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Nonadditive Gene Expression in Diploid and Triploid Hybrids of Maize

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ABSTRACT

The molecular basis of hybrid vigor (heterosis) has remained unknown despite the importance of this phenomenon in evolution and in practical breeding programs. To formulate a molecular basis of heterosis, an understanding of gene expression in inbred and hybrid states is needed. In this study, we examined the amount of various transcripts in hybrid and inbred individuals (B73 and Mo17) to determine whether the quantities of specific messenger RNAs were additive or nonadditive in the hybrids. Further, we examined the levels of the same transcripts in hybrid triploid individuals that had received unequal genomic contributions, one haploid genome from one parent and two from the other. If allelic expression were merely the additive value in hybrids from the two parents, the midparent values would be observed. Our study revealed that a substantial number of genes do not exhibit the midparent value of expression in hybrids. Instead, transcript levels in the diploid hybrids correlate negatively with the levels in diploid inbreds. Although transcript levels were clearly nonadditive, transcript levels in triploid hybrids were affected by genomic dosage.

HETEROSIS refers to the phenomenon in which hybrid offspring of two inbred varieties or lines exhibit characteristics that lie outside the range of the parents (Shull 1908). Although, the phenomenon has been known for centuries, the underlying basis remains elusive. The two classical explanations for this phenomenon, dominance and overdominance, are usually framed in classical genetic terms and, as such, may be inadequate to address the underlying molecular events that result in heterosis (Birchler *et al.* 2003). The dominance hypothesis of heterosis posits that slightly deleterious alleles, which are homozygous in the respective parents, are complemented in the hybrids by superior alleles (Bruce 1910; Jones 1917). If the complementation is additive among loci, the performance of the hybrid would exceed either parent. With overdominance, unlike alleles are postulated to result in a stimulating effect, so that genetic heterozygosity *per se* produces heterosis (East 1936; Hull 1945).

At the gene transcript level, one possibility is that a hybrid will exhibit the cumulative levels of expression of each allele contributed from the respective parents. Alternatively, the hybrid may exhibit nonadditive patterns of expression levels. An increasing number of studies in both the plant and animal kingdoms indicate that nonadditive gene expression is quite common in various types of hybrid situations (Adams *et al.* 2003; Hämmerle and Ferrús 2003; Michalak and Noor 2003; Song and Messing 2003; Gibson *et al.* 2004; Ranz *et al.* 2004). Whether such gene expression patterns are solely responsible for heterosis is not known, but they must certainly contribute to hybrid effects to some degree and thus are deserving of further investigation.

In this study, we examine how mRNA transcript levels of a sample of genes differ in hybrids relative to the inbreds from which they were derived. We have assembled eight genetic constitutions. Two are unrelated inbreds (B73 and Mo17) and two conditions are reciprocal hybrids in which the two inbreds are used alternatively as female and male parents. To assist in defining the effect that the genome of each inbred has upon the hybrid, we have included two classes of triploid hybrids that necessarily have received unequal genomic contributions from the inbred parents. To distinguish the effects of genomic dosage from the effects of ploidy *per se*, two classes of triploids were included that were derived from the original inbred lines. Here we present data on the steady-state transcript levels of a sample of genes in leaf tissue in the eight genotypes. The results indicate the prevalence of nonadditive hybrid expression and that this effect is

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modulated by genomic dosage, which we postulate is a reflection of dosage-dependent gene regulatory mechanisms.

MATERIALS AND METHODS

Stock source and production of genotypes: The initial stocks were obtained from E. Coe, U.S. Department of Agriculture and University of Missouri. Inbred lines B73 and Mo17 in their normal colorless background (*r1* and *c1*) as well as colored versions, in which *R1-scm2* and *C1* color alleles had been introduced, were used. The latter stocks exhibit anthocyanin production in the embryo and aleurone of the kernel.

Production of triploids: Triploids were produced using the trifluralin treatment developed by Kato (1997a, 1999a). Eight to 10 days prior to flowering, the main spikes of tassels of Mo17 *R1-scm2* or B73 *R1-scm2* were exposed by cutting through the enveloping sheaths and sprayed with a solution of 0.2% Treflan H. F. P. (DowElanco, Indianapolis) containing 0.1% Triton X-100 (Fisher Scientific, Pittsburgh). Treflan H. F. P. is an herbicide with 43% trifluralin as the active ingredient. Trifluralin interferes with the microtubules and can cause the second pollen mitosis to fail. As a result, some of the pollen grains possess one diploid sperm rather than the normal two haploid sperm. The consequences of pollinations using such pollen are illustrated in Figure 1 and explained below.

Colorless inbred plants were crossed with pollen from trifluralin-treated *R1-scm2* inbred plants. Kernels that resulted from fertilizations with normal pollen grains with two haploid sperm developed color in both the embryo and the endosperm (Figure 2A). Plants developing from these kernels were diploid and were designated BB if both parents were B73 in origin or MM if both parents were Mo17 in origin. If the female parent was B73 and the pollen parent was Mo17, then the resulting plant was designated BM. Alternatively, if the female parent was Mo17 and the pollen parent was B73, the plant was designated MB.

Pollen grains with one diploid sperm are competent to fertilize only the egg or the central cell. To ensure completion of double fertilization, all ears were pollinated again a day later using pollen from untreated colorless plants (*r1*, *c1*). Therefore, if a single diploid sperm fertilized an egg on the first day, a normal haploid sperm recessive for *r1* and *c1* would have fertilized the central cell on the second day. In this case the embryo was triploid, having received two genomes from the pollen and one genome from the egg. They were easily identified since the embryo was colored and the endosperm was colorless (Figure 2A). These kernels were designated BBB if both parents were B73 in origin and MMM if both parents were Mo17. Hybrids were designated BMM if the female was B73 and MBB if the female was Mo17. When the single diploid sperm fertilized the central cell, it resulted in a tetraploid endosperm that aborted during kernel development (Figure 2A). The pollination procedure calls for the treated pollen to be applied sparsely to the silks; therefore many embryo sacs remained unfertilized on the first day. Fully colorless kernels (Figure 2A) that resulted exclusively from the second pollination were excluded from this study.

