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# Nonadditive Expression of Homoeologous Genes Is Established Upon Polyploidization in Hexaploid Wheat

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ABSTRACT

Effects of polyploidy in allohexaploid wheat (*Triticum aestivum* L.) have primarily been ascribed to increases in coding sequence variation and potential to acquire new gene functions through mutation of redundant loci. However, regulatory variation that arises through new promoter and transcription factor combinations or epigenetic events may also contribute to the effects of polyploidization. In this study, gene expression was characterized in a synthetic *T. aestivum* line and the *T. turgidum* and *Aegilops tauschii* parents to establish a timeline for such regulatory changes and estimate the frequency of nonadditive expression of homoeologous transcripts in newly formed *T. aestivum*. Large-scale analysis of nonadditive gene expression was assayed by microarray expression experiments, where synthetic *T. aestivum* gene expression was compared to additive model values (mid-parent) calculated from parental *T. turgidum* and *Ae. tauschii* expression levels. Approximately 16% of genes were estimated to display nonadditive expression in synthetic *T. aestivum*. A certain fraction of the genes (2.9%) showed overdominance or underdominance. cDNA–single strand conformation polymorphism analysis was applied to measure expression of homoeologous transcripts and further verify microarray data. The results demonstrate that allopolyploidization, *per se*, results in rapid initiation of differential expression of homoeologous loci and nonadditive gene expression in *T. aestivum*.

THE prevalence of polyploidization events in angiosperm evolution (Masterson 1994) has raised many questions about the ecological and genetic consequences of genome doubling. The distribution of polyploid species often exceeds that of their diploid counterparts, suggesting that polyploidy may confer enhanced fitness (Stebbins 1950). Possible genetic mechanisms that may contribute to a polyploid advantage include: (1) greater gene and gene expression diversity (heterosis), (2) genome buffering (increased capacity to tolerate mutation events), and (3) increased potential for genes to evolve novel functions (sub-/neofunctionalization) (reviewed by Udall and Wendel 2006; Chen 2007).

Most answers to a polyploidy advantage have been sought in the initial polyploidization process that may require rapid changes in genome structure, composition, and gene expression that are essential for a new species to persevere (Wendel 2000; Chen and Ni 2006). Increasing evidence of novel gene expression patterns in several genera of neopolyploids suggests that differential regulation of genes is indeed a common feature following polyploidization (Comai *et al.* 2000; Kashkush *et al.* 2002; He *et al.* 2003; Adams *et al.* 2004; Wang *et al.* 2004, 2006; Flagel *et al.* 2008). Analysis of global expression levels in Arabidopsis synthetic allotetraploids by microarray experiments revealed that ~6% of transcripts (and potentially as high as ~40% depending on statistical methods employed) are expressed in a nonadditive manner, relative to mid-parent expression levels (Wang *et al.* 2006). Albertin *et al.* (2006) used a proteomics approach to show nonadditive accumulation of >25% of 1600 polypeptides measured in newly synthesized *Brassica napus* allotetraploids. At least for the Brassica system, their study established that substantial changes in protein expression occur in new polyploids. Recently, Flagel *et al.* (2008) used a novel homoeolog-specific mircroarray platform to conclude that ~24% of biased homoeolog expression events detected in natural *Gossypium hirsutum* are immediately established upon polyploidization.

Triticum species are an exemplary model of the polyploid advantage concept. *Triticum aestivum* (allohexaploid, $2n = 6x = 42$, known as common or bread wheat) arose as recently as 8000 years ago from hybridization between cultivated *T. turgidum* (allotetraploid, $2n = 4x = 28$, known as durum or macaroni wheat) and diploid

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goatgrass *Aegilops tauschii* (Kihara 1944; McFadden and Sears 1946; Nesbitt and Samuel 1996). Yet, *T. aestivum* accounts for 95% of world wheat production due to broader global adaptability to a range of abiotic and biotic stress factors and environmental conditions. The reasons for this polyploid advantage have been discussed (Faris *et al.* 2002; Dubcovsky and Dvorak 2007) but exact genetic mechanisms remain unknown. Differential gene expression in newly formed allohexaploid wheat has been the subject of only one investigation, despite the global importance of this species. He *et al.* (2003) analyzed transcriptional changes in a synthetic allohexaploid wheat line compared to its *T. turgidum* and *Ae. tauschii* parents. Although no evidence of gene deletion or rearrangement events was observed, 7.7% of transcripts showed nonadditive expression. Interestingly, the vast majority of differentially expressed transcripts (~95%) were reduced or absent in the allohexaploid, and only a few transcripts were induced.

The magnitude and significance of subfunctionalization in hexaploid wheat is unknown at present, though attempts have been made to estimate the frequency of differential expression of homoeologous transcripts (Mochida *et al.* 2003; Bottley *et al.* 2006). However, to date there has been no global analysis of gene expression patterns in spite of extensive classic observations of numerous wheat traits that are known to be affected by polyploidy (see discussion). Here we report results of a microarray experiment using a 17K feature microarray designed to test the hypothesis that nonadditive gene expression is rapidly established following polyploidization in *T. aestivum*. The expression of 30 arbitrarily selected sets of homoeologous transcripts was also monitored in the synthetic wheat and parental *Ae. tauschii* and *T. turgidum* lines by cDNA–single strand conformation polymorphism (SSCP). We observed many instances of nonadditive and overdominant expression that may partly account for evolutionary novelty following polyploidization.

## MATERIALS AND METHODS

**Plant materials:** The neopolyploid stable synthetic hexaploid wheat line TA4152 L3 (AABBDD) and parental genotypes *T. turgidum* (cultivar Altar 84, accession TA2970, AABB) and *Ae. tauschii* (accession TA1651, DD) were used for cDNA–SSCP and microarray expression experiments. The synthetic hexaploid was self-pollinated to $S_{5\text{-}6}$ generations after synthesis at the International Maize and Wheat Improvement Center (CIMMYT) and demonstrates disomic meiotic chromosome pairing behavior (He *et al.* 2003). One plant per pot was grown in ~600 g of Metro-Mix 200 potting media (Sun Gro Horticulture, Bellevue, WA) in 3.5-liter square plastic pots. Six grams of Osmocote 14-14-14 (Scotts, Marysville, OH) slow-release fertilizer were incorporated with potting media prior to planting. Plants were grown in a growth chamber with 16-hour day lengths at 20° daytime and 18° nighttime temperatures.

**cDNA–SSCP:** Leaves of seedling plants grown for 30 days were harvested into liquid nitrogen prior to extraction of total RNA or DNA. Total RNA was isolated from each of three plants per genotype using the TRIzol reagent (Invitrogen, Carlsbad CA) and cDNA synthesis was performed as desribed by He *et al.* (2003). DNase treatment was not performed; however, amplification with >10 intron-spanning primers revealed that genomic DNA (gDNA) contamination was undetectable in all samples. Genomic DNA extraction was performed as described by Liu *et al.* (2006).

