BAR-D.

Measurements of heading date, plant height, grain yield, grain test weight, and 100-kernel weight (Tables 1–3) showed that most of the HMW-GS transgenic lines did not show significant differences from Bobwhite as determined by Dunnett's comparisons. For heading date and plant height, a small fraction of the lines showed delayed maturity and were shorter than Bobwhite. Four lines—Dx5+Dy10-A, Dx5+Dy10-E, Hybrid-E,

and LongDx5-E short—were significantly shorter than Bobwhite at both locations where they were measured (Tables 1 and 2). The protein content of grain grown in Aberdeen in 2003 was significantly increased for five lines (Table 2).

More examples of substantial deviation from Bobwhite performance were noted for yield, test weight, and 100-kernel weight. Still, lines showing significant differ-

Table 1. Agronomic performance of transgenic wheat lines, null segregants, and Bobwhite at Aberdeen, 2002 and 2003.

Line	Heading date (d after 1 January)	Plant height	Grain yield	Grain protein†	Grain test weight	100 kernel weight
		cm	kg ha ⁻¹	%	kg m ⁻³	g
Dy10-B	170.9	89	6688	12.8	794	3.63
ShortDx5-B	170.8	84	6624	12.8	789	3.49
Dy10-D	170.8	89	6597	12.9	794	3.73
ShortDx5-D null	170.1	87	6597	13.0	787	3.45
ShortDx5-A	170.4	86	6490	12.4	791	3.49
Bobwhite	170.6	85	6486	12.5	792	3.60
LongDx5-B null	169.8	85	6476	12.9	796	3.53
Dx5+Dy10-F	171.1	84	6475	12.1	791	3.25*
LongDx5-D	170.1	88	6375	12.3	790	3.65
BAR-D	169.5	82	6346	13.3	793	3.58
Dx5+Dy10-C null	170.0	84	6346	12.6	788	3.35
Dy10-A	171.3	88	6281	12.7	798	3.64
Dx5-J	170.4	89	6263	12.2	794	3.50
Dx5+Dy10-G	170.5	83	6255	12.7	781	3.50
Dx5-G	170.6	85	6247	13.0	793	3.61
LongDx5-F	168.9	85	6229	12.4	793	3.54
LongDx5-F null	169.1	85	6226	13.0	795	3.46
LongDx5-H null	170.9	85	6217	13.0	793	3.46
Dx5+Dy10-B	170.9	89	6213	12.6	791	3.64
ShortDx5-G	171.5	84	6168	13.3	787	3.39
ShortDx5-D	171.9	86	6164	12.6	783	3.43
LongDx5-E tall	171.9	84	6130	12.9	788	3.40
ShortDx5-C null	170.1	82	6113	13.8	792	3.41
ShortDx5-E	171.8	84	6110	13.0	781	3.39
ShortDx5-H null	171.4	82	6082	13.9	792	3.51
Dx5-C‡	169.6	87	6058	12.2	792	3.59
LongDx5-A‡	170.1	85	6043	13.8	786	3.68
Hybrid-B null1	170.8	85	6023	13.0	790	3.59
Dx5-B‡	171.0	84	5985	12.6	786	3.53
Dx5-A‡	170.1	86	5984	12.6	791	3.56
BAR-C	170.9	85	5983	13.0	793	3.45
LongDx5-I null	168.8	83	5982	12.9	792	3.56
Hybrid-B null2	171.1	88	5974	13.1	802	3.64
Ax2*-A	169.3	86	5959	13.0	790	3.48
Dy10-C	169.8	84	5869	13.4	794	3.41
LongDx5-G	169.5	89	5840	12.4	789	3.56
Dy10-F	170.3	86	5839	12.7	791	3.48
Dy10-E	170.4	89	5837	13.5	784	3.46
Hybrid-G	170.6	88	5783	12.8	787	3.65
LongDx5-B	170.3	85	5783	12.7	797	3.33
ShortDx5-C	170.1	84	5727	12.9	789	3.33
BAR-A	170.3	86	5704	12.7	794	3.69
Dx5-K	169.6	80	5679	13.4	768**	3.26
BAR-B	169.8	73***	5659	12.9	774	3.69
Dx5+Dy10-D	172.5	83	5642	12.8	792	3.43
Hybrid-F	171.1	86	5579	12.9	785	3.59
Hybrid-E	170.0	72***	5535	12.8	783	3.34
Dx5-I	172.3	83	5389	12.5	787	3.23*
Hybrid-A‡	170.3	73***	5339	12.2	785	3.38
Hybrid-D	171.5	83	5321	12.7	780	3.50
Dx5-H	171.5	84	5165*	13.8	782	3.34
Dx5+Dy10-A	170.6	64***	5165*	13.3	786	3.48
Hybrid-H	170.0	79	5121**	12.7	792	3.58
Hybrid-I	170.4	81	5101**	13.6	782	3.34
Dx5-F‡	172.6	76*	5051**	14.4*	791	3.13**
Dx5-D‡	173.3	83	4983**	12.5	776	3.38
Dx5-E‡	170.9	76*	4896**	14.4*	795	3.38
ShortDx5-F	172.0	81	4821***	13.4	733***	2.99***
Hybrid-B‡	174.4***	81	4706***	13.6	733***	3.05***
Dx5+Dy10-C	173.3	83	4592***	13.6	787	3.48
LongDx5-C	173.3	87	4509***	14.6*	770**	3.39
Hybrid-C	171.9	82	3902***	13.8	767***	2.95***
Dx5-L‡	174.0**	77	3526***	14.4*	773*	3.15**
LongDx5-E short short	173.9*	59***	3468***	14.6*	774	3.33
Dx5+Dy10-E	175.4***	68***	3362***	14.1	782	2.88***