Cytological confirmation of ploidy: The chromosome number of each plant was confirmed by examination of root tip spreads (Figure 2B). Ten kernels from each of the eight genotypes (*i.e.*, BB, BBB, *etc.*) were germinated in moist vermiculite at 30°. Root tips were subjected to 1000 kPa of N_2O treatment for 1–3 hr prior to slide preparation according to the method of Kato (1997b, 1999b). The chromosomes were stained with acetic orcein and visualized with a light microscope. Individuals with the incorrect ploidy relative to the genetic classification were replaced. The germinated kernels were transferred to potting soil and grown to maturity in a greenhouse.

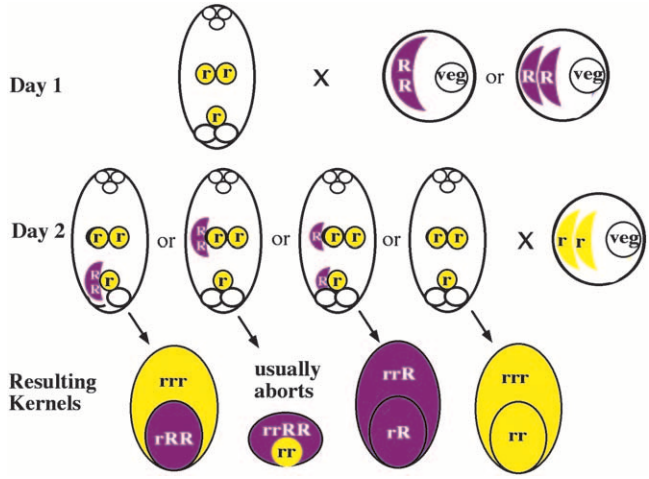

Figure 1.—Diagram of the procedure to generate triploids. Day 1, females of the colorless version of an inbred line homozygous for the recessive *r1* gene receive pollen from plants treated with trifluralin and that carry the dominant *R1-scm2* allele, which conditions full color in the embryo and aleurone of the kernel. (A genome possessing the recessive alleles for color is signified by an r; a genome possessing dominant alleles for color is signified by an R.) On the left is depicted the female gametophyte, *i.e.*, the embryo sac. The egg is portrayed as a single yellow circle toward the bottom and the central cell as a pair of yellow circles. On the right are the two types of pollen: one with a single diploid sperm and one with two normal haploid sperm. Day 2, the four embryo sacs on the left show the types of fertilizations that result from the pollinations on the previous day. Pollen grains possessing a single diploid sperm can effect only a single fertilization, either of the egg (far left) or of the central cell (second embryo sac). The normal pollen grains with two haploid sperm will accomplish double fertilization of both the egg and the central cell (third embryo sac). The pollination procedure uses a sparse application of pollen on day 1, so some embryo sacs remain unfertilized (fourth embryo sac). Double fertilization in the two left embryo sacs is accomplished by a second pollination. For this purpose untreated pollen carrying the recessive allele of *r1* is used. Therefore, these fertilizations will result in tissue that does not produce anthocyanin pigment. The resulting kernels are portrayed at the bottom, indicating the pigment distribution in each.

Of the 80 kernels originally selected for this study, only 3 had ploidy levels that were not predicted from the kernel phenotype: one putative BM was trisomic, one putative MB diploid was triploid, and one putative MMM was diploid rather than triploid. All were immediately replaced in the analysis. Some seedlings died but these were not replaced. It was deemed that there would be too great an age difference between the replacements and their cohorts. Consequently, two genotypes had <10 members: MM had 8 and MMM had 7. It remains a technical possibility that individuals with the correct chromosome number could have been monosomic/trisomic combinations. However, we consider this scenario unlikely because such individuals would be highly defective phenotypically and were not observed in the experiment.

RNA isolation: RNA was extracted from frozen leaf tissue. All tissue was harvested within a 2-hr period on the same day

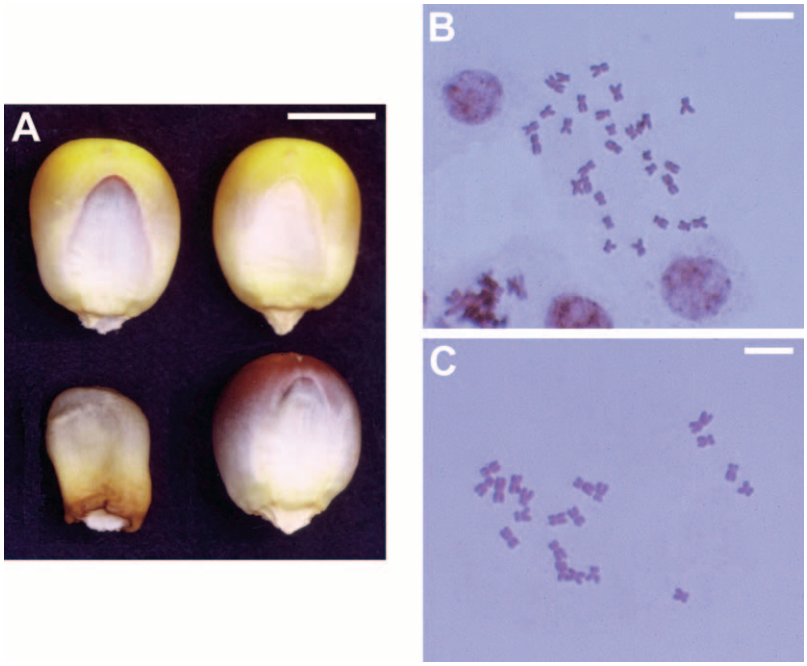

FIGURE 2.—Kernels resulting from trifluralin treatment. (A) The four classes of kernels diagrammatically depicted in Figure 1 are pictured here. The top left kernel resulted from a diploid sperm fertilizing the egg, resulting in a triploid embryo. The bottom left kernel resulted from a diploid sperm fertilizing the central cell, resulting in an abortive tetraploid endosperm. The bottom right-hand kernel resulted from a normal pollen grain with two haploid sperm fertilizing both the egg and the central cell; the embryo is diploid. The top right-hand kernel was the result of both the egg and central cells being fertilized on the second day by normal pollen carrying no color factors. Only the top left and bottom right types of kernels were used in this study. (B) Cytological analysis of a triploid seedling root tip. (C) Cytological analysis of a diploid sibling root tip.