Low-copy expressed sequence tags (ESTs) were arbitrarily selected by mining results of the National Science Foundation-supported wheat deletion-mapping project (Qi *et al.* 2004). Low-copy loci were parsed from the complete wEST-SQL database of deletion-bin mapped ESTs (http://wheat.pw.usda.gov/westsql/index.html) by a custom query requiring the number of bands detected by Southern blot hybridization to equal the same number of deletion bins (*e.g.*, three bands, with one band from each homoeologous locus in a group of chromosomes). The resulting subset of low-copy ESTs was then manually verified by viewing Southern blot images for each EST, and 30 high-confidence homoeoloci were chosen. Conserved primers for the 30 homoeologous loci were designed using the Primer 3 program (Rozen and Skaletsky 2000) based on sequence alignments of contigs (TC) containing each EST previously assembled by the Institute for Genomics Research (supplemental Table 1).

PCR reactions were performed in a total volume of 25 μl with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) using the manufacturer's instructions. Template gDNA (60 ng) and cDNA (20 ng) were PCR amplified with 32 cycles of 94° for 60 sec, 55°–60° for 60 sec, and 72° for 90 sec. cDNA–SSCP analyses were only performed with thirty genes (primer pairs) that amplified from both *T. turgidum* and *Ae. tauschii* gDNA. cDNA "*in vitro* synthetic" controls were included by equally mixing cDNA from the parental species to account for amplification bias and mobility shifts in electrophoresis. Initially, cDNA–SSCP analyses were done on each of the three replicates of cDNA synthesized from separate RNA isolations. However, lack of detectable differences between biological replicates supported mixing equal amounts of cDNA (1:1:1) from each replicate to conservatively streamline the experimental procedure.

Following PCR amplification, products were diluted 1:5 (v/v) in formamide loading dye (95% formamide, 10 mm EDTA, bromophenol blue, xylene cyanol), denatured for 5 min at 94°, and chilled on ice. Denatured products were run in MDE polyacrylamide gels (Cambrex, Rockland, ME) according to the manufacturer's instructions at room temperature using BioRad Sequi-Gen GT sequencing systems (Bio-Rad Laboratories, Hercules, CA). Gels were run at 4–5 W constant power for 15 hr and visualized by silver staining using the protocol of Bassam *et al.* (1991). To validate this modified cDNA–SSCP technique, analysis was first performed on differentially expressed cDNA–AFLP fragment "AFLP-23" (identified by He *et al.* 2003) for comparison.

**Microarray procedures:** Microarray hybridizations were conducted using RNA from three biological replicates each of the *Ae. tauschii* (TA1651), *T. turgidum* (Altar 84), and synthetic *T. aestivum* (TA4152L3) genotypes in each pairwise combination (treatment 1, *Ae. tauschii vs. T. turgidum*; treatment 2, *Ae. tauschii vs. T. aestivum*; treatment 3, *T. turgidum vs. T. aestivum*; three biological replicates per treatment for a total of nine slides; Figure 1). Total RNA was independently purified from the three biological replicates of each genotype by using an RNeasy Plant Mini kit (QIAGEN, Valencia, CA). RNA concentration was quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was confirmed prior to microarray probe synthesis and labeling on an Agilent Bioanalyzer 2100 (Agilent