* Significant at $P = 0.05$ as determined by Dunnett's comparisons to Bobwhite.** Significant at $P = 0.01$ as determined by Dunnett's comparisons to Bobwhite.*** Significant at $P = 0.001$ as determined by Dunnett's comparisons to Bobwhite.

† 2003 samples only.

‡ Lines exhibiting transgene-mediated cosuppression of HMW-GS genes.

ences from Bobwhite were in the minority. For these traits measured at Aberdeen and Davis (Tables 1 and 2, respectively), all lines with significant differences from the control showed reduced performance. Furthermore, despite the relatively few instances of significant dif-

ferences between individual HMW-GS transgenic lines and Bobwhite, their average performance as a group showed a trend toward lower performance (see yield summaries in Table 4). Although the five lines with significantly increased grain protein at Aberdeen also

Table 2. Agronomic performance of transgenic wheat lines, null segregants, and Bobwhite in Davis, 2002 and 2003.

Line	Heading date (d after Jan. 1)	Plant height	Grain yield	100 kernel weight
		cm	kg ha ⁻¹	g
Ax2*-A	129.3	85	2757	3.11
LongDx5-G	128.5	87	2651	3.30
Hybrid-G	125.8	87	2602	3.25
ShortDx5-C null	127.0	84	2599	3.31
Dx5-G	126.9	84	2569	3.29
Dx5+Dy10-C null	128.3	86	2547	3.25
Dx5+Dy10-D	131.4	84	2510	3.31
LongDx5-D	128.3	84	2506	3.51
BAR-D	128.3	84	2485	3.31
Bobwhite	128.1	84	2481	3.34
Dy10-B	129.0	82	2463	3.55
Dy10-A	128.4	84	2418	3.61
Dy10-D	129.4	84	2411	3.42
LongDx5-F	128.1	84	2405	3.25
ShortDx5-D null	129.1	84	2399	3.09
Dy10-E	130.0	82	2388	3.28
Dx5+Dy10-B	128.3	86	2368	3.50
Dx5+Dy10-F	128.5	83	2343	3.29
Hybrid-B null-2	128.1	85	2333	3.10
Dx5-I	128.1	82	2328	3.16
ShortDx5-A	127.8	84	2283	3.22
Hybrid-B null-1	127.9	84	2281	3.35
Dy10-C	127.5	84	2277	3.32
ShortDx5-C	128.4	82	2250	3.18
Dx5+Dy10-G	129.3	82	2184	3.44
ShortDx5-B	127.1	82	2174	3.38
Dx5-A†	127.5	85	2165	3.20
Dx5-J	126.5	84	2155	3.34
Dx5-D†	131.9	83	2085	3.19
BAR-A	129.0	85	2074	3.31
LongDx5-H null	127.4	84	2064	3.41
Dx5-C	127.1	83	2043	3.34
ShortDx5-G	127.8	81	2027	3.02
LongDx5-E tall	126.4	81	2025	3.14
Hybrid-F	126.3	83	2019	3.41
Dx5-B†	129.0	83	1961	3.09
ShortDx5-D	130.0	83	1959	3.11
Dx5-E†	127.4	81	1934	3.10
ShortDx5-H null	128.4	83	1928	3.21
LongDx5-F-null	129.0	85	1927	3.19
LongDx5-I null	127.9	83	1905	3.34
BAR-C	125.4	81	1841	3.24
Dy10-F	127.3	84	1827	3.23
LongDx5-A†	128.9	84	1817	3.26
LongDx5-B	129.3	83	1784	3.07
ShortDx5-E	130.0	80	1784	3.04
LongDx5-B null	127.4	83	1780	3.20
BAR-B	124.3	77	1775	3.42
Hybrid-H	125.5	81	1700	3.43
Hybrid-A†	128.8	73	1694	2.91*
LongDx5-C	131.9	82	1680	3.10
Dx5-F†	129.9	77	1604	3.10
Hybrid-E	128.1	70*	1600	3.21
Dx5-H	129.3	82	1595	3.03
Hybrid-I	124.5	80	1522	3.12
Hybrid-D	126.5	81	1482	3.14
Dx5-K	128.4	81	1481	3.14
Dx5+Dy10-C	129.5	83	1441	3.19
Hybrid-C	131.6	81	1384*	3.01
ShortDx5-F	130.6	77	1262**	2.75***
Dx5-L†	132.8	74	1042***	2.98
Hybrid-B†	132.4	77	1007***	2.73***
Dx5+Dy10-A	126.6	58***	973***	3.38
LongDx5-E short short	130.0	69*	914***	3.11
Dx5+Dy10-E	132.9	62***	598***	2.90

