Allele-Specific Expression Patterns Reveal Biases and Embryo-Specific Parent-of-Origin Effects in Hybrid Maize

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We employed allele-specific expression (ASE) analyses to document biased allelic expression in maize ("Zea mays"). A set of 316 quantitative ASE assays were used to profile the relative allelic expression in seedling tissue derived from five maize hybrids. The different hybrids included in this study exhibit a range of heterosis levels; however, we did not observe differences in the frequencies of allelic bias. Allelic biases in gene expression were consistently observed for \( \sim 50\% \) of the genes assayed in hybrid seedlings. The relative proportion of genes that exhibit cis- or trans-acting regulatory variation was very similar among the different genotypes. The cis-acting regulatory variation was more prevalent and resulted in greater expression differences than trans-acting regulatory variation for these genes. The ASE assays were further used to compare the relative expression of the B73 and Mo17 alleles in three tissue types (seedling, immature ear, and embryo) derived from reciprocal hybrids. These comparisons provided evidence for tissue-specific cis-acting variation and for a slight maternal expression bias in \( \sim 20\% \) of genes in embryo tissue. Collectively, these data provide evidence for prevalent cis-acting regulatory variation that contributes to biased allelic expression between genotypes and between tissues.

INTRODUCTION

Variation within a species provides the basis for natural selection or breeding efforts. This variation can be the result of differences in the nature, or in the level, of gene products that are produced. Maize ("Zea mays") exhibits high levels of phenotypic (Flint-Garcia et al., 2005), sequence (Tenailleon et al., 2001; Vroh Bi et al., 2005; Zhao et al., 2006), and transcriptional variation (Ma et al., 2006; Stupar and Springer, 2006; Swanson-Wagner et al., 2006). A fundamental question in biology is how genetic changes lead to transcriptional variation and how genetic and transcriptional variation influences phenotypic variation. For example, the relative contributions and interactions of alleles in an F1 hybrid have been proposed to play a role in heterosis (Birchler et al., 2003; Song and Messing, 2003; Guo et al., 2004; Springer and Stupar, 2007). Similarly, the differential transcription of homoeologous genes in polyploids has been extensively documented (reviewed in Adams, 2007) and may be an important source of novel phenotypic variation.

The regulatory variations that cause differential gene expression can be broadly grouped into two categories: cis-acting and trans-acting variation. A differentially expressed gene exhibits cis-acting variation when the differential expression is caused by factors linked to the differentially expressed alleles, such as differences in promoter sequences or chromatin state. There is a growing list of examples in which cis-acting regulatory variation plays an important role in phenotypic variation (Cong et al., 2002; Clark et al., 2006; Doebley et al., 2006; Konishi et al., 2006; Wray, 2007). A differentially expressed gene exhibits trans-acting variation when the differential expression is caused by factors unlinked to the differentially expressed alleles, such as differences caused by genetic background and regulatory networks.

Approaches for studying allelic differences and identifying cis- and trans-acting regulatory variation include expression quantitative trait loci (eQTL) analysis (Schadt et al., 2003; Kirst et al., 2005; DeCook et al., 2006; West et al., 2007; Keurentjes et al., 2007) and expression profiling of near-isogenic lines (Juenger et al., 2008). Both of these methods assess the total level of transcripts derived from a gene in different genetic lines to assay regulatory variation. Allele-specific expression (ASE) analysis is another method used to study cis- and trans-acting regulatory variation. In ASE analyses, the ratio of the transcripts derived from the two alleles is measured in F1 hybrid and parental mix RNAs to infer regulatory variation (Wittkopp et al., 2004). Both alleles in the F1 hybrid sample are present in the same nucleus and have access to the same regulatory factors; therefore, genes that exhibit a biased ASE ratio in the hybrid are inferred to possess cis-acting regulatory variation. Furthermore, genes that exhibit ASE ratios that are different in the F1 hybrid compared with the parental mixes are inferred to possess trans-acting regulatory variation

ASE techniques are also valuable for identifying tissue- or condition-specific allelic variation or parent-of-origin effects. There is evidence for allelic variation during development (Adams et al., 2004; Adams and Wendel, 2005) and following abiotic stress (Guo et al., 2004). These studies indicate that two alleles may exhibit cis-acting regulatory variation that results in differential responses to developmental or environmental cues. Furthermore, analyses of maternal and paternal allelic expression in reciprocal hybrids have revealed parent-of-origin effects on gene expression in embryo tissues (Vielle-Calzada et al., 2000;
Grimanelli et al., 2005). It has been suggested that these parent-of-origin effects are the result of delayed activation of the paternal alleles. However, there is evidence that at least some genes exhibit biallelic expression at very early stages of vegetative tissue development (Weijers et al., 2001).

We were interested in performing a large-scale screen of ASE in maize to assess the prevalence of cis- and trans-acting regulatory variation, tissue-specific transcriptional variation, and parent-of-origin effects. Understanding the mechanisms and prevalence of natural regulatory variation present within the species will provide further insight into the selectable variation extant within the maize germplasm. In this study, we have monitored the ASE ratios in three vegetative tissues of reciprocal hybrids B73 × Mo17 and Mo17 × B73. Furthermore, given the potential role of allelic variation in heterosis (Guo et al., 2004, 2006; Springer and Stupar, 2007), we monitored the ASE variation in the seedling tissue of four additional maize F1 hybrids, spanning a range of heterotic phenotypes. These data were analyzed to document the prevalence of different regulatory modes, tissue-specific alterations in allelic expression, and parent-of-origin effects observed in maize gene expression.