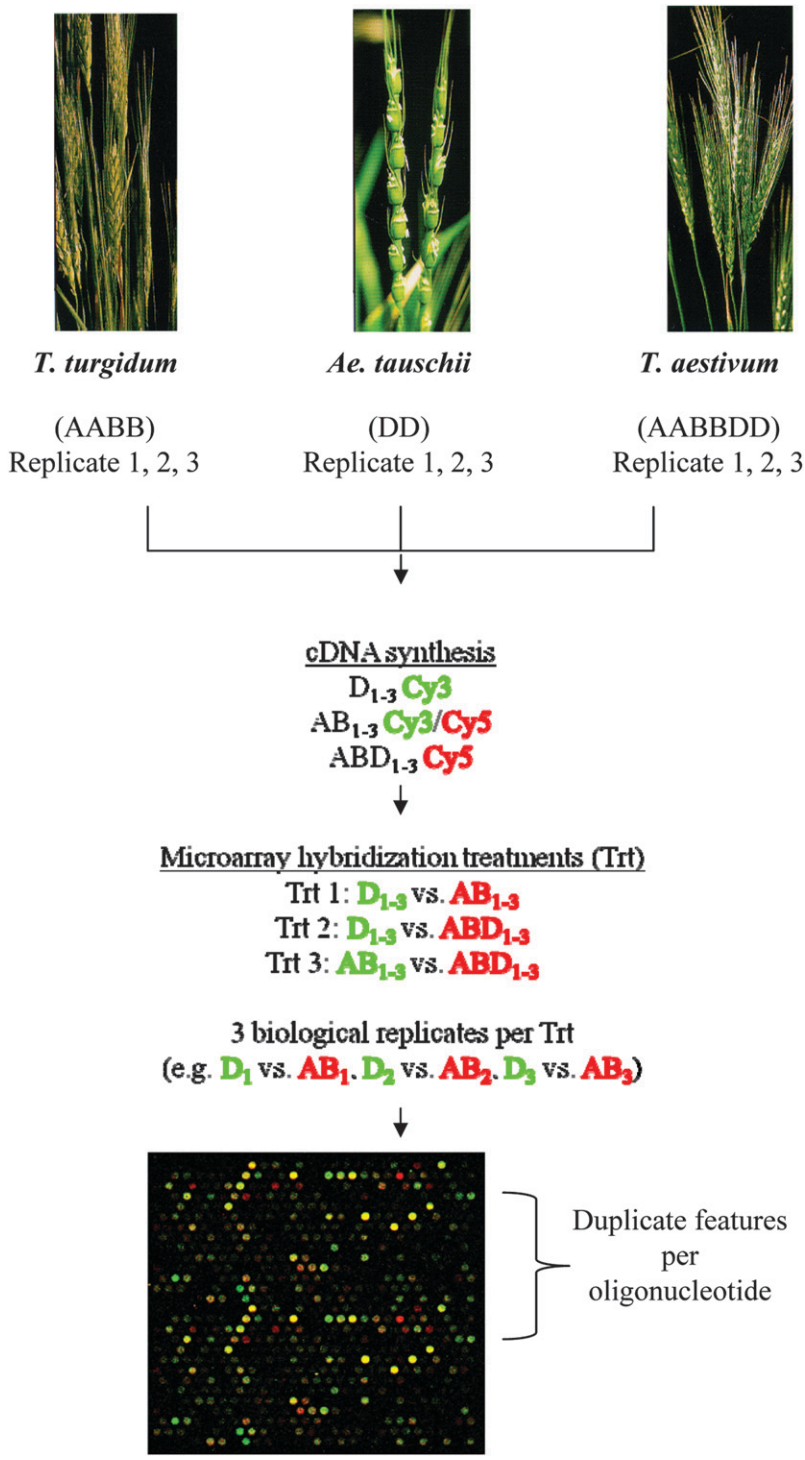


FIGURE 1.—Microarray experimental design. RNAs from each of three biological replicates of genotypes *T. turgidum* (AB genome), *Ae. tauschii* (D genome), and their derived synthetic hexaploid *T. aestivum* (ABD) were used in microarray hybridation experiments on a spotted 70-mer oligonucleotide array. Rather than mixing RNAs from *Ae. tauschii* and *T. turgidum* to create an *in vitro* synthetic for competitive hybridizations against synthetic *T. aestivum*, each parental line was independently cohybridized and later averaged to estimate mid-parent expression levels. For treatment 1 (Trt 1), *Ae. tauschii* transcripts were hybridized against *T. turgidum* transcripts; for treatment 2 (Trt 2), *Ae. tauschii* transcripts were hybridized against *T. aestivum*; for treatment 3, *T. turgidum* transcripts were hybridized against *T. aestivum*. Three biological replicates were used per treatment for a total of nine slides. Each feature is replicated on the 17K microarray, as indicated by the nearly identical hybridization patterns observed on a sample of our experimental slides.

Technologies, Palo Alto, CA). Rather than mixing RNAs from *Ae. tauschii* and *T. turgidum* to create an *in vitro* synthetic for competitive hybridizations against synthetic *T. aestivum*, each parental line was independently cohybridized and later averaged to estimate mid-parent expression levels. This was done to reduce potential homoeolog labeling/hybridization bias and because there is no empirical data on suitable ratios of parental RNAs to imitate allohexaploid gene expression (*i.e.*, in an allohexaploid nucleus, two of the three component genomes are from one parent while one genome is from another parent, suggesting a 2:1 ratio of tetraploid to diploid RNAs; however, from a parental/species perspective, a 1:1 ratio may be appropriate).

The *T. aestivum* 17K 70-mer oligoarray contains 35,568 features representing 17, 279 potentially unique wheat genes and 50 control sequences. The oligoprobes were double spotted in a single amino-silane coated Ultra GAPS slide (Corning, NY) in 48 grids with 27 × 28 spots/grid and with an average spot size of 100 μm. The oligoprobes used in this array were designed from a set of 246K wheat sequences. This set includes 223K EST sequences (with sequence tracefiles) generated by the Genome Canada-funded Functional

Genomics of Abiotic Stress (FGAS prefix) grant and the National Science Foundation-funded Wheat Genome grant (USDA_WHE prefix; http://wheat.pw.usda.gov/NSF/); and 23K wheat mRNA and EST sequences (flatfiles) from private and pubic databases provided by collaborators including 504 sequences derived from an experiment using the Curagen GeneCalling technique. The control sequences include 12 randomly generated 70-mer oligoprobes, 10 Stratagene SpotReport Alien OligoArray Validation System controls (http://www.stratagene.com/products/showProduct.aspx?pid=527), 10 Arabidopsis Functional Genomics Consortium spiking controls (http://www.arabidopsis.info/info/Clone_Information_NASC.pdf), 11 "housekeeping genes," the whale myoglobin (SYNWHLMG) gene, and 8 genes commonly used as plant transformation markers. The 70-mer oligos were designed using Sigoligo, software developed by William Crosby's group (University of Windsor, Windsor, Ontario, Canada) and the set of 70-mer oligoprobes was synthesized by QIAGEN (http://www1.qiagen.com), before array printing by Microarrays (http://www.microarrays.com). The wheat 17K 70-mer oligoarray was developed with the international collaboration of three partners, the United States Department of Agriculture–Agricultural Research Service (USDA–ARS), Genomics and Gene Discovery Research Unit (Albany, CA), the Genome Canada/Genome Prairie/Genome Quebec program through the FGAS grant, and the Australian Centre Plant Functional Genomics. The 17K 70-mer probe sequences, the source sequences used to design each probe, and the gal file for the array can be found at http://wheat.pw.usda.gov/~dlc/MI.downloads/.

RNAs were reverse transcribed (RT) and indirectly labeled with Cy3 or Cy5 fluorophores using the Array 900 system (Genisphere, Hatfield, PA) according to the manufacturer's instructions. RT reactions were purified using a CyScribe GFX purification kit (GE Healthcare, Piscataway, NJ) to remove unincorporated primer and then combined equally in hybridization reaction mixtures per manufacturer's instructions. Hybridization mixtures were incubated on microarray slides for 16 hr at 50°. Slides were washed in a 2× saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (w/v) solution for 10 min at 50° with gentle agitation, followed by 0.2× SSC and finally 0.1× SSC washing steps for 10 min each at room temperature. Cy3/Cy5 fluorophores were then hybridized for 4 hr at 50°, washed as above, and slides scanned using an Axon GenePix 4000B (Molecular Devices, Union City, CA). Channel intensity was balanced with 100% laser power for each wavelength with photomultiplier tube (PMT) gain set at 760–800 for the 635-nm (red) laser, and 680–720 for the 532-nm (green) laser. Each slide was scanned twice at different PMT gain settings so that scans with similar intensity values were obtained for across-treatment comparisons.

Fluorescence intensity data were recorded using GenePix Pro v6.0 (Molecular Devices). Lower-quality and weakly hybridizing features were stringently flagged for exclusion (by requiring a circularity ratio $\geq$0.8, signal intensity >75% of background plus one standard deviation, and the sum of median intensity from each channel >900) to initially reduce printing and hybridization errors and exclude oligonucleotide probes with poor hybridization characteristics. Data normalization was performed to reduce nonbiologically significant variation and further equilibrate channel intensities within and between microarray hybridizations. Slides were normalized individually to reduce potential interdependency among replicates. GeneSpring GX v7.3 (Agilent Technologies) was used for one-way analysis of variance (ANOVA), applying the Benjamini and Hochberg multiple testing correction procedure to control the false discovery rate ($\alpha = 0.05$). Mid-parent expression values (MPV) were calculated by averaging across treatments, assuming either 1:1 (*T. turgidum*: *Ae. tauschii*, $MPV_{1:1}$) or 2:1 (*T. turgidum*: *Ae. tauschii*, $MPV_{2:1}$) ratios of RNA abundance in the allohexaploid nucleus. Pearson's correlation coefficients among gene expression levels were calculated between and across genotypes/treatments in Microsoft Excel.

