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Disruption of Imprinting by *Mutator* Transposon Insertions in the 5′ Proximal Regions of the *Zea mays Mez1* Locus

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ABSTRACT

Imprinting is a form of epigenetic gene regulation in which alleles are differentially regulated according to the parent of origin. The *Mez1* gene in maize is imprinted such that the maternal allele is expressed in the endosperm while the paternal allele is not expressed. Three novel *Mez1* alleles containing *Mutator* transposon insertions within the promoter were identified. These *mez1-mu* alleles do not affect vegetative expression levels or result in morphological phenotypes. However, these alleles can disrupt imprinted expression of *Mez1*. Maternal inheritance of the *mez-m1* or *mez1-m4* alleles results in activation of the normally silenced paternal allele of *Mez1*. Paternal inheritance of the *mez1-m2* or *mez1-m4* alleles can also result in a loss of silencing of the paternal *Mez1* allele. The paternal disruption of imprinting by transposon insertions may reflect a requirement for sequence elements involved in targeting silencing of the paternal allele. The maternal disruption of imprinting by transposon insertions within the *Mez1* promoter suggests that maternally produced MEZ1 protein may be involved in silencing of the paternal *Mez1* allele. The endosperms with impaired imprinting did not exhibit phenotypic consequences associated with bi-allelic *Mez1* expression.

GENOMIC imprinting is an epigenetic phenomenon that results in the mono-allelic expression of a gene based on its parent of origin. Studies in plants (reviewed in Köhler and Makarevich 2006) and mammals (reviewed in Edwards and Ferguson-Smith 2007) have identified some of the key factors that are involved in the regulation of imprinted expression. In both plants and mammals, epigenetic marks such as DNA methylation and histone modifications are central components of the imprinting mechanism. For example, the DNA methyltransferase *MET1* and the DNA glycosylase *DEMETER* play crucial roles in regulating the imprinted expression of *MEA* in *Arabidopsis thaliana* (Choi *et al.* 2002, 2004; Xiao *et al.* 2003; Gehring *et al.* 2006; Jullien *et al.* 2006a). The putative histone methyltransferase encoded by the maternal *MEA* locus is thought to be important for the silencing of the paternal allele of *MEA*, likely through methylation of histone H3 at lysine 27 (Baroux *et al.* 2006; Gehring *et al.* 2006; Jullien *et al.* 2006b). This and other information has been formulated into a model of an imprinting mechanism in plants (reviewed in Huh *et al.* 2007). However, there are still significant gaps in our understanding of imprinting. In plants, the primary DNA sequences that are responsible for recruiting the epigenetic modifier proteins remain elusive. In addition, there is very little information on the phenotypic consequences of bi-allelic expression for loci that are usually imprinted.

Several sequence motifs in both Arabidopsis and *Zea mays* (maize) have been proposed as important *cis*-regulatory domains for imprinting. Transposons and direct repeats at the *MEA* locus display differential DNA methylation (Gehring *et al.* 2006); however, those regions are not required for imprinting (Luo *et al.* 2000; Spillane *et al.* 2004). A tandemly repeated SINE element is present in the promoter of the imprinted gene *FWA* (Kinoshita *et al.* 2007). Although this sequence is important for imprinting, the presence of a tandem duplication is not required (Fujimoto *et al.* 2008). Regions in the maize imprinted genes *Mez1*, *ZmFie1*, and *ZmFie2* also display differential DNA methylation between the parental alleles in the imprinted tissue. For the *ZmFie1* and *ZmFie2* genes, transposon and repeat sequences, respectively, are present in the 5′-proximal region but are not targeted for differential DNA methylation (Gutierrez-Marcos *et al.* 2006; Hermon *et al.* 2007). Additionally, Gutierrez-Marcos *et al.* (2006) demonstrated imprinted expression and differential DNA methylation of *ZmFie1*-GUS and *ZmFie2*-GFP transcriptional fusions containing ~4 kb of promoter and downstream sequence elements. Danilevskaya *et al.* (2003) proposed a two-CpG island rule as a "mark" for genomic imprinting; however, some other imprinted plant genes do not contain more than one CpG island (Haun *et al.* 2007). A comparison of the primary sequences required for imprinting as defined by trans-

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genic fusions or differential methylation did not identify any conserved sequence motifs.