RESULTS

ASE Assay Development

A set of 355 highly quantitative ASE assays were developed to determine the relative expression of B73 and Mo17 alleles (see Methods for details on assay design and validation; see Supplemental Table 1 online for details on primers, accession numbers, and annotations). These assays were designed based on the availability of single nucleotide polymorphism (SNP) data from Panzea (Zhao et al., 2006), on 454-based cDNA sequencing (Emrich et al., 2007), and on evidence for expression in B73 and Mo17 seedlings (based on Affymetrix microarray analysis in Stupar and Springer, 2006). Each of the assays used in this study passed a quality control test that involved measurements for a series of standards to identify assays that provide quantitative measures for the relative proportion of the two alleles (see ASE assays section in Methods).

The ASE assays were then employed to examine the relative proportion of the two alleles in several tissues and genotypes (Table 1). Three biological replicates were used for each of the different tissues and each of the genotypes. For each tissue by genotype combination, we measured the relative proportion of the two alleles in an F1 genomic DNA control (to determine the ASE ratio derived from an equal mix of the two alleles) provides an allele-specific detection is performed for three different sample

<p>| Table 1. Number of Informative ASE Assays for Each Genotype by Tissue Combination Studied |
|-----------------------------------------------|------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue</th>
<th>ASE Assaysa</th>
<th>DE Genes (% of Genes)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>B73 × Mo17</td>
<td>Immature ear</td>
<td>290</td>
<td>125 (43%)</td>
</tr>
<tr>
<td>B73 × Mo17</td>
<td>Embryo (19 DAP)</td>
<td>285</td>
<td>150 (53%)</td>
</tr>
<tr>
<td>B73 × Mo17</td>
<td>Seedling</td>
<td>266</td>
<td>124 (47%)</td>
</tr>
<tr>
<td>B37 × B73</td>
<td>Seedling</td>
<td>96</td>
<td>48 (50%)</td>
</tr>
<tr>
<td>B37 × B73</td>
<td>Seedling</td>
<td>67</td>
<td>37 (55%)</td>
</tr>
<tr>
<td>Oh43 × B73</td>
<td>Seedling</td>
<td>101</td>
<td>50 (50%)</td>
</tr>
<tr>
<td>Oh43 × Mo17</td>
<td>Seedling</td>
<td>125</td>
<td>62 (50%)</td>
</tr>
</tbody>
</table>

aThe number of ASE assays refers to the number of assays that are quantitative, polymorphic, and expressed in the tissue and genotype. The B73 × Mo17 crosses have a higher number of informative assays because the assays were designed based on sequence polymorphisms between these two genotypes.

bNumber of genes that exhibit differential accumulation in the inbred parent lines based on ASE reading from 1:1 RNA mix sample not equal to ASE reading from F1 genomic DNA (number in parentheses is percentage of genes that exhibit differential expression), DE, differentially expressed.

cFor the B73 × Mo17 genotype, the number of assays and number of differentially expressed genes include data derived from both reciprocal hybrid (B73 × Mo17 and Mo17 × B73) genotypes.

Gene Regulatory Modes Are Similar among Three Different Tissues

ASE expression data can be useful for characterizing the frequency and modes of allelic regulatory variation. To do this, allele-specific detection is performed for three different sample types: F1 hybrid genomic DNA, a 1:1 parental mix RNA, and F1 hybrid RNA. Measurement of ASE from hybrid genomic DNA (which contains equal amounts of the two alleles) provides an experimental control that establishes the ASE value that will be derived from equal inputs of the two alleles. Thus, there is evidence for differential expression (differential accumulation of the gene transcripts) between the inbred lines whenever the ASE ratio from the 1:1 parental mix RNA is different from the F1 genomic DNA control. All genes were divided into differentially expressed and nondifferentially expressed groups based on the comparison of the 1:1 parental mix RNA and the F1 genomic DNA controls (Table 1). We found that ~50% of genes exhibit differential expression in each of the three tissues that were analyzed. To cross-validate the differential expression calls made using ASE data, we compared the ASE ratios between inbreds B73 and Mo17 with Affymetrix microarray data that were previously generated using the same B73 and Mo17 RNAs.
(Stupar and Springer, 2006). The majority of genes that exhibited differential expression between the inbred lines in the Affymetrix analysis also exhibited differential expression (in the same direction) based on the ASE ratios of the 1:1 parental mix RNA samples: 36/45 for seedlings, 22/29 for immature ear, and 56/66 for embryo (see Supplemental Figure 1 online).

The genes that were called differentially expressed by the ASE data were further subclassified according to putative mechanism of allelic regulatory variation. The differentially expressed genes could be classified into four different groups based on the comparisons between the F1 RNA, the 1:1 parental RNA mix, and the F1 DNA control sample (Figure 1, Table 2). A gene that is differentially expressed could be subject to cis- and/or trans-acting regulatory variation. Genes that exhibited similar allelic ratios in the F1 RNA and the F1 DNA control samples but a statistically different allelic ratio in the 1:1 parental RNA mix sample were classified as possessing trans-acting regulatory variation. Genes that exhibited similar allelic ratios in the F1 and 1:1 parental RNA mix samples but a statistically different allelic ratio in the F1 DNA control sample were classified as possessing cis-acting regulatory variation. Genes in which the three samples were all statistically different from one another were classified as possessing both cis- and trans-acting regulatory variation. Additionally, a small number of genes exhibited a significant difference between the 1:1 parental RNA mix and the F1 DNA control sample but no significant difference between the F1 RNA and either of the two other samples. These were classified as “unknown” as they do not logically fit any of the other three classifications. These genes typically displayed low levels of differential expression, thus making them difficult to classify based on statistical tests (Figure 2A).