## RESULTS

**Detection of differential expression of homoeoloci by cDNA–SSCP:** Expression levels of individual homoeoloci were assayed by cDNA–SSCP on seedling leaf tissue of *Ae. tauschii* (DD), *T. turgidum* (AABB), and synthetic *T. aestivum* (AABBDD). Because the quantitative cDNA–SSCP technique reported by Cronn and Adams (2003) was modified, a well-characterized transcript (AFLP-23) that was differentially expressed in the same plant materials (He *et al.* 2003) was first used for comparison and validation. AFLP-23 provided a particularly useful comparison; He *et al.* (2003) sequenced each AFLP-23 homoeoallele and conducted competitive RT–PCR experiments to quantify differential homoeologous expression of this locus. The quantitative cDNA–SSCP technique used in this study was suitable to confirm differential expression of AFLP-23 homoeologous transcripts (Figure 2A).

Four of the 30 arbitrarily selected sets of homoeologous transcripts (~13%) showed altered expression patterns in the synthetic wheat. Suppression of *Ae. tauschii* D genome homoeologs was observed for two differentially expressed transcripts, TC270558 and TC273936 (Figure 2B). TC253445 displayed upregulation of the *Ae. tauschii* homoeolog and/or concurrent downregulation of a *T. turgidum* homoeolog on the basis of comparison to the *in vitro* synthetic control. The same general pattern was found for TC264908, where the *Ae. tauschii* homoeolog was clearly more abundant, while a *T. turgidum* transcript appeared to be silenced. Amplification and electrophoresis were repeated for the four genes classified as differentially expressed and all results were consistent.

**Estimation of nonadditive gene expression by microarray analysis:** Global gene expression levels were quantified in the same lines by microarray expression experiments. Synthetic allohexaploid expression levels were compared to mid-parent expression model values calculated from the parental species to estimate the frequency of nonadditive gene expression in neoallohexaploid wheat.

*Data analysis:* Stringent filtering of low-quality features and normalization were employed to reduce nonbiological variation. Due to the balanced channel intensities maintained through slide scanning and data normalization, channel median intensities were very similar (differences less than ±10% of experiment-wide mean) between and within slides. Therefore, no median centering calculations were applied. Expression of 825 high-quality features (supplemental Table 2; data available at http://www.genetics.org/supplemental) was

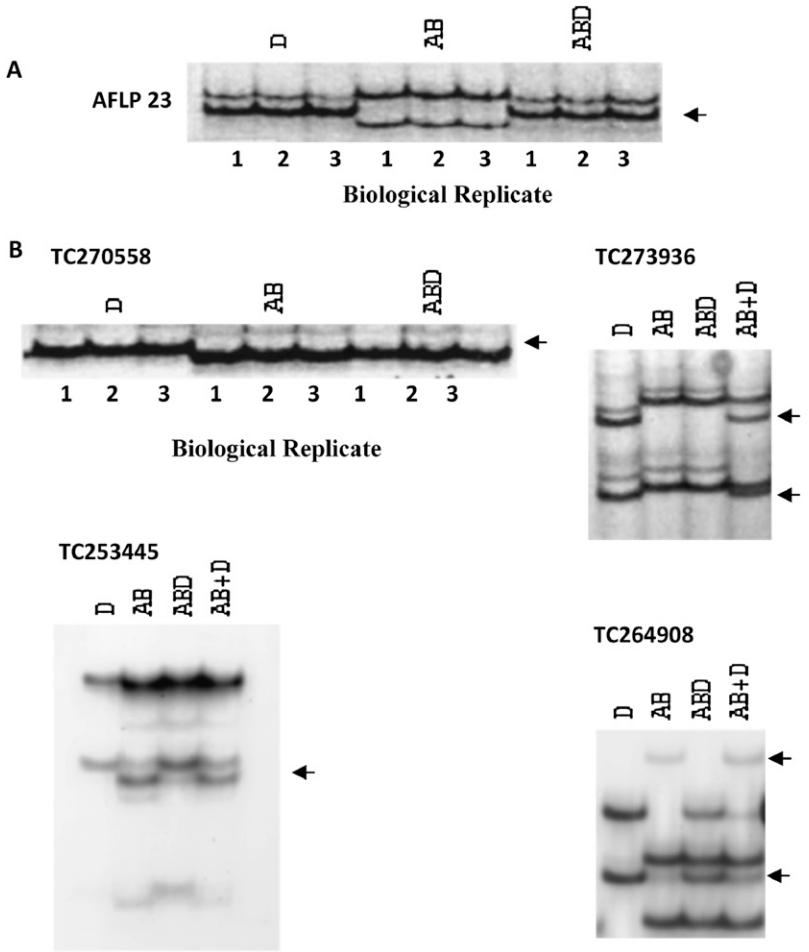


FIGURE 2.—(A) cDNA–SSCP analysis of cDNA–AFLP differentially expressed transcript AFLP-23. Although total AFLP-23 transcript levels are similar between diploid *Ae. tauschii*, tetraploid *T. turgidum*, and the derived synthetic *T. aestivum*, homoeologous transcripts are differentially expressed in seedling leaves following polyploidization (He *et al.* 2003). Equal amounts of second-strand cDNAs from *Ae. tauschii* (D), *T. turgidum* (AB), and synthetic *T. aestivum* (ABD) were PCR amplified with AFLP-23 primers and run on MDE polyacrylamide gels. The cDNA–SSCP technique was suitable to identify suppression of a *T. turgidum* homoeoallele (arrow). (B) Images of the four differentially expressed homoeologous transcripts identified by cDNA–SSCP analysis of 30 arbitrary homoeologs. Equal amounts of second-strand cDNAs from *Ae. tauschii* (D), *T. turgidum* (AB), and synthetic *T. aestivum* (ABD), as well as an *in vitro* synthetic control (AB + D cDNAs equally mixed) were PCR amplified with conserved primers for each locus and run on MDE polyacrylamide gels. TC270558 was run prior to streamlining the procedure by equally mixing RNAs from three biological replicates (due to lack of observable differences between replicates). Arrows indicate homoeoalleles with differential expression.

analyzed using GeneSpring one-way ANOVA. Putative annotations of the list of 825 transcripts revealed a diverse representation of genes involved in all aspects of plant growth and development, including: biosynthesis, carbohydrate metabolism, cell division and structure, nucleic acid metabolism, photosynthesis, protein synthesis and metabolism, signal transduction, transport, and ~17% unknown or hypothetical. The strong agreement among expression levels across treatments was sufficiently high for each genotype to indicate that both dye and hybridization bias were minimal in the normalized data set and data may be analyzed across treatments (Table 1).