soon after all plants had ceased shedding pollen. Leaf blade tissue, exclusive of midrib and ligule, was taken from three leaves of each plant: the leaf subtending the primary ear and leaves immediately above and below. Leaf tissue from genotypically identical plants (*e.g.*, BB) was pooled. Total RNA was isolated using a method described by CONE *et al.* (1986) with modifications. Tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. About 10 ml of lysis buffer was added per gram of frozen ground tissue. The lysis buffer was 2% sodium dodecyl sulfate (SDS), 0.1 M sodium chloride (NaCl), 50 mM Tris-HCl (pH 7.4), 50 mM disodium ethylenediaminetetraacetate (EDTA, pH 8.0), and 200 μg/ml proteinase K. The supernatant was subjected to two extractions with phenol (pH 7.9):chloroform (1:1) and one with pure chloroform. Samples were centrifuged at 12,000 × *g* for 10 min after each extraction. RNA was precipitated with LiCl (2 M final solution). The RNA was dissolved in formamide. The RNA was diluted 1:9 in water prior to a second precipitation with 2.5 volumes absolute ethanol and 0.1 volume 3 M sodium acetate (pH 5.4). The RNA from the second precipitation was also dissolved in formamide and the concentration was determined by measuring absorbance at 260 nm.

Electrophoresis and blotting: Samples were subjected to electrophoresis in formaldehyde-agarose (1.5%) gels. Each well was loaded with 7.5 μg of total RNA. RNA was transferred to uncharged nylon membranes by capillary blotting. RNA was immobilized on the membranes by UV cross-linking.

Methylene blue staining: Blots were stained with methylene blue (HERRIN and SCHMIDT 1988) to visualize the rRNA, which was used as the loading control. After a 15-min immersion in 5% acetic acid, the blots were placed in the staining solution (0.5 M sodium acetate, pH 5.4; 0.04% methylene blue) and gently agitated for 10 min. Blots were rinsed in deionized distilled water. The moist blots were wrapped in clear plastic film and scanned on a conventional flat bed scanner. The intensities of the 26S rRNA bands were quantified using Image Gauge V3.3 (Fuji Photo Film, Tokyo). Prior to hybridizations, the blots were destained in a solution of 1% SDS and 1× SSPE.

Probe sources: Twenty-nine probes were used to sample differences of steady-state RNA transcript levels among the various genotypes (Table 1). The probes were selected to represent a heterogeneous sample of expressed genes. Many were the same as those used in previous studies of the effects of genetic dosage upon gene expression (GUO and BIRCHLER 1994; GUO *et al.* 1996; AUGER *et al.* 2001; COOPER and BIRCHLER 2001). All probes were derived from clones, which were inserted into plasmids that permit *in vitro* transcription. The sources of the clones are listed in Table 1. No probe was used for the chloroplast rRNA (ct rRNA) because these bands were readily visualized with the methylene blue stain.

Hybridization: The hybridization and labeling procedures used were similar to those reported by BIRCHLER and HIEBERT (1989) with modifications. The blots were prehybridized for up to 4 hr at 65°. A ^{32}P-radiolabeled antisense RNA probe was added and allowed to hybridize for an additional 20 hr at the same temperature. The antisense RNA probes were prepared by *in vitro* transcription. The hybridization solution was 50% formamide, 5× SSC (0.75 M NaCl; 0.075 M sodium citrate, pH 7.0), 10× Denhardt's solution, 0.5% SDS, 10% dextran sulfate, and 0.2 mg/ml fragmented salmon sperm DNA. The blots were washed for 2 hr at 70° with four changes of wash solution. The wash solution was 0.2× SSC and 0.05% SDS.

Measurement and data analysis: To ensure that 26S rRNA was a reliable loading control, three minigels were run (TAE, 1% agarose). Each gel was loaded with total nucleic acids, *i.e.*, DNA and RNA, from each of the genotypes. The total nucleic acids used in each minigel were from separate extractions according to the method described in VERWOERD *et al.* (1989). Instead of precipitating with LiCl, total nucleic acids were precipitated using 2.5 volumes of ethanol. The gels were stained in 0.5 μg/μl solution of ethidium bromide and visualized on an ultraviolet light box. Images were made with a Kodak (Rochester, NY) EDAS camera system and analyzed with Image Gauge V3.3 (Fuji Photo Film). Per ANOVA (P = 0.86) no change in the level of 26 rRNA relative to DNA was detected among the eight genotypes considered in this study (Table 2). By using 26S rRNA as a loading control, the relative RNA quantities determined in each experiment are relative