Although there has been significant progress toward understanding the mechanisms that control imprinted gene expression, our understanding of the biological role of imprinting during seed development remains somewhat obscure. There are examples of paternal activation of imprinted genes in *mea*, *dme*, *fie*, and *met1* mutant backgrounds (Choi *et al.* 2002; Gehring *et al.* 2006; Jullien *et al.* 2006a). However, in these studies the disruption of imprinting is the result of mutation or ectopic expression of one or more crucial regulatory genes. In many of these studies, there is evidence for strong morphological consequences. However, it is unclear whether these phenotypic differences are associated with bi-allelic expression of normally imprinted loci or with altered regulation at other genomic loci. A recent study in Arabidopsis demonstrated that seed production could occur without the contribution of a paternal genome in the endosperm by bypassing genomic imprinting (Nowack *et al.* 2007). The resulting seeds were significantly smaller than wild-type seeds, which the authors suggested was an indication of early angiosperm seed development prior to the evolution of imprinting (Nowack *et al.* 2007). A paternal contribution often increases overall seed size, but may introduce genetic information that results in a misallocation of maternal resources. Imprinting may have evolved as way to capture the advantages of having a paternal genome while still leaving resource allocation under the control of the maternal parent (Haig 2004). The generation of seeds without genomic imprinting is an interesting result; however, it does not address the phenotypic consequence of activating the silent paternal allele of an imprinted gene.

The maize gene *Mez1* encodes a putative histone methyltransferase gene related to the Drosophila PcG gene *Enhancer of zeste* and the Arabidopsis *MEA*, *CLF*, and *SWN* genes (Springer *et al.* 2002). *Mez1* is imprinted in endosperm tissue but exhibits bi-allelic expression in other plant tissues (Haun *et al.* 2007). A differentially methylated region (DMR) was identified in the 5′ proximal sequences of *Mez1* and may play a role in the imprinted expression of *Mez1*. We identified several alleles that contain *Mu* transposon insertions in the 5′ proximal sequences of the *Mez1* gene. These transposon insertion alleles were used to probe the mechanism and effects of imprinted expression at the *Mez1* locus.

MATERIALS AND METHODS

Plant materials and tissue collection: Maize inbred and *mez1-mu* insertion lines were grown using standard field conditions in the summer of 2006 and 2007 in St. Paul. All three *mez1-mu* insertion alleles were independently backcrossed to B73 and Mo17 for three to five generations prior to use in loss-of-imprinting assays. Reciprocal crosses between B73 or Mo17 and the heterozygous *mez1-mu* individuals were performed to generate endosperm tissue in which all kernels were polymorphic for coding-region single nucleotide polymorphisms (SNPs) (see below). Heterozygous individuals were used for the crosses because they produce wild-type kernels for comparisons with the kernels that inherit a *Mu* insertion allele. A total of 67 crosses were made for *mez1-m1* to or by Mo17, for *mez1-m2* to or by B73, and for *mez1-m4* to or by B73. Ears were harvested at 14–16 days after pollination and endosperm tissue from individual kernels was collected by dissection. For each ear, we collected a pooled sample of 12–16 endosperms and an additional 10 individual endosperms. All isolated endosperm tissue was flash frozen in liquid nitrogen and stored at −80° until processed.