We classified the regulatory mode for each gene that was sampled in each of the tissue types (Table 2). The relative prevalence of the different regulatory modes was quite similar in the different tissues that were examined. We were able to classify the majority of the differentially expressed genes according to the cis/trans paradigm (Table 2). Strikingly, cis-acting regulatory variation was found to account for differential expression in ~70% of the differentially expressed genes (Table 2). By contrast, only ~5% of the differentially expressed genes were classified as regulated solely by trans-acting regulatory variation. Another ~15% of the genes studied exhibited evidence for both cis-acting and trans-acting regulatory variation (Table 2). Visual examination of the correlation between the F1 RNA and the 1:1 parental RNA mix data suggests that many of the genes classified as possessing both cis- and trans-acting variation are more influenced by cis- than by trans-acting regulatory variation (Figure 2A). In many cases, a gene was assigned to the same mode of regulatory variation in multiple tissues (see Supplemental Table 1 online).

A visual examination of Figure 2A reveals several trends regarding the degree of biased expression of B73 and Mo17 alleles. First, there are several instances of monoaletic expression. Eight genes displayed monoaletic expression in at least one of the three tissues, and four of these genes displayed monoaletic expression in all three tissues. These are genes that are present in the genomic DNA of both inbreds but show cis-acting variation leading to monoaletic expression. Second, the genes exhibiting cis-acting regulatory variation often have high levels of biased expression between the two alleles, whereas the genes exhibiting trans-acting regulatory variation often have relatively minor expression differences. We examined the fold

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**Figure 1.** Definition of Regulatory Variation Based on a Comparison of 1:1 Parental Mix RNA and F1 Hybrid RNA.

Hypothetical examples of each type of regulatory variation are illustrated. The F1 genomic DNA exhibits a standardized value of 0.5 (solid black line). The first three genes are examples of differentially expressed genes such that the 1:1 parental mix RNA is statistically different from the F1 genomic DNA control. The three genes on the right are examples of genes that did not have evidence for differential expression (the 1:1 parental mix RNA is not significantly different from the F1 genomic DNA control). The first gene is an example of cis-acting regulatory variation since the 1:1 parental mix RNA and the F1 RNA both exhibit the same level of biased expression. The second gene is an example of trans-acting regulatory variation because the F1 RNA does not exhibit biased allelic variation. The third gene is an example in which the differential expression is caused partially by cis-acting variation and partially by trans-acting regulatory variation. The nondifferentially expressed genes include a gene with no expression variation between any samples, a gene with low levels of cis-variation, and a gene with variation only in the F1 RNA sample.
change of the higher expressed allele relative to the lower expressed allele for all genes classified as \textit{cis}-, \textit{trans}-, or \textit{cis}- and \textit{trans}-acting regulatory variation using the expression differences calculated from the 1:1 parental mRNA mix (see Supplemental Figure 2 online). All genes classified with \textit{trans}-acting variation exhibit <1.5-fold change in the inbred expression level of the two alleles. By contrast, genes with \textit{cis}- or \textit{cis}- and \textit{trans}-acting regulatory variation exhibit a wider range of fold change values, with >30% of these genes displaying a greater than twofold change between the two alleles.

We also subclassified the genes that did not exhibit differential expression between the parental inbred lines (those without significant evidence for differences between the 1:1 parental mRNA mix and the F1 DNA control samples) (Table 2, Figures 1 and 2B). The majority (~63%) of these genes did not exhibit a significant difference in any of the three comparisons and were classified as “no variation.” The second largest group of these genes (~23%) exhibited a significant difference between the F1 RNA and the F1 DNA control sample, but the F1 and the 1:1 parental mix RNA samples were not different from one another. Visual inspection of these genes (Figure 2B) suggests that many of these genes exhibit \textit{cis}-acting regulatory variation; however, they failed to exceed the statistical threshold for differential expression. Indeed, the majority of these genes display t test P values between 0.05 and 0.15 in a comparison of the 1:1 parental mRNA RNA and the F1 DNA control sample, indicating that they were nearly significant for differential expression between inbreds.

We were interested in determining whether maize hybrids with differing levels of diversity and heterosis would exhibit similar proportions of \textit{cis}- and \textit{trans}-acting regulatory variation. ASE was tested in a series of four additional hybrids that include a hybrid with relatively little diversity between parents (B84 × B73), a hybrid with moderate levels of diversity between parents (B37 × B73), and hybrids with higher levels of diversity between parents (Oh43 × B73 and Oh43 × Mo17). B37, B73, and B84 are all Stiff Stalk Synthetic lines that are in the same heterotic group, with B73 and B84 more closely related to one another than to B37. Oh43 and Mo17 are both Non-Stiff Stalk lines from a second heterotic group that are distantly related to one another. Nei minimum genetic distance estimates range from 0.57 for B84-B73,

<table>
<thead>
<tr>
<th>Result of t Test (P &lt; 0.05)$^a$</th>
<th>Inferred Mechanism</th>
<th>B73 × Mo17 (Immature Ear)$^b$</th>
<th>B73 × Mo17 (Embryo)$^b$</th>
<th>B73 × Mo17 (Seedling)$^b$</th>
<th>B84 × B73 (Seedling)</th>
<th>B73 × B37 (Seedling)</th>
<th>Oh43 × B73 (Seedling)</th>
<th>Oh43 × Mo17 (Seedling)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differentially expressed genes</td>
<td>(1:1 RNA mix ≠ F1 DNA)</td>
<td>$^*$ cis-regulatory variation</td>
<td>88</td>
<td>106</td>
<td>91</td>
<td>23</td>
<td>38</td>
<td>35</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≠ F1 DNA)</td>
<td>$^*$ trans-regulatory variation</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≠ F1 DNA)</td>
<td>cis- and trans-variation</td>
<td>22</td>
<td>24</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≠ F1 DNA)</td>
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<td>5</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≈ F1 DNA)</td>
<td>No variation</td>
<td>76</td>
<td>97</td>
<td>91</td>
<td>29</td>
<td>32</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≈ F1 DNA)</td>
<td>Low levels of cis-variation</td>
<td>54</td>
<td>25</td>
<td>36</td>
<td>5</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≈ F1 DNA)</td>
<td>F1 variation</td>
<td>17</td>
<td>8</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(1:1 RNA mix ≠ F1 DNA)</td>
<td>Unknown</td>
<td>18</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total by tissue and genotype</td>
<td></td>
<td>290</td>
<td>285</td>
<td>266</td>
<td>67</td>
<td>96</td>
<td>101</td>
<td>125</td>
<td>1230</td>
</tr>
</tbody>
</table>

$^a$1:1 RNA mix refers to an equal mix of the RNA from the parent inbreds; F1 refers to the RNA isolated from the F1 hybrid; F1 DNA refers to genomic DNA from the hybrid that will contain equal amounts of the two alleles.