*Expression divergence and nonadditive gene expression estimates:* Divergence in expression levels between *Ae. tauschii* and *T. turgidum* was substantial; *Ae. tauschii* expression levels accounted for only 11% of the variation in *T. turgidum* expression levels, or vice versa (Figure 3a). Approximately 78% (641/825) of genes were differentially expressed (threshold of greater than twofold higher/lower, $P \leq 0.05$) between these parental species on the basis of ANOVA. Expression level divergence was

**TABLE 1**

**Correlation coefficients, *r*, among gene expression levels observed for *Ae. tauschii* (D), *T. turgidum* (AB), and synthetic *T. aestivum* (ABD) across hybridization treatments one (Trt 1), two (Trt 2), and three (Trt 3)**

| | D-Trt 2 | AB-Trt 1 | AB-Trt 3 | ABD-Trt 2 | ABD-Trt 3 |
|---|---|---|---|---|---|
| D-Trt 1 | 0.94*** | 0.30*** | 0.26*** | 0.65*** | 0.67*** |
| D-Trt 2 | | 0.37*** | 0.34*** | 0.70*** | 0.70*** |
| AB-Trt 1 | | | 0.97***[a] | 0.75*** | 0.75*** |
| AB-Trt 3 | | | | 0.69*** | 0.71*** |
| ABD-Trt 2 | | | | | 0.97*** |

***Correlation significant at $P < 0.0001$.

[a] *T. turgidum* was labeled with Cy5 in treatment one; with Cy3 in treatment three.

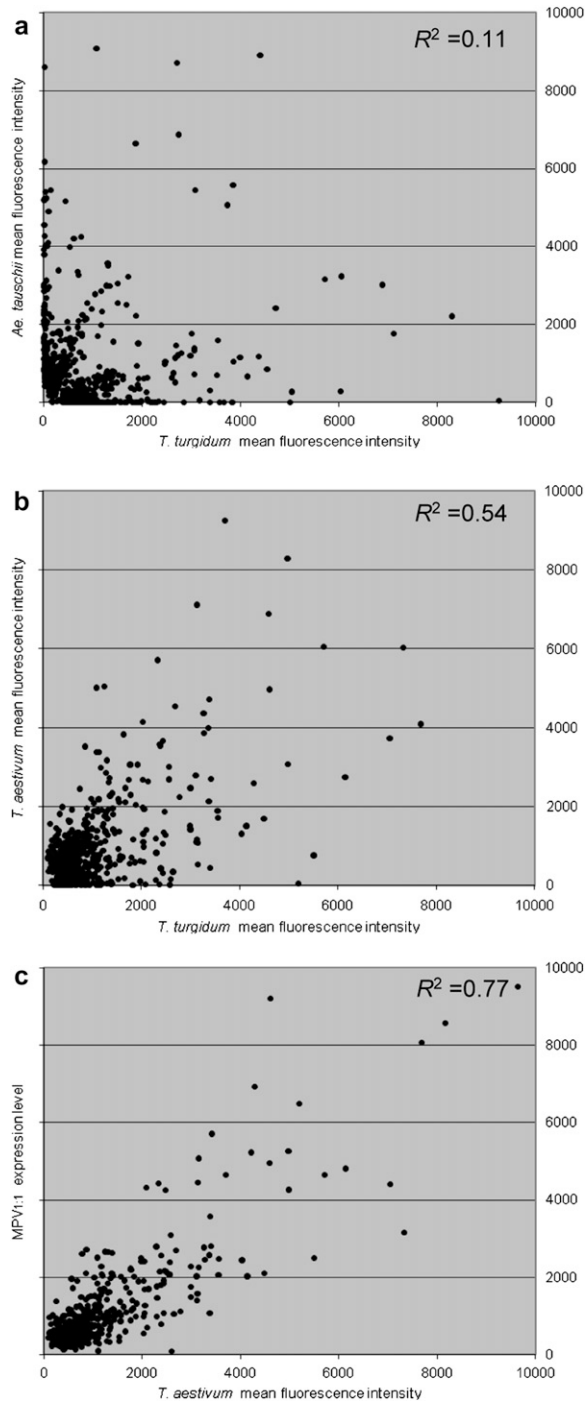


Figure 3.—Scatter plots showing correlation in microarray gene expression levels based on linear regression of (a) *Ae. tauschii* mean fluorescence intensities *vs. T. turgidum* mean fluorescence intensities, (b) *T. turgidum* mean fluorescence intensities *vs. T. aestivum* mean fluorescence intensities, and (c) *T. aestivum* mean fluorescence intensities *vs.* $MPV_{1:1}$ model estimates.

significantly lower between either *Ae. tauschii* or *T. turgidum* and the derived synthetic *T. aestivum* (Table 1). Linear regression of *Ae. tauschii* and *T. turgidum* expression levels explained 49 and 54%, respectively, of the variation in synthetic *T. aestivum* gene expression (Figure 3b; comparison of *T. turgidum* and *T. aestivum* shown).

Two mid-parent expression models were tested for their ability to predict synthetic allohexaploid gene expression levels: $MPV_{1:1}$, the average of *T. turgidum* and *Ae. tauschii* expression levels (assuming a 1:1 ratio of transcript abundance from each parental genome), and $MPV_{2:1}$, a weighted 2:1 average of *T. turgidum* to *Ae. tauschii* expression levels (assuming 2/3 of total transcription is from A and B genome homoeoalleles of *T. turgidum* and 1/3 is from the D genome homoeoallele of *Ae. tauschii* in a synthetic hexaploid). Linear regression on $MPV_{1:1}$ and $MPV_{2:1}$ expression values accounted for 77 and 74% of the variation in synthetic *T. aestivum* expression levels, respectively (Figure 3c; comparison of $MPV_{1:1}$ and *T. aestivum* shown). Thus, both MPV models are better predictors of hexaploid gene expression than actual expression levels observed for either parental species.

Nonadditive gene expression (threshold of greater than twofold higher/lower) was estimated for 16.2% of transcripts when *T. aestivum* levels were compared to $MPV_{1:1}$ additive model values (Table 2; supplemental Table 3). A slight majority of genes classified as differentially expressed, 8.5%, had higher expression levels in synthetic *T. aestivum* than expected, while 7.8% were lower and potentially downregulated (Table 2). $MPV_{2:1}$ model comparison yielded very similar results, with 15.5% of genes estimated to have nonadditive expression levels. The estimate of upregulated genes was similar with the $MPV_{2:1}$ model at 9.2% and downregulation estimates were slightly lower at 6.3% (Table 2). Approximately two-thirds of genes classified as downregulated and three-fourths of genes classified as upregulated were the same between the two models (supplemental Table 3). A third "high-parent" gene expression value was also used for comparison. Instead of averaging parental expression levels, the higher of the two was compared to observed *T. aestivum* levels, reflective of overdominant expression in heterosis literature. The fraction of genes categorized as having greater than twofold higher expression over high-parent estimates (overdominant) was 1.7% [14/825 (Table 3)]. Similarly, 1.2% (10/825) of transcripts had expression levels lower than the "low-parent" (underdominant).