Nucleic acid extractions: DNA and/or RNA were extracted for genotyping, expression, and DNA methylation analyses. For genotyping and characterization of the *mez1-mu* alleles, DNA was extracted from leaf tissue of segregating *mez1-mu* families using a CTAB extraction protocol (Springer 2007). For the *mez1-mu* seedling real-time PCR assays, DNA and RNA were extracted from greenhouse-grown 14-day-old seedlings. Seedlings were genotyped for *mez1-mu* insertion alleles using the protocols described below. All above-ground tissue was harvested, flash frozen in liquid nitrogen, and stored at −80°. Tissue was ground to a fine powder and RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. The endosperm tissue was ground in liquid nitrogen and the tissue was divided between the DNA and RNA extractions for the imprinting assays on pooled or individual endosperms. DNA was extracted using the Qiagen DNeasy plant mini kit (Valencia, CA) according to the manufacturer's protocol. RNA was isolated using a SDS/Trizol combination protocol as described previously (Stupar *et al.* 2007). RNA was resuspended in RNAse-free water and quantified on a NanoDrop Spectrophotometer (Wilmington, DE).

First-strand cDNA synthesis was performed to generate template for the real-time PCR assays and imprinting assays. Contaminating DNA was removed by digestion with RQ1 DNase (Promega, Madison, WI). Total RNA (1.5 μg for endosperm cDNA synthesis, 2.5 μg for seedling cDNA synthesis) was mixed with 0.5 μg oligo(dT) (Invitrogen, Carlsbad, CA) and heated to 70° for 5 min followed by 1 min on ice. First-strand cDNA synthesis was performed in a total volume of 30 μl by adding 6 μl 5× reaction buffer, 1.5 μl RNaseOut (Invitrogen), 0.8 μl 25 mm dNTPs, and 1 μl M-MLV reverse transcriptase (Invitrogen). This reaction was incubated at 42° for 50 min followed by 70° for 15 min. The resulting cDNA was purified by phenol:chloroform extraction followed by ethanol precipitation and resuspended in 30 μl ddH_2O.

Genotyping of *mez1-mu* insertion alleles: To facilitate backcrossing and downstream analyses, a PCR-based genotyping assay was developed to screen for the presence of the *Mu* insertions at the *Mez1* locus. To genotype field samples, DNA was extracted from leaf tissue of segregating *mez1-mu* families as described above. The 9242 primer (5′-AGAGAAGCCAACG CCAWCGCCTCYATTTCGTC-3′), specific to the *Mu* terminal inverted repeat (TIR), and the *Mez1*-specific primer Mez1R31 (5′-CATCACCCGTGGAAACCCTAGC-3′) downstream of the *Mu* insertions were used to assay the presence of the *Mu* element. To distinguish between heterozygous *mez1-mu* and homozygous *mez1-mu*, a separate PCR reaction was performed on each sample using the *Mez1*-specific primer Mez1F32 (5′-GACGAGCACACTGGTTTTTCCTACC-3′) upstream of the *Mu* insertion sites with the Mez1R31 primer. PCR reactions were performed in a 15-μl total volume, containing ~25 ng of DNA, 2.5 pmol of each primer, 0.33 units of HotStarTaq polymerase (Qiagen), 1.5 μl 10× reaction buffer, and 0.1 μl of 25 mm dNTPS. PCR conditions were as follows: 94° for 15 min,

35 cycles of 94° for 1 min, 65° for 30 sec, and 72° for 1 min, followed by 72° for 10 min. The PCR products were separated by electrophoresis in a 1% agarose TBE gel and observed by ethidium bromide staining.

To identify the location of each of the *mez1-mu* insertions, DNA was extracted from the leaf tissue of plants homozygous for each of the *mez1-mu* insertion alleles. DNA (1 μl) was used in PCR reactions as described above with 9242/Mez1R31 and 9242/Mez1F32 primers. The appropriate-sized band was verified by gel electrophoresis and the remaining PCR product was purified with a PCR purification kit (Qiagen). PCR products were sequenced using both Mez1R31 and Mez1F32 primers. On the basis of the sequence, the location of the *Mu* insertions were identified by examining where the *Mez1* sequence terminated and the *Mu* TIR sequence began. These same sequence reads were used to obtain ~45 bp of the *Mu* TIR sequence and used for identification of the *Mu* element responsible for each insertion allele.