* Significant at $P = 0.05$ as determined by Dunnett's comparisons to Bobwhite.

** Significant at $P = 0.01$ as determined by Dunnett's comparisons to Bobwhite.

*** Significant at $P = 0.001$ as determined by Dunnett's comparisons to Bobwhite.

† Lines exhibiting transgene-mediated cosuppression of HMW-GS genes.

Table 3. Agronomic performance of transgenic wheat lines, null segregants, and Bobwhite in El Centro, 2002.

Line	Grain yield	Grain test weight
	kg ha ⁻¹	kg m ⁻³
Dy10-F	11493	788*
Dx5-C†	11365	772
Dy10-B	11238	766
ShortDx5-B	11202	766
ShortDx5-D null	11078	756
Dx5+Dy10-C null	11006	766
Dy10-A	10974	769
ShortDx5-H null	10960	788*
LongDx5-I null	10735	775
ShortDx5-D	10683	756
LongDx5-F	10651	769
Dy10-C	10535	785*
Dx5-E†	10507	788*
BAR-D	10450	763
LongDx5-D	10436	766
ShortDx5-G	10399	759
ShortDx5-A	10359	769
Dx5+Dy10-G	10199	759
LongDx5-A†	10184	769
LongDx5-B null	10126	766
ShortDx5-C	10119	772
Dy10-E	10105	775
BAR-A	10095	775
LongDx5-F null	10095	775
Dx5+Dy10-D	10084	779
BAR-B	10055	750
Dx5+Dy10-F	10025	769
LongDx5-H null	9983	759
LongDx5-G	9959	775
Bobwhite	9936	746
Dx5-J	9934	769
Dx5+Dy10-B	9916	753
Dx5+Dy10-A	9842	756
Dy10-D	9795	763
Hybrid-F	9778	766
ShortDx5-C null	9771	766
Ax2*-A	9747	779
Dx5-A†	9699	759
Hybrid-H	9696	779
BAR-C	9684	769
Hybrid-A†	9598	775
Dx5-K	9464	763
Hybrid-I	9457	756
Dx5-B†	9428	750
Dx5-G	9391	753
Hybrid-D	9356	763
LongDx5-B	9356	772
Hybrid-B null-2	9323	766
ShortDx5-E	9259	763
Hybrid-G	9187	766
Hybrid-B null-1	9133	763
Dx5-F†	9126	782
LongDx5-C	8582	769
Dx5-H	8486	759
Dx5-D†	8427	763
Dx5-I	8162	753
Hybrid-E	7760*	730
ShortDx5-F	7651*	727
Dx5+Dy10-C	7592**	775
Hybrid-B†	7188**	676***
Dx5+Dy10-E	7075***	763
Dx5-L†	6533***	753
Hybrid-C	6307***	766

* Significant at $P = 0.05$ as determined by Dunnett's comparisons to Bobwhite.