*Characterization of genes classified as nonadditively expressed:* Expression of six genes with nonadditive expression levels on the basis of microarray experiments was characterized by cDNA–SSCP. cDNA–SSCP assays were performed on three sets of transcripts that were classified as upregulated (TC252860, TC267682, and TC262784) and three sets of transcripts that were classified as downregulated (TC267455, TC267082, and TC23840) in synthetic *T. aestivum* compared to $MPV_{1:1}$ values (Figure 4A). The weak expression levels of TC252860 (oligonucleotide feature FGAS.02071, 2.3-fold higher, annotated as a putative respiratory burst

**TABLE 2**

**Percentage of genes classified as nonadditively expressed in synthetic *Triticum aestivum* when compared to mid-parent expression level (MPV) estimates derived from *Aegilops tauschii* and *T. turgidum* expression values**

| | Higher in synthetic *T. aestivum* (%) | Lower in synthetic *T. aestivum* (%) | Total nonadditive (%) |
|---|---|---|---|
| $MPV_{1:1}$ | 8.5 (70/825) | 7.8 (64/825) | 16.2 (134/825) |
| $MPV_{2:1}$ | 9.2 (76/825) | 6.3 (52/825) | 15.5 (128/825) |

$MPV_{1:1}$ is the average of *T. turgidum* and *Ae. tauschii* expression levels, while $MPV_{2:1}$ is a weighted 2:1 average of *T. turgidum* to *Ae. tauschii* expression levels. A threshold of twofold increase/decrease was used for assigning genes to higher/lower categories, respectively.

oxidase homolog) were confirmed by cDNA–SSCP analysis; despite strong amplification products using gDNA, cDNA amplification products were relatively faint in the *Ae. tauschii* and *T. turgidum* parents. Elevated expression of a *T. turgidum* homoeolog(s) and/or suppression of the *Ae. tauschii*-derived transcript accounted for nonadditive expression of TC252860 in synthetic hexaploid wheat. The cDNA–SSCP analysis of TC267682 (oligonucleotide feature FGAS.03312, 3.7-fold higher, overdominant, putative sucrose synthase) supported higher expression levels in synthetic *T. aestivum*, as indicated by the saturated staining, most likely due to upregulation of a *T. turgidum* homoeoallele. TC262784 (oligonucleotide feature FGAS.00736, 2.6-fold higher, overdominant, putative transketolase) displayed an interesting electrophoresis profile. Comparison of cDNA and gDNA profiles revealed that the *Ae. tauschii*-derived transcript is suppressed, while a *T. turgidum* transcript appears more abundant in synthetic *T. aestivum*.

Amplification of TC267455, TC267082, and TC23840 on gDNA of each genotype revealed that gene deletion events had not occurred in the synthetic hexaploid for any of these genes (Figure 4B), and gene loss was most likely not responsible for the reduced expression levels measured by microarray hybridization. The *Ae. tauschii*-derived homoeoallele of TC267455 (oligonucleotide feature USDAWHE.02876, 3.4-fold lower, putative Hsp70 binding protein) was very faint or absent in the cDNA–SSCP profile, indicating silencing of this homoeoallele in synthetic *T. aestivum*. Repression of a *T. turgidum* homoeoallele and/or upregulation of the *Ae. tauschii* homoeoallele was observed for TC267082 (oligonucleotide feature FGAS.02280, 3.0-fold lower, underdominant, putative serine carboxypeptidase). For TC238480 (oligonucleotide feature USDAWHE.05946, 2.7-fold lower, underdominant, putative histidyl-tRNA synthetase), the cDNA profile had similar band intensities to the gDNA profile when compared across genotypes and did not support nonadditive expression of this locus. In summary, cDNA–SSCP analysis revealed that at least three of the six genes characterized show differential expression of homoeologous loci in the synthetic *T. aestivum*. Although not a purely quantitative assay, cDNA–SSCP also supported nonadditive overall transcript levels.

## DISCUSSION

The expression of homoeologous genes in a new allopolyploid nucleus may be altered from that of the parents by epigenetic mechanisms, interactions between diverged regulatory networks present in each progenitor species, deletion events, chromosomal rearrangements, or novel epistatic interactions (Chen and Ni 2006). In short, novel gene expression levels and patterns may be both qualitative and quantitative. In the only investigation documenting widespread gene expression changes in neoallohexaploid wheat to date, He *et al.* (2003) estimated that ~7% of transcripts are downregulated and only 0.4% of transcripts are more abundant in synthetic *T. aestivum* using the cDNA–AFLP technique. A limitation to this technique is the primarily qualitative nature of the results, which undoubtedly underestimates the magnitude of quantitative changes that occur upon polyploidization. Even so, repression or silencing appears by far to be the most frequent method of nonadditive gene regulation in new allopolyploids (reviewed by Udall and Wendel 2006).

In this investigation, additive mid-parent expression values were estimated, on the basis of averaging parental *Ae. tauschii* and *T. turgidum* microarray expression levels, and compared to those observed for the synthetic *T. aestivum*. Similar expression level divergence between synthetic *T. aestivum* and either of its parental lines indicates that neither parental species overwhelmingly biases hexaploid gene expression. This is supported by the better agreement between synthetic *T. aestivum* expression levels and hypothetical $MPV_{1:1}$ expression values, compared to the agreement in expression levels between synthetic *T. aestivum* and either parent (Figure 3, b and c). In other words, and not surprisingly, the substantial majority of genes show additive expression in the synthetic allohexaploid nucleus.

Approximately 78% of transcripts were classified as differentially expressed between the *Ae. tauschii* and *T. turgidum* parents on the basis of Genespring ANOVA. In comparison, ~43% of transcripts were classified as differentially expressed between diploid relatives *Arabidopsis thaliana* and *A. arenosa* using a per-gene variance model (Wang *et al.* 2006). Similarly, ~48% of genes were differentially expressed between diploid relatives *Drosophila melanogaster* and *D. simulans* (Ranz *et al.* 2003). The elevated estimate of differential expression between the *Ae. tauschii* and *T. turgidum* genotypes is not necessarily surprising considering the difference in ploidy levels and histories of these species. After Triticum/Aegilops divergence ~3 MYA, *T. turgidum* was formed ~0.5–1 MYA