TABLE 1

List of maize gene clones used as probes for quantitative Northern blotting

Gene	Description	Clone source
adh1	Alcohol dehydrogenase I	Sheldon *et al.* (1983)
sps34	Sucrose phosphate synthase	Pioneer HiBred International (unpublished data)
cox5b	Cytochrome c oxidase subunit 5b	Pioneer HiBred International (unpublished data)
csu5	Thiol protease	University of Missouri, Columbia, Maize RFLP Laboratory
csu17	31k ribonucleoprotein	University of Missouri, Columbia, Maize RFLP Laboratory
csu26	ATP/ADP translocator	University of Missouri, Columbia, Maize RFLP Laboratory
csu30	Vacuolar ATPase proteolipid subunit	University of Missouri, Columbia, Maize RFLP Laboratory
csu31	Unknown	University of Missouri, Columbia, Maize RFLP Laboratory
csu77	Malate dehydrogenase	University of Missouri, Columbia, Maize RFLP Laboratory
csu91	Unknown	University of Missouri, Columbia, Maize RFLP Laboratory
csu93	Unknown	University of Missouri, Columbia, Maize RFLP Laboratory
csu96	Thiol protease inhibitor	University of Missouri, Columbia, Maize RFLP Laboratory
csu137	MADS box gene	University of Missouri, Columbia, Maize RFLP Laboratory
csu140	Glyceraldehyde 3P dehydrogenase	University of Missouri, Columbia, Maize RFLP Laboratory
cyclin	Putative *cyclin delta-3*	Maize Gene Discovery Project, Stanford, California
rip3	Ribosome inactivating protein	R. Boston, North Carolina State University
sh1	Shrunken (sucrose synthase)	Sheldon *et al.* (1983)
sus1	Sucrose synthase I	McCarty *et al.* (1986)
xet2	Xyloglucan endotransglycosylase	Maize Gene Discovery Project, Stanford, California
rrn18	18S mitochondrial rRNA	Mulligan *et al.* (1988)
atpA	ATP synthase α-subunit	Mulligan *et al.* (1988)
cox1	Cytochrome c oxidase subunit 1	E. Kuzmin (unpublished data)
cox2	Cytochrome c oxidase subunit 2	Fox and Leaver (1981)
cox3	Cytochrome c oxidase subunit 3	Hiesel *et al.* (1987)
nad4	NADH dehydrogenase subunit 4	Marienfeld and Newton (1994)
rps3	Ribosomal protein S3	Hunt and Newton (1991)
petA	cytochrome f apoprotein	Barkan *et al.* (1986)
psaB	PSI P700 apoprotein A2	Rodermel and Bogorad (1985)
psaC	PSI-C	Rodermel and Bogorad (1985)

to the quantity of DNA and, therefore, comparable on a per genome basis.

The amounts of hybridized ^{32}P-labeled mRNA were quantified using a phosphorimager (FLA-2000; Fuji Photo Film). Four replicate blots were probed for each mRNA (Table 1). Each blot possessed one lane for each of the eight genotypes (BB, BBB, BM, BMM, MB, MBB, MM, and MMM). The mRNA signal intensity from each genotype was quantified relative to the midparent value of the diploid inbreds. Significance between transcript levels of any two genotypes was determined by *t*-test ($P \leq 0.05$ or $P \leq 0.01$).

TABLE 2

Relative amount of 26S rRNA to DNA for each genotype

Genotype	rRNA/DNA	SE
BB	1.13	0.07
BBB	0.87	0.14
BM	1.16	0.19
BMM	0.92	0.03
MB	0.92	0.08
MBB	1.04	0.11
MM	0.97	0.05
MMM	0.90	0.05

RESULTS

Gene transcript levels: The mRNA transcript levels from each hybridization experiment are compiled in Table 3. For comparison purposes, the mRNA transcript level for each genotype is presented relative to the midpoint between the B73 diploid and Mo17 diploid gene transcript levels. Significant differences between any genotype and the B73-Mo17 midpoints are indicated. To assist in visualizing the data in Table 3, the expression data for one gene, *sh1*, are illustrated (Figure 3). The transcript levels for *sh1* are portrayed in a bar graph, below which is shown the signals from a Northern blot.

Transcript levels in diploid hybrids relative to diploid inbreds: The BM hybrids had 19 of 30 transcript levels significantly different from the B73-Mo17 midpoint transcript level and for MB hybrids 20 were significantly different (Table 3). If gene transcript levels in the hybrids were strictly due to the additive contributions of the parental genomes, then they should reflect the midpoint transcript level of the inbred parents. Clearly, this is not the trend. Nor do transcript levels in hybrids necessarily follow phenotypic parameters, which typically exceed the midparent values. Of the 19 cases where

TABLE 3

Expression levels of various RNA transcripts in each genotype relative to midpoint expression of the B73 and Mo17 diploids