$^b$The regulatory modes for the B73 × Mo17 columns were actually determined using data from both of the reciprocal hybrids (B73 × Mo17 and Mo17 × B73).
0.99 for B37-B73, 1.64 for both Oh43-B73 and Oh43-Mo17, and 2.23 for B73-Mo17 (J. Doebley, personal communication). Hybrids derived from crossing inbreds with higher levels of diversity (i.e., crosses between heterotic groups, such as Oh43 × B73 or B73 × Mo17) generally exhibit higher levels of yield heterosis than hybrids derived from crossing inbreds within the same heterotic groups. For example, B84 × B73 exhibits only 25% better-parent heterosis for yield, while B73 × Mo17 exhibits 65% better-parent heterosis for yield (Zanoni and Dudley, 1989). Therefore, we could assess whether the proportion of biased allelic expression (as suggested in Guo et al., 2004) or the distribution of different modes of regulatory variation was altered in hybrids with altered levels of heterosis for specific traits.

The ASE assays were designed based on SNPs between the B73 and Mo17 inbreds; thus, not all assays were polymorphic in these additional crosses. However, a substantial number of assays were polymorphic in each hybrid (Table 1). As expected, the parental pairs within heterotic groups (B84 and B73; B37 and B73) had fewer polymorphic loci than the parental pairs among heterotic groups (Table 1).

A similar proportion of genes exhibiting biased allelic expression were observed in each of these hybrids compared with the B73 × Mo17 and Mo17 × B73 hybrids (~50% in all hybrids; Table 2). We used the same methods as described above to determine the relative proportions of genes that exhibit evidence for each of the potential modes of regulatory variation (Table 2). The observed distribution is quite similar to that observed for the different tissues of B73 × Mo17 hybrids (Table 2, Figure 2C; see Supplemental Figure 3 online); in all crosses, the majority of genes with biased allelic expression exhibited cis-acting regulatory variation. In many instances, we noted that a particular allele derived from B73 or Mo17 exhibited similar levels of cis-acting variation in multiple hybrids. These findings suggest there is not a direct relationship between the level of diversity/heterosis and the proportion of cis- and trans-modes of regulatory variation between different parental combinations.

(A) and (B) The proportion of the B73 allele detected in a 1:1 parental mix of inbred B73 and Mo17 RNA is graphed on the x axis, and the proportion of transcript derived from the B73 allele in F1 RNA is graphed on the y axis. All three tissues are displayed using different symbols to denote data derived from embryos (circles), immature ears (squares), and seedlings (triangles). Genes were divided into differentially expressed (A) or nondifferentially expressed (B) groups based on a comparison of allele-specific detection of the inbred 1:1 parental mix RNA versus the F1 DNA control samples. The genes are displayed with color coding to indicate their putative regulatory mode (see Table 2 for definition of regulatory classes). Trend lines that predict the expected location for cis- or trans-acting regulatory variation are shown in (A). Trend lines that show the predicted location for cis-acting or F1 regulatory variation are shown in (B).

(C) The genes that exhibit differential expression in the 11-d-old seedling tissues of four additional hybrids are plotted in a graph similar to (A). The proportion of the B73 allele (or the Oh43 allele in the Oh43 × Mo17 cross) was determined in the F1 RNA (y axis) and in a 1:1 mix of the parental inbred lines (x axis). The shape of the symbols denotes the hybrid that was tested, while the color denotes the putative regulatory mode (see Table 2 for definition of regulatory classes). These values represent the mean derived from three biological replicates, and the values have been scaled such that the F1 genomic DNA is equal to 0.5 for each assay.

Figure 2. ASE and Gene Regulatory Modes.
**cis-Acting Variation Affecting ASE Ratios between Tissues**

In addition to studying the regulatory variation, the ASE data can be used to identify examples of tissue-specific allelic variation. The B73 × Mo17 and Mo17 × B73 reciprocal hybrid ASE data were used to determine whether allelic expression varied across the 11-d-old seedlings, immature ear, and 19-DAP embryo tissue types. We used graphical analyses (Figure 3A) and pairwise statistical comparisons of the ASE ratios from the different tissues (Table 3) to search for tissue-specific differences in allelic transcription. A graphical comparison of the ASE ratios across tissue types reveals that the majority of genes exhibit similar ASE ratios in multiple tissues (Figure 3A); however, a substantial proportion on the genes (24 to 31%) exhibited altered allelic ratios in the pairwise tissue comparisons (black spots in Figure 3A; Table 3). Approximately half of the genes (112/233; ~48%) that are expressed in all three tissues displayed a difference in ASE in at least one of the three pairwise tissue comparisons (Figure 3B).