** Significant at $P = 0.01$ as determined by Dunnett's comparisons to Bobwhite.

*** Significant at $P = 0.001$ as determined by Dunnett's comparisons to Bobwhite.

† Lines exhibiting transgene-mediated co-suppression of HMW-GS genes.

had significant reductions in yield, other lines with decreased yield did not have significant increases in grain protein, and no general relationship between grain protein content and grain yield could be detected. At El

Table 4. Summary of grain yields for Bobwhite and transgenic wheat lines containing high molecular weight glutenin subunit (HMW-GS) and/or UBI:BAR transgenes.

Plasmid class	No. lines evaluated (no. showing cosuppression)	Mean yield at Aberdeen	No. lines with significant yield reductions at Aberdeen†	Mean grain yield at Davis	No. lines with significant yield reductions at Davis†	Mean yield at El Centro	No. lines with significant yield reductions at El Centro†	No. lines with significant yield reductions at all locations†
		kg ha ⁻¹		kg ha ⁻¹		kg ha ⁻¹		
Dx5	12 (7)	5436	5	1914	1	9210	1	1
Dy10	6 (0)	6185	0	2297	0	10690	0	0
Dx5 + Dy10	7 (0)	5386	3	1774	2	9248	2	1
Long Dx5	8 (1)‡	5547	2	1973	1	9861	0‡	0‡
Short Dx5	7 (0)	6015	1	1963	1	9953	1	1
Hybrid	9 (2)	5154	4	1668	2	8703	3	2
Ax2*	1 (0)	5959	0	2757	0	9747	0	0
All HMW-GS transgenic lines	50 (10)	5688	15	1975	7	9630	7	5
BAR	4 (0)	5923	0	2044	0	10071	0	0
Null	10	6204	0	2183	0	10221	0	0
Bobwhite	–	6486	–	2481	–	9936	–	–

† Significantly different from Bobwhite grain yields at $P < 0.05$ level.

‡ Only seven lines were evaluated at El Centro. The tall and short sublines of LongDx5-E were not grown. The latter had significantly lower yields at both Aberdeen and Davis.

Centro, all lines that were significantly different from Bobwhite for yield showed reductions (Table 3). However, when considered as a group, the yields of transgenic lines showed a relatively normal distribution about the mean of Bobwhite, and their mean yield was more similar to Bobwhite than at the other locations (Table 4). There was an upward trend for test weight relative to Bobwhite, and four of the five lines significantly different in test weight from Bobwhite had higher values (Table 3).

Deviations from control performance may have been caused by altered HMW-GS expression patterns, *bar* expression, or SCV. Since our primary interest was to determine the effects of altered HMW-GS expression, several controls were used to estimate the relative contributions of these other potential sources of variability. To assess the effects of *bar* expression in the absence of HMW-GS transgenes, comparisons were made of the four BAR lines to Bobwhite. None showed significant differences in any of the evaluated traits, although deviations from the mean yields of Bobwhite were skewed downward at two locations. At Aberdeen, Davis, and El Centro, respectively, the mean yields for the BAR lines were 91, 82, and 101% of Bobwhite (Table 4). Although these data might indicate a negative effect of *bar* expression that did not reach statistical significance in this study, further evidence that *bar* expression had negligible effects on performance came from a comparison of yield data and *bar* expression data from the 49 HMW-GS lines. While all contained the *bar* gene, approximately one-quarter were sensitive to glufosinate-ammonium applied as a spray (0.05% active ingredient) (data not shown). No correlation between resistance or sensitivity and yield could be detected (data not shown).