Transcript		BB		BBB		BM		BMM		MB		MBB		MM		MMM	
Name	Genome	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
adh1	Nucl.	1.11*	0.02	1.39**	0.05	0.99	0.05	0.89	0.04	0.92	0.04	0.86*	0.04	0.89	0.07	0.99	0.07
cox5b	Nucl.	1.00	0.02	1.10*	0.02	1.24**	0.05	1.17*	0.07	1.10*	0.01	1.10**	0.01	1.00	0.04	0.93	0.03
csu5	Nucl.	1.09*	0.01	1.36**	0.03	1.03	0.03	1.13*	0.03	0.98	0.07	1.18**	0.02	0.91	0.08	1.02	0.04
csu17	Nucl.	1.07*	0.02	1.14**	0.04	1.06	0.04	1.23**	0.03	1.00	0.02	1.17**	0.01	0.93	0.03	0.99	0.02
csu26	Nucl.	0.98	0.02	1.12*	0.03	0.76**	0.03	1.05	0.06	0.95	0.07	1.03	0.04	1.02	0.04	0.97	0.06
csu30	Nucl.	0.97	0.02	1.09**	0.02	1.09*	0.03	1.07**	0.01	1.24**	0.03	0.96*	0.00	1.03	0.02	1.01	0.03
csu31	Nucl.	0.95	0.04	1.04	0.02	0.74**	0.03	0.81**	0.01	0.74**	0.04	0.91	0.04	1.05	0.05	0.95	0.02
csu77	Nucl.	1.05	0.01	1.11	0.05	0.60**	0.04	0.80**	0.03	0.81**	0.04	0.87**	0.02	0.95	0.06	0.86*	0.03
csu91	Nucl.	0.90*	0.02	0.96	0.01	1.10*	0.02	0.99	0.03	1.02	0.02	0.93	0.03	1.10	0.05	1.04	0.03
csu93	Nucl.	0.97	0.03	1.03	0.06	1.00	0.01	1.15	0.07	1.09	0.06	1.05	0.06	1.03	0.09	0.91	0.07
csu96	Nucl.	0.60**	0.02	0.68**	0.02	1.03	0.02	1.08*	0.02	1.10**	0.00	0.81**	0.01	1.40**	0.04	1.22**	0.04
csu137	Nucl.	0.91**	0.02	1.10**	0.01	1.10**	0.02	1.21**	0.01	1.28**	0.01	1.33**	0.01	1.09*	0.03	0.99	0.03
csu140	Nucl.	0.91**	0.01	0.96	0.02	0.58**	0.03	0.61**	0.01	0.52**	0.01	0.71**	0.01	1.09*	0.03	1.05	0.02
delta3	Nucl.	0.85**	0.03	1.01	0.02	0.64**	0.03	0.80**	0.02	0.68**	0.00	0.75**	0.03	1.15*	0.05	1.10*	0.04
rip3	Nucl.	1.01	0.02	1.05**	0.01	1.08*	0.03	1.07*	0.02	1.07**	0.02	1.06	0.02	0.99	0.03	0.98	0.01
sh1	Nucl.	0.90	0.02	1.70**	0.02	2.50**	0.06	1.75**	0.03	2.32**	0.04	1.24**	0.02	1.03	0.03	1.07	0.05
sps34	Nucl.	0.97	0.03	0.92*	0.02	0.82**	0.03	0.83*	0.05	0.83**	0.03	0.86*	0.04	1.03	0.03	0.97	0.01
sus1	Nucl.	0.73**	0.06	0.69**	0.01	0.88**	0.01	0.99	0.02	0.83**	0.03	0.86**	0.00	1.27**	0.02	1.20**	0.01
xet	Nucl.	0.76**	0.02	0.95	0.02	1.32**	0.07	1.63**	0.04	2.07**	0.05	0.93*	0.01	1.24**	0.04	1.16**	0.01
mt rRNA	Mito.	1.02	0.02	1.03	0.02	1.22**	0.03	1.17*	0.05	1.11	0.04	1.09	0.02	0.98	0.08	0.93	0.02
atpA	Mito.	0.65**	0.02	0.68**	0.03	0.95	0.08	1.00	0.07	0.91*	0.03	0.75**	0.01	1.35**	0.07	1.44**	0.02
cox1	Mito.	0.88**	0.01	0.86**	0.02	0.95	0.05	0.85**	0.03	0.81**	0.01	0.73**	0.03	1.12*	0.05	1.08*	0.02
cox2	Mito.	0.96	0.06	0.85*	0.04	0.87*	0.03	0.85*	0.05	0.76**	0.03	0.69**	0.03	1.04	0.03	0.99	0.05
cox3	Mito.	0.99	0.01	1.08*	0.03	1.06	0.03	0.97	0.01	0.95	0.02	0.91	0.04	1.01	0.05	1.05	0.02
nad4	Mito.	1.00	0.06	1.01	0.04	1.01	0.05	0.96	0.07	0.98	0.03	0.93	0.05	1.00	0.06	1.01	0.06
rps3	Mito.	0.88**	0.02	0.72**	0.01	0.72**	0.02	0.85**	0.02	0.67**	0.02	0.73**	0.01	1.12**	0.02	0.99	0.02
ct rRNA	Chlor.	1.06	0.03	1.05	0.04	1.06	0.02	1.02	0.01	1.04	0.01	1.00	0.01	0.94	0.03	0.99	0.02
petA	Chlor.	0.68**	0.01	0.63**	0.02	0.74	0.03	0.95	0.05	0.70**	0.03	0.71**	0.04	1.32**	0.06	1.32**	0.04
psaB	Chlor.	0.63**	0.01	0.55**	0.02	0.85**	0.02	0.97	0.04	0.82**	0.02	0.71**	0.03	1.37**	0.07	1.37**	0.03
psaC	Chlor.	1.07	0.04	0.95	0.03	0.75**	0.02	0.81**	0.03	0.80**	0.02	0.80**	0.02	0.93	0.05	0.99	0.02

BB, B73 diploid; BBB, B73 triploid; BM, B73/Mo17 diploid; BMM, B73 Mo17 triploid; MB, Mo17/B73 diploid; MBB, Mo17/B73 triploid; MM, Mo17 diploid; MMM, Mo17 triploid; Nucl., nuclear gene; Mito., mitochondrial gene; Chlor., chloroplast gene. *Significantly different from midpoint expression of B73 and Mo17 diploids ($P < 0.05$); **significantly different ($P < 0.01$).

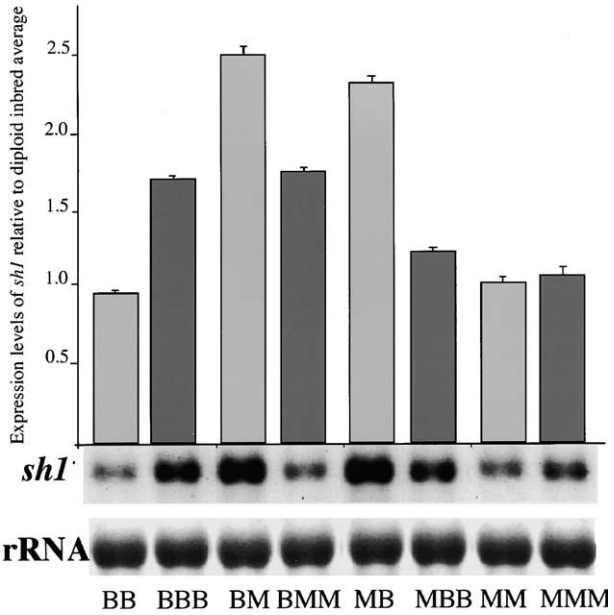

FIGURE 3.—Northern analysis of the transcript levels of the *sh1* locus in the eight genotypes analyzed in this study. The bar graph shows the average expression level of the *sh1* transcript in each genotype. Bars with light shading reflect expression levels in the diploids; Bars with dark shading reflect triploids. The error bars reflect the standard errors of four replicates. The *sh1* mRNA shows a nonadditive expression in diploid and triploid hybrids. The two types of diploid hybrids differ only slightly from each other; however, the two types of triploid hybrids differ much more.

the BM hybrids differed in transcript level from the B73-Mo17 midpoint, 8 exceeded the midpoint, while 11 had lower transcript levels. Of the 20 MB hybrids, 7 were greater and 13 were lower. A lower hybrid expression is found more often with organellar than with nuclear-encoded genes.