In Supplemental Figure 4 online, we display the ASE ratios in the three different tissue types for 12 representative genes that exhibit variation in the hybrid ASE ratio between tissue types. The six genes displayed in Supplemental Figure 4A online showed similar ASE ratios in two of the tissues, while the third tissue showed significant variation. The six genes in Supplemental Figure 4B online showed significant variation for hybrid ASE ratios in all three tissues studied. We assessed the annotations for the genes that exhibit tissue-specific biases in ASE patterns but did not note any similarities that might point to a common mechanism. These genes include a Cys protease (CA405132), an RNase III domain protein (BM073847), a DnaJ protein (CF627442), a ribosomal protein (CK370651), a mannitol transporter (AY111942), a lethal-leaf spot gene (CO520423), a UBA domain protein (BM347950), and three hypothetical proteins of unknown function.

**Maternal Effects on Allelic Expression in 19-DAP Embryo Tissue**

Parent-of-origin effects have been noted in endosperm tissues for total gene expression levels and for allelic expression levels resulting in imprinting (reviewed in Dilkes and Comai, 2004; Gehring et al., 2004). There is also limited evidence for the potential of delayed transcriptional activation of the paternal genome during embryo development (Vielle-Calzada et al., 2000; Grimamelli et al., 2005). We tested for parent-of-origin effects on gene expression levels by comparing the proportion of gene transcript derived from the B73 allele in reciprocal hybrids. In the absence of parent-of-origin effects on gene expression, the ASE ratio would be unaffected by the directionality of a given cross; for example, the proportion of the B73 allele transcript would the same in reciprocal hybrids B73×Mo17 and Mo17×B73. A t test applying a Benjamini-Hochberg false-discovery correction rate (15%) was used to determine significant differences between reciprocal hybrids for any given gene. No examples of genes with parent-of-origin effects on gene expression were found in seedling or immature ear tissue. However, 57 of the 285 genes that were assayed in 19-DAP embryo tissue displayed statistically significant allelic expression differences in reciprocal hybrids. Parent-of-origin effects could result in a maternal or paternal bias in the expression of the two alleles. Interestingly, all 57 examples of parent-of-origin effects displayed evidence of a maternal bias such that the proportion of the transcript derived from the B73 allele is higher in B73×Mo17 than in Mo17×B73. None of the examples of parent-of-origin bias displayed mono-allelic expression, in which the paternal allele is completely silent.
Instead, these are instances of biallelic expression that show slight bias toward the maternal parent in the embryo tissues (Figure 4A).

To confirm this finding, we computed the difference between the B73 transcript proportion in the maternal hybrid (B73 £ Mo17) versus the paternal hybrid (Mo17 £ B73); this calculation was performed for all expressed genes in each of the three tissue types (Figure 4B). In the absence of parent-of-origin effects, this calculation should reveal no consistent differences between reciprocal hybrids (the distribution would center around 0.0 in Figure 4B). While the distribution for seedling and immature ear did not show a parent-of-origin trend, the distribution for the 19-DAP embryo showed a small but distinct maternal allele expression bias. This suggests that there is a widespread trend toward a slight maternal allele expression bias in 19-DAP embryo tissues, beyond the genes that were identified in our statistical analysis.

**DISCUSSION**

ASE approaches offer important advantages for studying gene regulation. First, the comparison of the two alleles within the same individual allows for control over environmental factors. Both alleles are present within an identical environment and are subjected to identical experimental manipulations in the extraction and analysis of nucleic acids. Second, because we are using hybrid genotypes, the ASE approach allows us to investigate the relative transcription of two alleles in a competitive fashion. Both alleles are subjected to the same genetic background and regulatory networks; therefore, any variation in expression is attributable to allelic differences.

Comparisons between mixed inbred and hybrid RNA samples are powerful because they can distinguish between cis- and trans-acting forms of allelic variation. Furthermore, these comparisons allow for the identification of genes that experience complex transcriptional regulation, such as genes affected by altered regulatory networks or feedback regulation. For example, a gene may be subject to feedback regulation such that the total level of expression is held near a constant level; two different inbreds and their F1 hybrid may exhibit similar overall expression levels for this gene, but ASE analysis may reveal that one of the two alleles is preferentially expressed in the hybrid. In reaching the constant total level of expression, one allele outcompetes the other and represents a larger proportion of transcripts. In this example, the cis-acting regulatory variation between alleles is masked when comparing total gene expression levels in the inbred and hybrid plants.
Analysis of ASE patterns for a set of 316 maize genes revealed several interesting findings regarding allelic variation and gene expression. A significant portion of the genes exhibit biased allelic expression patterns in F1 hybrid plants. Our experimental design allowed us to investigate the causes of biased allelic transcriptional variation in multiple tissues and multiple genotypes. These data shed light upon the prevalence of regulatory variation between maize lines, hybridization effects, allelic variation in different tissues, and parental effects.

Relative Contributions of cis- and trans-Acting Variation

We noted a prevalent representation of cis-acting regulatory variation in maize intraspecific allelic comparisons. Similar to Guo et al. (2004), we noted that a large proportion of these genes exhibit allelic expression biases in the hybrids. Of the genes for which we were able to assign a mode of regulatory variation, we found cis-acting variation far more frequently than trans-acting variation. In addition, the genes with cis-acting regulatory variation exhibit a wide range of expression variation with many examples of greater than twofold change in the expression between the two parental inbred lines. However, the genes with trans-acting regulatory variation exhibit relatively minor differences in gene expression levels. It is worthwhile to note that the exact proportions of cis- and trans-acting variation depend upon the choice of statistical criteria and the number of replications that are employed. For example, further replication may increase the power of statistically detecting small differences between the ASE values of the hybrid and 1:1 parental mix RNAs, thus reducing the proportion of genes classified as cis-acting variation and increasing the proportion classified as a combination of cis- and trans-acting variation. However, while increasing replication may affect the coloration of data in Figures 2A and 2C, it is unlikely to affect the strong positive correlation between the ASE values of the hybrid and the 1:1 parental mixes; therefore, our conclusions about the prevalence of cis-acting effects would not change.