To assess the effect of SCV in the absence of transgenes, various comparisons were made of null segregant lines, transgenic lines, and Bobwhite. Since SCV might be only partially heritable, leading to increased performance during generation advance, the data were examined for relationships between the grain yield and the generation of line derivation and/or line testing, and none were found (data not shown). A more powerful

approach to determine the impact of SCV was to assess the performance of null segregant controls relative to Bobwhite. Because of their early generation derivations, each null segregant line was relatively heterogeneous and the genetic variability caused by SCV present in their original T_0 parent was well represented and expected to be manifested as performance reductions relative to the Bobwhite control. This requires the assumption—which is reasonable based on previous studies of SCV—that SCV will consist of predominantly random genetic changes that are more likely to reduce than they are to increase performance. As seen for the UBI:BAR lines, none of the null segregants showed differences from Bobwhite for any measured trait either individually (Tables 1–3) or as a group (data not shown), as examined by Dunnett's comparisons ($P < 0.05$). Because the heterogeneity of the null segregant lines, it is possible that significant SCV-caused performance reductions could have occurred in a small percentage of plants within any given null segregant line, a situation which might not reduce the overall performance of that line enough to lead to significant yield reductions. Similar (but slightly less strong) overall trends were observed as for the UBI:BAR lines: lower average performance at Aberdeen (96% of Bobwhite) and at Davis (88% of Bobwhite), but not at El Centro (103% of Bobwhite) (Table 4). Overall, except for the short variant of Long-Dx5E, these data indicate that SCV had little impact on agronomic performance.

The data were examined to evaluate the effects of particular HMW-GS transgenes and/or their expression levels on agronomic traits. An examination of the mean performance of lines for each of the HMW-GS transgenes showed that there may have been an association with some of the transgenes and reduced yield (Table 4). However, perusal of the data (Tables 1–3) clearly shows large line $\times$ plasmid interactions, and divergent performance among lines for each plasmid class (except for plasmid Ax2* for which there was only one tested line). One possible exception may have been for transgenic lines containing the hybrid glutenin transgene, which showed relatively poor mean yields and few instances of

Fig. 3. SDS-PAGE of seed proteins in the lower-yielding transgenic wheat lines (numbered lanes) and their nontransgenic parent Bobwhite (C). The gel positions and names of the high molecular weight glutenin subunit (HMW-GS) native to Bobwhite are indicated to the left. Numbered lanes contain protein extracts from field-grown seeds from homozygous transgenic wheat lines T₁₁ Hybrid-C (1), T₇ Hybrid-B (2), T₆ Dx5 + Dy10-E (3), T₅ Dx5-L (4), T₅ ShortDx5-F (5), T₈ LongDx5-E short (6), and T₅ Dx5 + Dy10-C (7). Arrowheads to the right of the individual lanes indicate expected locations of bands corresponding to transgene-encoded HMW-GS. Open circles to the right of individual lanes mark locations of bands of unexpected sizes and unknown identity.

lines ranked in the top 50% for yield. Even so, only two lines containing the hybrid glutenin transgene showed consistently significant differences from Bobwhite at all locations. These data suggest that unidentified genetic changes that were associated with specific transformation events, rather than the expression of specific HMW-GS transgenes, were the primary cause of reductions in agronomic performance.

This conclusion is further supported by HMW-GS expression data, which generally showed no consistent relationships between yield (based on means of all locations and years) and the extent of HMW-GS alterations. For example, in comparisons among lines transformed with the same plasmid, lines with relatively larger HMW-GS deviations from Bobwhite such as Dx5-J (Fig. 2A, lane 5) and Long Dx5-G (Fig. 2B, lane 4) had mean yields (6117 and 6150 kg ha⁻¹, respectively) that were more similar to Bobwhite (6301 kg ha⁻¹) than were lines with relatively smaller HMW-GS alterations such as Dx5-G (Fig. 2A, lane 4; 6069 kg ha⁻¹) and Long Dx5-C (Fig. 2B, lane 3; 4924 kg ha⁻¹). For the Dx5 example, neither of the cited lines showed significant yield differences from Bobwhite despite divergent HMW-GS expression patterns, a situation that was seen in other cases such as Short Dx5-A vs. Short Dx5-D (Fig. 2B, lanes 5 and 6). Most importantly, however, was that the lines that were shown to have significant yield reductions did not have a consistent pattern of alterations in HMW-GS expression. For instance, the lowest yielding lines (Fig. 3, Tables 1–3) includes those transformed with five different classes of HMW-GS genes. Comparisons of the banding patterns shown in Fig. 3 with those shown in Fig. 2 show that these lines share HMW-GS expression patterns with lines with higher yields (e.g., both low and high yielding lines show cosuppression) or that they do not have highest additive accumulation of transgenic HWM-GS for their respective plasmid class (e.g., Hybrid-C, Dx5+Dy10-E). Furthermore, the short and tall lines selected from Long-Dx5E,

which had very different performance characteristics, had identical HMW-GS compositions (data not shown). The low-yielding lines Short Dx5-F and Dx5+Dy10-C each contained a seed protein band with an unexpected size (lanes 5 and 7, respectively, of Fig. 3), but whether or not these novel proteins are related to the yield reductions is unknown.