However, the transcript levels in the hybrids are not random. Correlations were computed between each pair of genotypes for transcript levels across the 30 genes assayed (Table 4). Transcript levels in the diploid inbreds did not correlate with each other (BB:MM, $r = -0.17$, $P > 0.05$), but they each correlate negatively with either of the diploid hybrids (BB:BM, $r = -0.50$; BB:MB, $r = -0.59$; MM:BM, $r = -0.53$; MM:MB, $r = -0.52$; all $P < 0.01$). Indeed, if transcript levels of the diploid hybrids are correlated against the sum of the diploid inbred transcript levels, then the correlations are much stronger (BM:BB + MM, $r = -0.83$; MB:BB + MM, $r = -0.86$; both $P < 0.01$). The correlation between transcript levels of BM and MB is $r = 0.67$.

Transcript levels in triploids relative to diploids: Before examining the transcript levels in triploid hybrids, the effects of triploidy *per se* need to be considered. The transcript levels of both inbred triploids correlate highly with their diploid counterparts (BBB:BB, $r = 0.72$; MMM:MM, $r = 0.94$; Table 3). The relative differences in transcript levels are summarized in Table 5. The triploid transcript levels are shown as a proportion of their corresponding diploid transcript level. When the triploid transcript level is higher, the proportion exceeds one; when lower, the proportion is less than one. Asterisks indicate those cases where the triploid and diploid transcript levels were significantly different. This was true in almost 40% of the comparisons. Triploids usually had transcript levels that were higher than those of diploid counterparts. This was especially true in the B73 inbred background (BBB/BB) and the hybrids where B73 served as the female (BMM/BM). These results are similar to those of GUO *et al.* (1996) in which 9 of 18 assayed genes had significantly higher transcript levels in the triploid and only one was significantly higher in the diploid. In contrast, in the Mo17 background (MMM/MM), there were fewer significant differences and most of these showed lower transcript levels in the triploids. The hybrids in which Mo17 served as the female showed numerous differences between the triploid and the diploid (MBB/MB) but with no apparent trends. These results illustrate that variation exists in maize for the impact of ploidy on gene expression.

In addition to the effects of triploidy *per se*, we must also consider the effect of parental transmission because the extra genome was delivered through the male in producing the triploids as a necessary condition of the procedure used to create them. If transmission of the

TABLE 4

Correlations of 30 transcript levels between various genotypes

	BBB	BM	BMM	MB	MBB	MM	MMM
BB	0.72**	−0.50**	−0.70**	−0.59**	0.39*	−0.19	−0.17
BBB		−0.16	−0.54**	−0.20	0.45**	−0.59**	−0.54**
BM			0.47**	0.67**	−0.16	−0.53**	−0.49**
BMM				0.71**	0.02	−0.22	−0.26
MB					−0.13	−0.52**	−0.56**
MBB						−0.48**	−0.53**
MM							0.94**

*$P \leq 0.05$; **$P \leq 0.01$.

TABLE 5

Gene transcript levels of triploid plants given as a proportion of their corresponding diploids

Transcript	BBB/BB	BMM/BM	MBB/MB	MMM/MM
adh1	1.25**	0.90	0.93	1.11
cox5b	1.10**	0.94	1.00	0.93
csu5	1.25**	1.10	1.20*	1.12*
csu17	1.07	1.16*	1.17**	1.06
csu26	1.14*	1.38**	1.08	0.95
csu30	1.12*	0.98	0.77**	0.98
csu31	1.09	1.09	1.23*	0.90
csu77	1.06	1.33**	1.07	0.91
csu91	1.07*	0.90*	0.91	0.95
csu93	1.06	1.15	0.96	0.88
csu96	1.13*	1.05	0.74**	0.87*
csu137	1.21**	1.10**	1.04*	0.91*
csu140	1.05	1.05	1.37**	0.96
delta3	1.19**	1.25**	1.10	0.96
rip3	1.04**	0.99	0.99	0.99
sh1	1.75**	0.70**	0.53**	1.04
sps34	0.95	1.01	1.04	0.94**
sus1	0.95**	1.13**	1.04*	0.94*
xet	1.25**	1.23**	0.45**	0.94
mt rRNA	1.01	0.96	0.98	0.95
atpA	1.05	1.05	0.82**	1.07
cox1	0.98	0.89	0.90*	0.96
cox2	0.89	0.98	0.91	0.95
cox3	1.09*	0.92*	0.96	1.04
nad4	1.01	0.95	0.95	1.01
rps3	0.82**	1.18**	1.09	0.88**
ct rRNA	0.99	0.96	0.96	1.05
petA	0.93	1.28*	1.01	1.00
psaB	0.87*	1.14*	0.87*	1.00
psaC	0.89*	1.08	1.00	1.06

*Diploid and triploid were significantly different (*t*-test, $P < 0.05$); **diploid and triploid were significantly different (*t*-test, $P < 0.01$).

TABLE 6

Gene expression transcript levels of various maize genotypes relative to other genotypes

Transcript	BM/MB	BMM/MBB	MM/BB
adh1	1.08	1.03	0.80*
cox5b	1.13	1.06	1.00
csu5	1.05	0.96	0.83**
csu17	1.06	1.05	0.88*
csu26	0.80	1.02	1.03
csu30	0.88*	1.11**	1.06
csu31	1.00	0.89*	1.09
csu77	0.74**	0.92*	0.90*
csu91	1.08	1.06	1.22*
csu93	0.92	1.10	1.07
csu96	0.94*	1.33**	2.33**
csu137	0.86**	0.91**	1.20**
csu140	1.12	0.86**	1.19**
delta3	0.94	1.07	1.35**
rip3	1.01	1.01	0.98
sh1	1.08*	1.41**	1.07
sps34	0.99	0.97	1.06*
sus1	1.06*	1.15**	1.75**
xet	0.64**	1.75**	1.64**
mt rRNA	1.10*	1.07	0.96
atpA	1.04	1.33*	2.08**
cox1	1.17*	1.16*	1.28**
cox2	1.14	1.23*	1.08
cox3	1.12*	1.07	1.02
nad4	1.03	1.03	1.00
rps3	1.07	1.16**	1.27**
ct rRNA	1.02	1.02	0.88*
petA	1.06	1.34*	1.96**
psaB	1.04	1.37**	2.19**
psaC	0.94	1.01	0.86

*Transcript levels of numerator and denominator were significantly different (*t*-test, $P < 0.05$); **transcript levels of numerator and denominator were significantly different (*t*-test, $P < 0.01$).

genome via male *vs.* female differentially affects gene transcript levels, it could obscure the effects of genomic dosage. Transcript levels in BM correlated ($r = 0.67$, $P < 0.01$) with transcript levels in MB. The relative differences between BM and MB for gene transcript levels are shown in Table 6 under BM/MB, where the relative level for each transcript in the BM genotype is shown proportionately to the level in the MB genotype. Clearly, parental transmission did have some effect since one-third of the genes showed significant differences in transcript levels.