There are similarities and differences between our findings and those of eQTL studies on plants and animals. The finding that cis-acting variation often results in greater differences in total transcript accumulation than trans-acting variation has been noted by several groups performing eQTL analyses (Schadt et al., 2003; Wayne et al., 2004; Hughes et al., 2006; Keurentjes et al., 2007; West et al., 2007). For example, Schadt et al. (2003) reported that 80% of the eQTLs in maize with a logarithm of odds (LOD) score >7.0 mapped in cis. A major difference between our study and previous eQTL studies is the very low proportion of trans-acting regulatory variation that we observed. In many of the eQTL studies of plants (Schadt et al., 2003; Kirst et al., 2005; DeCook et al., 2006; Keurentjes et al., 2007; West et al., 2007), a much larger frequency of trans-acting eQTL were identified; in many cases, trans-acting eQTL were more common than cis-acting eQTL. This difference may be attributed to technical differences between the different types of studies or biological differences between the species examined.

There are several fundamental differences between ASE and eQTL studies that may contribute to the different findings. First, ASE approaches examine the biased allelic expression in the F1 compared with the level of transcript accumulation in the two parents to assign regulatory modes, while eQTL approaches examine the total transcript accumulation in a series of genetically distinct lines to assign regulation modes. Second, it is worth noting that ASE approaches can only study alleles that contain a polymorphism within the coding region, while eQTL approaches are capable of studying polymorphic or nonpolymorphic genes. It is possible that polymorphic genes that can be monitored by ASE may contain a higher rate of linked polymorphisms that result in cis-acting regulatory variation. Conversely, nonpolymorphic genes are likely to have a lower rate of polymorphisms in cis-linked regulatory regions, and some of these genes, which cannot be studied by ASE approaches, are likely to exhibit trans-acting variation. This implies that the ASE approach, when applied on a transcriptome-wide scale, may overestimate the frequency of cis-acting regulatory variation and underestimate the relative frequency of trans-acting variation on gene expression. In addition, eQTL studies will identify epistatic interactions and transgressive segregation, both of which are attributable at least in part to trans-acting variation. Many of the trans-acting eQTL exhibit lower statistical significance than the cis-acting eQTL. In general, the use of a larger population size and more accurate measurement techniques results in the discovery of more trans-acting eQTL that typically explain a small percentage of expression level variance. Therefore, the experimental design can have a significant effect on the proportion of cis- and trans-acting regulatory variation that is detected. Indeed, evidence from using a slightly different experimental design, expression profiling of near-isogenic lines, found that cis-acting variation was more prevalent than trans-acting variation (Juenger et al., 2006).

It is intriguing that cis-acting regulatory variation often exhibits stronger effects than trans-acting regulatory variation in both this study and previous eQTL studies. There are well-known examples (such as the maize anthocyanin biosynthesis pathway) in which alterations in trans-acting factors can have major effects on the expression level of the target genes (Dooner et al., 1991). In addition, changes in a single transcription factor can affect a large number of genes. However, it is likely that trans-acting variants will oftentimes result in greater phenotypic variation due to the affects on multiple target genes and will thus provide a basis for natural or artificial selection (Doebley and Luken, 1998; West et al., 2007). In an artificially selected crop, such as maize, these trans-acting variants that lead to major phenotypic variation might be under strong purifying selection and would become fixed within a population, and these major effects would not segregate in an eQTL or ASE experiment. Alternatively, regulatory networks might display a high degree of redundancy and robustness such that perturbations of a single component only result in minor changes.

It is also possible that different reproductive strategies (inbreeding versus outcrossing), different historical selection pressures (such as domestication), or different rates and mechanisms of genome plasticity will effect the specific types of regulatory variation that persist within each species. It is possible that our finding of predominant cis-acting regulatory variation, which often has strong effects, is partially due to the reproductive mode and selective pressures that act on maize. For instance, the inbred lines used in this study are the products of intense
artificial selection to generate useful inbred lines that will grow in similar regions of the United States. Many of the \textit{trans}-acting allelic variants with major phenotypic effects may have been similarly fixed in these inbred lines, leaving the localized \textit{cis}-acting allelic variants with minor phenotypic effects as the primary sources of variation between the lines. Furthermore, maize genome plasticity and the high levels of variation in the intergenic spaces in the maize genome (Fu and Dooner, 2002; Brunner et al., 2005; Messing and Dooner, 2006; Wang and Dooner, 2006; Springer and Stupar, 2007) may result in prevalent \textit{cis}-acting regulatory variation. By contrast, \textit{Arabidopsis thaliana} is an undomesticated selfing species in which there is significantly less variation in genome microstructure. These attributes may largely determine the modes and rates of allelic regulatory variation, suggesting that our observations in maize may not apply to \textit{Arabidopsis} and other species with these characteristics.

\textbf{Maternal Effects on Embryo Allelic Transcription}

The prevalence of maternal effects on gene expression in 19-DAP embryo tissue was unexpected. Most reports of imprinting phenomena in plants have been restricted to endosperm tissue, and in general there are very few, if any, phenotypic differences between reciprocal hybrids. Indeed, we did not note any significant differences in the allelic expression ratios of reciprocal hybrids in seedling or immature ear tissue. However, in the embryo tissue, we found that 57 (20\%) of the genes exhibited statistically significant differential ASE patterns depending on which parent was the male and the female. In all 57 examples of biased allelic expression patterns, we observed a slight bias toward higher expression of the maternally inherited allele.