The kernel traits we measured (test weight and kernel weight) might be influenced by changes in endosperm HMW-GS composition, but these traits were only significantly and consistently different from the control in two lines: ShortDx5-F at two locations and Hybrid-B at all locations. The transgene(s) in ShortDx5-F exhibits high levels of additive expression while that in Hybrid-B exhibits co-suppression (Fig. 3), so total quantity of HMW-GS does not appear to be a factor in determining kernel size, shape, or density.

DISCUSSION

The main objective of this study was to determine whether and to what extent wheat agronomic performance was affected by introduction and expression of HMW-GS transgenes. Controls were included to estimate the effects of other likely sources of variability, SCV, and effects of *bar* expression. Fifty-four transgenic and 10 null segregant wheat lines were evaluated in field trials in three locations over 2 yr. Most of these lines were not significantly different from Bobwhite for heading date, height, grain yield, grain protein content, test weight, or 100 kernel weight.

Of the traits measured, yield showed the greatest variability among lines, as might be expected because the expression of this trait depends on many others. As a group, the transgenic lines tended to yield less at Aberdeen and Davis than the nontransgenic controls (Bobwhite and null segregants). In contrast, at the El Centro location, the yields of the transgenic lines were more normally distributed about the mean of Bobwhite.

Nevertheless, the relative rankings for the performance of most of the lines were similar at all three locations. For the few lines that showed extreme line-rank $\times$ environment interactions for yield, their performance was not significantly different from Bobwhite at any of the locations, and thus one explanation for these apparent interactions may simply be experimental error. Alternatively, the source of these interactions may trace, at least in part, to transformation-induced genomic variability, either as a result of SCV or of transgene integration and/or expression. If the effects of such alterations were slight, their phenotypic manifestations might be negligible when the plants were grown under highly favorable conditions. El Centro may have been such an environment: every line had higher yields at El Centro than at either of the other two locations.

Five lines exhibited significant reductions in yield at all locations, and a sixth that was tested only at two locations showed substantial yield reductions at both locations, compared to their nontransformed parent. The sources of these differences could be SCV associated with the transformation process, the presence of *bar* and/or HMW-GS transgenes, and/or the expression of either or both types of transgenes. The performance of 10 null segregant lines and four lines containing only *bar* were not significantly different from Bobwhite, indicating that neither SCV nor the expression of *bar* was a significant source of variability. With the possible exception of the hybrid HMW-GS, no consistent associations could be found between agronomic performance and the type of transgenic HMW-GS plasmid nor with the level of HMW-GS expression alterations. Total grain protein content also appeared unrelated to agronomic performance, except for five low-yielding lines that had elevated protein contents. Such elevations may be related to the overall vigor of the plants, rather than a consequence of the HMW-GS alterations. Tissue culture-derived elevations in grain protein concentrations coupled with severe yield reductions have been observed for barley (Bregitzer et al., 1995). Thus, the data indicate that changes in agronomic performance likely trace primarily to other sources that are specific to individual transformation events (e.g., pleiotropic effects of integration site differences).

The tendency toward slightly reduced performance of transgenic lines overall, and the presence of lines with essentially unchanged performance, is in agreement with previous studies of transgenic wheat (Hanson et al., 1994; Barro et al., 2002; Zhou et al., 2003). The lack of evidence for significant SCV in the tested wheat lines stands in contrast with our experience with transgenic barley, where the phenotypic manifestations of SCV such as delayed maturity and yield reductions have been severe (Bregitzer et al., 1998, 2002). It is likely SCV occurs in bread wheat at a similar rate as has been shown for other species, but the polyploid, genetically redundant wheat genome likely buffers the effect and masks the presence of SCV. Thus, although it would be prudent to include hybridization and selection steps in the process of developing transgenic wheat cultivars, simple selection within transgenic populations may also be successful.

In conclusion, significant changes to the HMW-GS proteins were made via biolistic transformation techniques, and many of the transgenic lines performed well under a variety of environmental conditions. Thus, it would appear that significant alterations in the expression of major proteins involved with breadmaking quality are compatible with the development of agronomically competitive cultivars.

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