In contrast to the diploid hybrids, the triploid hybrids did not correlate with each other (Table 4). In the same fashion as hybrid diploids, the gene transcript levels in the BMM genotype relative to MBB are shown in Table 6. One-half of the genes show significant differences in transcript levels between BMM and MBB. It does not appear that these differences are related to parental transmission to any substantial degree because the relative transcript levels of BM/MB do not correlate with BMM/MBB ($r = 0.18$). In contrast the data do suggest that gene transcript level in the hybrid triploids is related to genomic dosage. Table 6 also shows gene transcript levels in the MM genotype relative to the BB genotype levels. As mentioned above, the transcript levels in these two genotypes do not correlate and for most genes transcript levels are significantly different. The MM/BB and BMM/MBB proportions correlate ($r = 0.621$, $P < 0.01$), which is consistent with the effect of genomic dosage upon gene transcript levels.

DISCUSSION

Our study reveals that a substantial number of genes are not expressed at the midparent level in hybrids. If

gene transcript level were merely the average in hybrids from the two parents, the midparent values would be observed. Clearly, gene expression mechanisms in the hybrid situation do not follow this rule. Similar findings of nonadditive gene expression in a hybrid situation were found by Hämmerle and Ferrús (2003) using enhancer trap lines in Drosophila and by Song and Messing (2003) analyzing zein expression in hybrid endosperms and on protein levels in maize root tips (Romagnoli *et al.* 1990; Leonardi *et al.* 1991). Adams *et al.* (2003) also found unequal contributions from the two genomes in newly synthesized allopolyploids of cotton. Thus, a growing body of data indicates that when diverse genomes are brought together in a hybrid, the gene expression patterns will not be predicted by simply averaging the expression of the parental lines. The work of Hämmerle and Ferrús (2003) argues that this nonadditive expression is a reflection of regulatory interactions in the hybrid given that a single reporter gene is influenced by different enhancers, which would react differently in different positions in the genome.

In this study we examined how gene expression is affected in the two types of triploid hybrids. Our rationale was to test the impact of allele dosage on the nonadditive gene expression. It has previously been noted that gene regulatory hierarchies in multicellular eukaryotes tend to show a dosage-dependent nature and that their genetics behave in a manner similar to quantitative traits (Birchler 1979; Birchler and Newton 1981; Guo and Birchler 1994; Lee *et al.* 1996; Birchler *et al.* 2001; Birchler and Auger 2005). The dosage dependency of gene regulatory mechanisms is likely a reflection of their action within molecular complexes (Veitia 2002; Papp *et al.* 2003). The nonadditive effects in hybrids are impacted by allelic dosage and cannot result from a dominant/recessive complementation of regulatory gene variation, so the sum of the findings is consistent with regulatory interactions producing novel effects on target genes under hybrid conditions.

Besides nonadditivity, the two types of triploid hybrids usually differed from each other in a way that appeared to be related to genomic dosage. As noted above, gene expression levels in diploid hybrids are negatively related to expression levels in diploid inbreds. The relationships among the triploid hybrids and the inbreds, diploid or triploid, are more complex. Gene transcript levels in the BMM genotype correlate negatively with BB ($r = -0.70$, $P < 0.01$) and BBB ($r = -0.54$, $P < 0.01$) but the correlations with MM ($r = -0.21$, $P > 0.05$) and MMM ($r = -0.26$, $P > 0.05$) are not significant. Gene transcript levels in the MBB genotype correlate positively with BB ($r = 0.39$, $P < 0.01$) and BBB ($r = 0.45$, $P < 0.01$) and negatively with MM ($r = -0.52$, $P < 0.01$) and MMM ($r = -0.56$, $P < 0.01$). It appears that in the triploid hybrid the single-copy genome contributes in similar fashion as it does in the hybrid (negative correlation to inbred), while the two-copy genome does not.

These data provide the realization that nonadditive gene expression is quite prevalent in hybrids. The question arises as to whether and how these nonadditive expression levels contribute to heterosis. The triploid data indicate that allelic dosage affects the nonadditivity and therefore that gene regulatory interactions are involved. Further work will be required to determine what spectrum of gene expression, if any, is correlated with heterosis.

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LITERATURE CITED

Adams, K. L., R. Cronn, R. Percifield and J. F. Wendel, 2003 Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. Proc. Natl. Acad. Sci. USA **100:** 4649–4654.

Auger, D. L., K. J. Newton and J. A. Birchler, 2001 Nuclear gene dosage effects upon the expression of maize mitochondrial genes. Genetics **157:** 1711–1721.

Barkan, A., D. Miles and W. C. Taylor, 1986 Chloroplast gene expression in nuclear, photosynthetic mutants of maize. EMBO J. **5:** 1421–1427.

Birchler, J. A., 1979 A study of enzyme activities in a dosage series of the long arm of chromosome one in maize. Genetics **92:** 1211–1229.

Birchler, J. A., and D. L. Auger, 2005 Biological consequences of dosage dependent gene regulatory mechanisms in multicellular eukaryotes, in *Biology of Dominance*, edited by R. Veitia. Landes Bioscience, Georgetown, TX (in press).

Birchler, J. A., and J. C. Hiebert, 1989 Interaction of the *Enhancer of white-apricot* with transposable element alleles at the *white* locus in *Drosophila melanogaster*. Genetics **122:** 129–138.

Birchler, J. A., and K. J. Newton, 1981 Modulation of protein levels in chromosomal dosage series of maize: the biochemical basis of aneuploid syndromes. Genetics **99:** 247–266.