**TABLE 3**

**Transcripts displaying over-/underdominant expression (twofold or more) in microarray experiments, their putative annotation (function), and expression fold change in synthetic *T. aestivum* relative to high-/low-parent levels**

| Oligonucleotide | Blast annotation/putative function | Expect | Over-/underdominant fold |
|---|---|---|---|
| FGAS.00200 | Putative arabinogalactan-like protein [*Oryza sativa*] (signaling, development, cell-wall anchored) | $4 \times 10^{-41}$ | 2.3↓ |
| FGAS.00291 | Putative class III chitinase [*Oryza sativa*] (PR protein, defense, stress response, development) | $1 \times 10^{-123}$ | 2.4↑ |
| FGAS.00484 | OSJNBa0036B21.19 [*Oryza sativa*] (hypothetical noncell-autonomous protein pathway2; cell to cell communication) | $9 \times 10^{-90}$ | 2.9↓ |
| FGAS.00736 | Putative transketolase 1 [*Oryza sativa*] (Calvin cycle) | 0 | 2.4↑ |
| FGAS.01252 | Predicted protein [*Arabidopsis thaliana*] (adh1-adh2 region, novel transposable element, contains TIR) | 5 | 2.9↑ |
| FGAS.02082 | Putative GDSL-like lipase/acylhydrolase [*Oryza sativa*] (lipid metabolism) | $8 \times 10^{-59}$ | 2.4↑ |
| FGAS.02280 | Serine carboxypeptidase I CP-MI [*Hordeum vulgare*] (protein turnover) | 0 | 2.6↓ |
| FGAS.02689 | Putative NADPH-dependent reductase [*Oryza sativa*] | 0 | 2.9↑ |
| FGAS.02907 | AT3g20810/MOE17_10 [*Arabidopsis thaliana*] (hypothetical; possible transcription factor; transferase activity) | $4 \times 10^{-77}$ | 3.4↑ |
| FGAS.03312 | Sucrose synthase [*Zea mays*] | $1 \times 10^{-179}$ | 2.1↑ |
| FGAS.03426 | B1139B11.9 [*Oryza sativa*] (hypothetical; putative regulator of gene silencing; EF-hand, calcium binding motif) | $1 \times 10^{-47}$ | 3.1↓ |
| FGAS.04295 | Dentin sialophosphoprotein-related [*Arabidopsis thaliana*] (expressed protein) | $2 \times 10^{-06}$ | 5.1↓ |
| FGAS.05144 | Putative endoxyloglucan transferase [*Oryza sativa*] (cell-wall structure/remodeling) | $2 \times 10^{-69}$ | 2.0↑ |
| FGAS.06913 | Pseudo-response regulator-like [*Oryza sativa*] (hypothetical signal transduction; transcriptional regulation) | $9 \times 10^{-16}$ | 2.1↑ |
| FGAS.06924 | None (Repeat database: Jura 2 DNA transposon, MITE, tourist) | $8 \times 10^{-50}$ | 16.5↑ |
| FGAS.07985 | Unknown protein [*Oryza sativa*] (hypothetical) | $4 \times 10^{-32}$ | 2.5↑ |
| FGAS.08506 | Similar to vesicle transport protein [*Oryza sativa*] (protein trafficking) | $1 \times 10^{-92}$ | 2.6↓ |
| FGAS.09294 | Unknown protein [*Oryza sativa*] (hypothetical) | $1 \times 10^{-06}$ | 2.3↑ |
| USDAWHE.00320 | DNA topoisomerase II [*Arabidopsis thaliana*] | $3 \times 10^{-55}$ | 3.2↓ |
| USDAWHE.01509 | Plastid sigma factor SIG5 [*Oryza sativa*] (plastid RNA polymerase subunit) | $1 \times 10^{-61}$ | 2.5↓ |
| USDAWHE.02888 | Unnamed protein product [*Oryza sativa*] (hypothetical helicase-related) | $3 \times 10^{-22}$ | 6.1↑ |
| USDAWHE.03577 | Putative high-affinity potassium transporter [*Hordeum vulgare*] | $3 \times 10^{-12}$ | 3.2↓ |
| USDAWHE.04169 | Unnamed protein product [*Oryza sativa*] (hypothetical invertase/pectin methylesterase inhibitor) | $4 \times 10^{-32}$ | 2.1↑ |
| USDAWHE.05946 | Histidyl-tRNA synthetase [*Triticum aestivum*] (protein synthesis) | $1 \times 10^{-73}$ | 2.6↓ |

Down and up arrows indicate lower and higher expression, respectively, in synthetic *T. aestivum* than expected based on high-/low-parent values of *Ae. tauschii* or *T. turgidum.*

as the result of a polyploidization event between *T. urartu* and yet another Aegilops species (Huang *et al.* 2002). *T. turgidum* gene expression levels have undoubtedly significantly diverged from *Ae. tauschii* due to the "genomic shock" (McClintock 1984) caused by this polyploidization event, in addition to mutation and selection during wild tetraploid evolution and subsequent domestication and plant breeding efforts.

Comparisons to MPV estimates suggest that ~16% of genes have nonadditive expression in seedling leaves of the synthetic *T. aestivum,* using a threshold of twofold change. Approximately 7% of genes show reduced expression levels. This estimate is in remarkable agreement with the cDNA–AFLP estimate of ~7.3% in the same synthetic wheat line (He *et al.* 2003) and with other neoallopolyploid gene expression investigations (Adams