There are several potential mechanisms that could contribute to these maternal effects. Differential imprinting would result in biased allelic expression patterns (Dilkes and Comai, 2004). However, there is little evidence for imprinting in vegetative tissues, and in endosperm, there are examples of both maternal and paternal biases in expression (Dilkes and Comai, 2004; Gehring et al., 2004). While gene-specific imprinting has not been widely reported for diploid plant tissues, there are several reports of delayed transcriptional activation of the paternal genome during early development of \textit{Arabidopsis} and maize endosperm and embryo tissues (Vielle-Calzada et al., 2000; Danilevskaya et al., 2003; Grimanelli et al., 2005). At very early embryo development stages, only the maternal alleles are expressed and the activation of the paternal genome may occur in a stochastic fashion. There is some evidence that different genes may experience paternal activation at various stages. It is possible that the minor maternal effects that we observed in 19-DAP embryo tissue are the result of incomplete paternal activation.

\textbf{Implications for Hybridization Effects and Heterosis}

The results of this work allow us to speculate on the potential for hybridization effects and how allelic variation may interact with heterosis. We noted a small but interesting set of genes that do not display differential transcript accumulation in the inbred parents but do display biased allelic expression specifically in the F1 ("F1 variation class" in Figure 2B, Table 2). These allelic expression differences could be the result of a response to hybridization of the two parental inbred lines or could be the result of feedback regulation masking \textit{cis}-acting variation in the two parents (described above). Although the genes that exhibit novel F1 expression patterns were relatively rare (~3\% of genes), it is possible that the altered allelic expression may contribute to phenotypic variation. Similarly, hybridization effects may play an important role in altered gene expression in newly formed allopolyploid species (Adams, 2007; Chen, 2007).

By assessing the ASE in several different genotypes, we could assess the correlation between allelic expression patterns and heterosis. The hybrids used for this study include a cross between highly related parents that exhibits low levels of heterosis (B84 × B73) and crosses with intermediate to high levels of diversity and heterosis (Zanoni and Dudley, 1989). As expected, the number of polymorphic assays is strongly correlated with the genetic diversity between the two parents and hybrid heterosis (for example, B84 and B73 have lower levels of polymorphic assays, genetic diversity, and hybrid heterosis than the other genotypes in this study). However, within the subsets of polymorphic assays, we noted that the relative proportion of different modes of regulatory variation was quite similar in each of the hybrids studied. Increased genetic distance between parents did not correlate with a greater amount of \textit{trans}-acting variation, as might be expected if heterosis is caused by alterations in the transcriptional regulatory networks of hybrids. Our findings suggest that the modes of regulatory variation between different maize crosses are similar regardless of the genetic distance between parents and thus may not correlate with the range of heterosis observed among different crosses.

A previous study by Guo et al. (2006) noted a correlation between hybrid yield and the level of mid-parent expression. In addition, Guo et al. (2006) found that hybrids with lower levels of heterosis exhibited higher levels of paternally biased expression. However, these findings were only present in one of two different growing seasons that were evaluated. We did not observe any consistent paternal allelic bias in our ASE analyses. This lack of paternal allelic bias is similar to that observed during the second growing season monitored by Guo et al. (2006) and may reflect relatively mild growth conditions.

It is difficult to compare the ASE expression studies on hybrids relative to inbred parents with microarray expression studies on similar materials. The microarray expression studies (including Huang et al., 2006; Stupar and Springer, 2006; Swanson-Wagner et al., 2006; Wang et al., 2006; Meyer et al., 2007; Uzarowska et al., 2007) compare the total level of transcript accumulation in hybrids relative to the inbreds to assess additivity or overdominance for expression phenotypes. ASE analyses compare the relative expression of the two alleles in the hybrid and can assess the frequency and levels of biased allelic expression.

\textbf{Tissue-Specific \textit{cis}-Acting Variation and Complementation}

By comparing the allelic expression ratios in several different tissues of F1 hybrid plants, we were able to test for allelic expression variation between different tissues. A substantial proportion of the genes (24 to 31\%) that were tested displayed
variation in allelic expression ratios between two different tissues. There is also evidence for severe differences such that only one of the two alleles is expressed in certain tissues (Figure 3A). The most likely explanation for the different ASE ratios among different tissues is that there is cis-acting variation between the two alleles that has tissue-specific effects on their relative expression levels.

We noted a significant level of ASE variation between different maize tissues. This finding is similar to studies of the homoeologous genes in cotton (Gossypium hirsutum) polyploids, in which genes derived from the A and D subgenomes show different relative expression levels among different tissue types (Adams et al., 2003; Udall et al., 2006; Adams, 2007). Evidence for relative changes in homoeologous gene expression among tissue types have also been identified in soybean (Glycine max; Schlueuter et al., 2006). Additionally, a cotton diploid interspecific hybrid exhibited variable expression ratios of two AdhA alleles among different tissue types (Adams and Wendel, 2005). Similarly, Guo et al. (2004) noted variation for maize alleles in their transcriptional responsiveness to density stress. These studies indicate that the relative transcription level of homologous or homoeologous alleles may be affected by changes in development or environmental conditions.

The tissue-specific ASE differences may also be considered from a perspective of complementation. Maize inbred alleles may acquire different regulatory expression patterns, some of which may be detrimental (i.e., underexpressed or overexpressed) in a specific tissue type. In hybrids, alleles with detrimental tissue-specific expression states may be complemented by the expression state of the homologous allele (Springer and Stupar, 2007). This combination of alleles may provide a more complete suite of transcripts within each tissue type, collectively contributing to whole-plant heterosis. The combination of alleles may also provide enhanced response to environmental stresses as suggested by Guo et al. (2004).