Birchler, J. A., U. Bhadra, M. Pal-Bhadra and D. L. Auger, 2001 Dosage dependent gene regulation in higher eukaryotes: implications for dosage compensation, aneuploid syndromes and quantitative traits. Dev. Biol. **234:** 275–288.

Birchler, J. A., D. L. Auger and N. C. Riddle, 2003 In search of a molecular basis of heterosis. Plant Cell **15:** 2236–2239.

Bruce, A. B., 1910 The Mendelian theory of heredity and the augmentation of vigor. Science **32:** 627–628.

Cone, K. C., F. A. Burr and B. Burr, 1986 Molecular analysis of the maize anthocyanin regulatory locus C1. Proc. Natl. Acad. Sci. USA **83:** 9631–9635.

Cooper, J. L., and J. A. Birchler, 2001 Developmental impact on trans-acting dosage effects in maize aneuploids. Genesis **31:** 64–71.

East, E. M., 1936 Heterosis. Genetics **21:** 375–397.

Fox, T. D., and C. J. Leaver, 1981 The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell **26:** 315–323.

Gibson, G., R. Riley-Berger, L. Harshman, A. Kopp, S. Vacha *et al.*, 2004 Extensive sex-specific non-additivity of gene expression in *Drosophila melanogaster*. Genetics **167:** 1791–1799.

Guo, M., and J. A. Birchler, 1994 Trans-acting dosage effects on the expression of model gene systems in maize aneuploids. Science **266:** 1999–2002.

Guo, M., D. Davis and J. A. Birchler, 1996 Dosage effects on gene expression in a maize ploidy series. Genetics **142:** 1349–1355.
Hämmerle, B., and A. Ferrús, 2003 Expression of enhancers is altered in *Drosophila melanogaster* hybrids. Evol. Dev. **5:** 221–230.
Herrin, D. L., and G. W. Schmidt, 1988 Rapid, reversible staining of northern blots prior to hybridization. Biotechniques **6:** 196–197, 199–200.
Hiesel, R., W. Schobel, W. Schuster and A. Brennicke, 1987 The cytochrome oxidase subunit I and subunit III genes in Oenothera mitochondria are transcribed from identical promoter sequences. EMBO J. **6:** 29–34.
Hull, F. H., 1945 Recurrent selection for specific combining ability in corn. J. Am. Soc. Agron. **37:** 134–135.
Hunt, M., and K. J. Newton, 1991 The NCS3 mutation: genetic evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria. EMBO J. **10:** 1045–1052.
Jones, D. F., 1917 Dominace of linked factors as a means of accounting for heterosis. Genetics **2:** 466–479.
Kato, A., 1997a Induced single fertilization in maize. Sex Plant Reprod. **10:** 96–100.
Kato, A., 1997b An improved method for chromosome counting in maize. Biotech. Histochem. **72:** 249–252.
Kato, A., 1999a Induction of bicellular pollen by trifluralin treatment and occurrence of triploids and aneuploids after fertilization in maize. Genome **42:** 154–157.
Kato, A., 1999b Air drying method using nitrous oxide for chromosome counting in maize. Biotech. Histochem. **74:** 160–166.
Lee, E. A., L. L. Darrah and E. H. Coe, 1996 Dosage effects on morphological and quantitative traits in maize aneuploids. Genome **39:** 898–908.
Leonardi, A., C. Damerval, Y. Herbert, A. Gallais and D. de Vienne, 1991 Association of protein amount polymorphisms (PAP) among maize lines with performances of their hybrids. Theor. Appl. Genet. **82:** 552–560.
Marienfeld, J. R., and K. J. Newton, 1994 The maize NCS2 abnormal growth mutant has a chimeric *nad4-nad7* mitochondrial gene and is associated with reduced complex I function. Genetics **138:** 855–863.
McCarty, D., J. R. Shaw and L. C. Hannah, 1986 The cloning, genetic mapping, and expression of the constitutive sucrose synthase locus of maize. Proc. Natl. Acad. Sci. USA **83:** 9099–9103.
Michalak, P., and M. A. F. Noor, 2003 Genome-wide patterns of expression in Drosophila pure species and hybrid males. Mol. Biol. Evol. **20:** 1070–1076.
Mulligan, R. M., A. P. Maloney and V. Walbot, 1988 RNA processing and multiple transcription initiation sites result in transcript size heterogeneity in maize mitochondria. Mol. Gen. Genet. **211:** 373–380.
Papp, B., C. Pal and L. D. Hurst, 2003 Dosage sensitivity and the evolution of gene families in yeast. Nature **424:** 194–197.
Ranz, J. M., K. Namgyal, G. Gibson and D. L. Hartl, 2004 Anomalies in the expression profile of interspecific hybrids of *Drosophila melanogaster* and *D. simulans*. Genome Res. **14:** 373–379.
Rodermel, S., and L. Bogorad, 1985 Maize plastid photogenes: mapping and photoregulation of transcript levels during light-induced development. J. Cell Biol. **100:** 463–476.
Romagnoli, S., M. Maddaloni, C. Livini and M. Motto, 1990 Relationship between gene expression and hybrid vigor in primary root tips of young maize (*Zea mays* L.) plantlets. Theor. Appl. Genet. **80:** 769–775.
Sheldon, E., R. Ferl, N. Fedoroff and L. C. Hannah, 1983 Isolation and analysis of a genomic clone encoding sucrose synthetase in maize: evidence for two introns in *Sh*. Mol. Gen. Genet. **190:** 421–426.
Shull, G. H., 1908 The composition of a field of maize. Am. Breed. Assoc. Rep. **4:** 296–301.
Song, R., and J. Messing, 2003 Gene expression of a gene family in maize based on non-collinear haplotypes. Proc. Natl. Acad. Sci. USA **100:** 9055–9060.
Veitia, R. A., 2002 Exploring the etiology of haploinsufficiency. BioEssays **24:** 175–184.
Verwoerd, T. C., B. M. Dekker and A. Hoekema, 1989 A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. **17:** 2362.

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