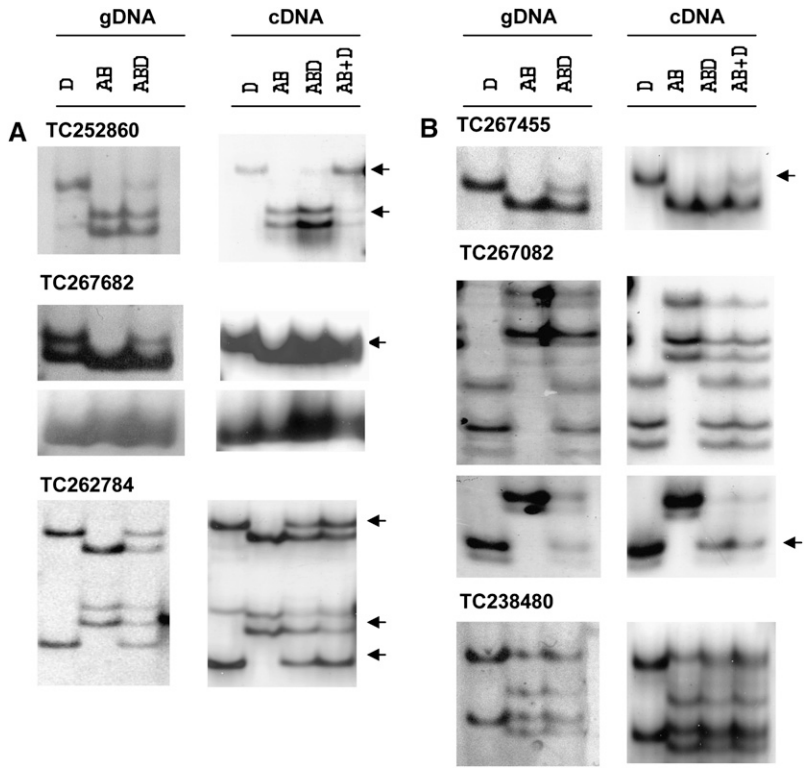


Figure 4.—cDNA–SSCP analysis of six genes with nonadditive expression levels based on microarray experiments. Assays were performed on three transcripts that were classified as upregulated (A) and three transcripts that were classified as downregulated (B) in synthetic *T. aestivum* compared to $MPV_{1:1}$ values. (A) TC252860 corresponds to oligonucleotide feature FGAS.02071 that was expressed 2.3-fold higher in synthetic *T. aestivum*. TC267682 corresponds to oligonucleotide feature FGAS.03312 that was expressed 3.7-fold higher. TC262784 corresponds to oligonucleotide feature FGAS.00736 that was expressed 2.6-fold higher. (B) TC267455 corresponds to oligonucleotide feature USDAWHE.02876 that was expressed 3.4-fold lower in synthetic *T. aestivum*. TC267082 corresponds to oligonucleotide feature FGAS.02280 that was expressed 3.0-fold lower in synthetic *T. aestivum*. TC238480 corresponds to oligonucleotide feature USDAWHE.05946 that was expressed 2.7-fold lower. Arrows indicate differential expression of homoeologs. Two independent replicates were in agreement; only one is pictured for the homoeoloci in each group. Images of both single-stranded and heteroduplex conformations are shown for products of cDNA amplification with TC267682 and TC267082.

*et al.* 2004; Wang *et al.* 2006). Approximately 9% of genes show higher expression levels than expected on the basis of MPV estimates. Even when compared to high-parent expression levels, a considerable fraction of genes (~1.7%) were expressed greater than twofold higher (overdominant expression). Considering many significant expression changes likely fall below a twofold threshold (Birchler *et al.* 2003) and would be ignored in this study, we speculate that this is still a gross underestimate of the extent to which relevant changes in gene expression occur.

The frequency of differential homoeologous expression we observed (~13%) in seedling leaf tissue by analyzing transcription of 30 homoeoloci by cDNA–SSCP supports the hypothesis that allopolyploidization results in rapid initiation of subfunctionalization (Udall and Wendel 2006) and nonadditive gene expression in synthetic *T. aestivum*. While it is likely that the frequent preferential expression of homoeoalleles observed to date in natural hexaploid wheat (Mochida *et al.* 2003; Bottley *et al.* 2006) have continued to accumulate due to genetic [*e.g.*, mutation in promoter or enhancer regions after a homoeolog is freed from selective constraints by genome duplication (Kellogg 2003; Vietia 2005)] and epigenetic changes in the brief evolution of hexaploid wheat, our data indicate that allopolyploid formation, *per se*, establishes much of these changes. A similar conclusion was recently drawn on the basis of work with allotetraploids of Gossypium (Flagel *et al.* 2008).

Although this investigation was not conducted to identify particular genes or biological processes affected by polyploidization, the putative annotations of nonadditively expressed genes and their similarity to genes differentially expressed in other neoallopolyploid systems warrants discussion. In their genomwide survey of nonadditive gene expression in Arabidopsis allotetraploids, Wang *et al.* (2006) showed that 33 out of 97 heat shock proteins (*HSP*s) in the Arabidopsis genome were differentially expressed relative to mid-parent values. More interestingly, 31 of the 33 were repressed. Nine putative *HSP*s were indentified from the 825 genes in this study, six of which were repressed greater than twofold relative to $MPV_{1:1}$ and/or $MPV_{2:1}$ values (supplemental Table 3). The agreement between this investigation and the results of Wang *et al.* (2006) suggest that *HSP*s are specifically targeted in early stages of allopolyploidization across diverse species. The role of *HSP90* in modulating expression of genetic variation in both plant and animal species is well established (Rutherford and Lindquist 1998; Queitsch *et al.* 2002) and may be partially attributed to an epigenetic mechanism (Sollars *et al.* 2002). Nonadditive expression of glutathione *S*-transferases (GSTs) was documented in allotetraploids of both Arabidopsis and Brassica (Wang *et al.* 2004; Albertin *et al.* 2006), as well as, in natural hexaploid wheat (Xu *et al.* 2002). Two of the four putative GSTs included in the 825 genes presently studied were classified as nonadditively expressed. Additional examples of nonadditively expressed gene families also identified in other allopolyploid systems include putative peptide/protein transporters, cellulose synthase catalytic subunits, kinesin-related proteins, ribosomal

proteins, and chlorophyll a/b binding proteins (Wang *et al.* 2004; Hegarty *et al.* 2005; Albertin *et al.* 2006).

Although many polyploidization-induced regulatory changes may be conserved across several independent polyploidization events and maintained on an evolutionary time frame, a significant fraction of changes are expected to be random or genotype-specific and may provide additional layers of regulatory polymorphism (Kashkush *et al.* 2002; Adams *et al.* 2003, 2004; He *et al.* 2003;Wang *et al.* 2004, 2006; Liu and Adams 2007; Flagel *et al.* 2008). Because natural hexaploid wheat likely arose from only two chance hybridization events between *T. turgidum* and *Ae. tauschii* (Talbert *et al.* 1998), the amount of regulatory variation captured is expected to be significantly less than what exists in the progenitor species. Systematic efforts to harness additional genetic diversity in wheat breeding programs by utilizing synthetic wheats (Warburton *et al.* 2006) and other wild relatives (Gill *et al.* 2006) have undoubtedly provided a wealth of new regulatory variation. A rapid onset of differential expression of homoeologous loci may also have specific relevance to trait improvement efforts. Several studies utilizing synthetic *T. aestivum* or other amphiploid materials have documented the suppression of disease resistance genes that are highly effective in parental lines (Kerber and Green 1980; Kerber 1983; Innes and Kerber 1994; Kema *et al.* 1995; Ma *et al.* 1995; Ren *et al.* 1996). We speculate that in some cases this is due to differential regulation of homoeologous loci.

Quantitative determination and comparison of gene expression among ploidy levels is a challenge in the synthetic allohexaploid *T. aestivum* system, due to the unbalanced composition of the progenitor diploid and tetraploid genomes. Identification of an infallible approach to estimate and classify quantitative gene expression changes in a new allohexaploid is daunting, if not impossible, especially given the magnitude of gene expression differences observed between maize inbreds and their $F_1$ hybrid progeny [where underdominant, low-parent dominant, high-parent dominant, and overdominant expression patterns are relatively frequent (Swanson-Wagner *et al.* 2006)]. Restating a view presented by Birchler *et al.* (2003) in reference to current efforts to define a molecular basis for heterosis: such difficulties in deciphering how genes behave and interact in an allohexaploid nucleus may lead some to conclude that such an investigation should not be attempted at present; however, the only way to advance is to "chip away at alternatives."

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