**METHODS**

**Biological Materials**

For seedling tissue, three biological replicates of five maize (Zea mays) inbred lines (B73, Mo17, B84, B37, and Oh43) and six maize F1 hybrids (B73 × Mo17, Mo17 × B73, B37 × B73, B84 × B73, Oh43 × B73, and Oh43 × Mo17; female listed first for each hybrid genotype) were grown for 30 days from seed to well-developed seedlings. All seeds (including the apical meristem) were flash-frozen in liquid nitrogen and stored at −80°C. Tissue samples were pooled within each genotype using tissue-specific ASE differences to monitor allelic heterogeneity. The combination of alleles may provide a more complete suite of transcripts within each tissue type, collectively contributing to whole-plant heterosis. The combination of alleles may also provide enhanced response to environmental stresses.

**Nucleic Acid Isolation and cDNA Synthesis**

Genomic DNA samples were isolated from hybrid B73 × Mo17 seedlings (1:1 genotype mix), B73 × Mo17 endosperm (2:1 genotype mix), Mo17 × B73 endosperm (1:2 genotype mix), and inbred B73 and Mo17 seedlings (to create 1:4 and 4:1 mixes) using Qiagen DNeasy kits (Qiagen). The same methodology was used to isolate genomic DNA from hybrid B37 × B73, B84 × B73, Oh43 × B73, and Oh43 × Mo17 seedlings. All DNA samples were quantified using the Nanodrop spectrophotometer (Nanodrop Technologies).

RNA was isolated from immature ear and seedling tissue using Trizol reagent according to the manufacturer’s instructions (Invitrogen). RNA was isolated from embryo tissue using the plant RNeasy kit, according to the manufacturer’s instructions (Qiagen). All RNA samples were subsequently subjected to DNase treatment and phenol:chloroform extraction. RNA was precipitated with 0.1× volume sodium acetate, pH 5.5, and 2.5× volume ethanol. Resuspended RNAs were further purified using the RNeasy system (Qiagen). RNA samples were quantified using the Nanodrop spectrophotometer (Nanodrop Technologies) and agarose gel electrophoresis. An F1 and a 1:1 parental mix cDNA was synthesized for each biological replicate of each tissue collected. The F1 cDNA was synthesized from the hybrid RNA, while the 1:1 parental mix cDNA was synthesized from a mix of equal amounts of B73 and Mo17 RNA. The cDNAs were reverse transcribed using SuperScript III reverse transcriptase according to the manufacturer’s instructions (Invitrogen).

**ASE Assays**

A set of 557 ASE assays were designed using Sequenom assay design software. A large subset of the genes selected for the ASE analyses were essentially chosen at random; the only criteria being that the genes are expressed in seedling tissues (based on previously conducted microarray data; Stupar and Springer, 2006) and possess a known SNP between B73 and Mo17. The SNPs used to design these assays were derived from Panzea (Zhao et al., 2006) and 454-based cDNA sequencing (Emrich et al., 2007). These ASE assays used multiplex PCR reactions followed by single base extension of the multiplex PCR products. The extension products were then separated and quantified using matrix-assisted laser-desorption ionization time of flight mass spectrometry (Jurinke et al., 2005). Each of the ASE assays were then performed on three biological replicates of a series of genomic DNA standards that include mixes of B73:Mo17 at 4:1, 2:1, 1:1, 1:2, and 1:4 mixes. Analysis of allelic proportions within the DNA standards resulted in the removal of some assays that were not detected or were not polymorphic. Additionally, some assays were removed from the analyses because the measured allelic ratios of the DNA standard mix series did not correlate with the known input ratios. We found that 355 of the 557 assays (64%) passed these criteria, resulting in an r² correlation between the measured and known ratios >0.9 (see Supplemental Table 1 online for the primers and accession numbers for these 355 assays). The 203 unsuccessful assays included 82 for which no PCR products were obtained, 74 for which no polymorphism was detected, and 47 assays that were not quantitative. These 355 quantitative assays were also performed on the genomic DNA from the B37 × B73, B84 × B73, Oh43 × B73, and Oh43 × Mo17 hybrids to determine which assays were polymorphic in each of these hybrids. Following the screening and standardization of the assays, ASE reactions were performed on each of the F1 hybrid and 1:1 parental mix cDNAs. We were able to monitor the ASE ratios for all polymorphic biological replicates of at least one tissue for 316 of the 355 assays.

**Statistical Analyses**

A series of tests was applied to classify gene regulatory modes. First, a two-tailed homoscedastic variance t-test comparison of the inbred 1:1 parental mix RNA versus the F1 genomic DNA standard samples was employed to bin genes as “differentially expressed” (P < 0.05) or no evidence for differential expression (P > 0.05). We then proceeded to
perform two-tailed homoscedastic variance t tests to compare the F1 RNA versus the 1:1 parental mix RNA values and the F1 RNA versus the F1 genomic DNA values. Gene regulatory modes were then classified according to whether these tests were significant at P < 0.05 as described in Table 2 and by Stupar and Springer (2006).

Tissue-specific allelic variation was assessed by comparing the F1 or the 1:1 parental mix RNA ASE values from two different tissues using two-tailed homoscedastic variance t tests. Only genes for which there was a difference (P < 0.05) in the ASE ratio of the B73 and Mo17 alleles for both reciprocal hybrids were deemed significant.

To identify evidence of parental effects on allelic expression ratios, we performed a two-tailed homoscedastic variance t test to compare the proportion of the B73 allele in the B73 × Mo17 hybrid relative to the Mo17 × B73 hybrid. A Benjamini and Hochberg (1995) false discovery correction test (false discovery rate = 15%) was applied to the resulting distribution of P values to minimize type I errors.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Validation of ASE by Affymetrix Values.

Supplemental Figure 2. Analysis of Fold Change for cis- and trans-Acting Regulatory Variants.

Supplemental Figure 3. Analysis of Nondifferentially Expressed Genes in Four Additional Hybrids.

Supplemental Figure 4. Examples of Tissue-Specific Biased Allelic Expression.

Supplemental Table 1. List of All ASE Assays and Classifications.

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