To monitor imprinting or allele-specific DNA methylation patterns, sequence polymorphisms within the coding region and DMR of *Mez1* were identified in the B73, Mo17, *mez1-m1*, *mez1-m2*, and *mez1-m4* alleles. A region corresponding to the 3′ coding region and UTR was PCR amplified from leaf tissue as described above using the Mez1F33 (5′-CACAAAGAAGCAG ATAAGCGTGGAAAG-3′) and Mez1R33 (5′-TTGGGGCCTAT GAATGACAGGTTC-3′) primers. PCR products were purified using a Qiagen PCR purification kit and sequenced. The genotype for a previously identified cleaved amplified polymorphism sequence (CAPS) marker was determined for each sample (Haun *et al.* 2007). The *Mez1* CAPS assay relies upon a polymorphism in an *Alu*I restriction site. A second region in the DMR was also amplified and sequenced using the Mez1F55 (5′-GGGGGCTAAAACTTGATAATAAACA-3′) and Mez1R52 (5′-CGGTCTCTGTGCATTTGTCCTT-3′) PCR primers. The DMR CAPS assay relies upon a polymorphism in an *Aci*I restriction site.

Quantitative real-time PCR assays: Real-time PCR assays were performed on first-strand cDNA (described above) from seedling or endosperm tissue using the Applied Biosystems custom Taqman gene expression assay (Foster City, CA). cDNA (2 μl) was amplified using an Applied Biosystems 7900HT real-time PCR system in a 20-μl reaction volume. A twofold dilution series (undiluted, 1:2, 1:4, 1:8, and 1:16 dilutions) calibration line was made from B73 seedling cDNA, prepared in an identical manner to the seedling cDNA segregating for *mez1-mu* insertion alleles. Each sample was amplified with primers specific to *Mez1* (forward primer 5′-CCGGTCTCGCCCATCT AG-3′, reverse primer 5′-AGCTCGCACAGCCGAATTA-3′, probe sequence 5′-CCGCCCAGGTCACCAG-3′) and to the maize *GAPC1* gene (GenBank no. X15596; forward primer 5′-CCTCA CCGTCAGAATCGAGAAG-3′, reverse primer 5′-CACATAA CCCATGATACCCTTGAGT-3′, probe sequence 5′-CTCGGAA GCAGCCTTAAT-3′). Three technical replicates were performed for each sample. The average cycle threshold (Ct) values were determined for the three technical replicates. Variations in the amount of starting template were corrected for by calculating a ΔCt value on the basis of the differences between *Mez1* and *GAPC1* Ct values. For each experiment (a comparison of wild type and mutants for each allele), the highest ΔCt was subtracted from each remaining Ct value to generate a relative ΔΔCt. Fold change of *Mez1* expression within each experiment was calculated by the equation $2^{-\Delta\Delta Ct}$.

MassARRAY analysis of allele-specific expression: PCR-based assays for monitoring allele-specific expression on the basis of SNPs were designed using the MassARRAY platform from Sequenom (San Diego) (see Haun *et al.* 2007 for PCR and extension primers for these assays). First-strand cDNA was prepared from both pooled and individual endosperm RNA as described above. Two technical replicates were performed for pooled endosperm cDNA and three technical replicates were performed for individual endosperm cDNA. Mass spectrometry quantification of allele ratios was performed at the University of Minnesota Genotyping Facility. Multiple measurements of the ratio of the maternal and paternal alleles were performed for each of the technical replicates.

CAPS analysis of allele-specific expression: A 30-μl PCR reaction was performed on 2 μl of cDNA from sibling endosperm tissue that was heterozygous for the *mez1-mu* allele or lacked the *mez1-mu* allele with primers corresponding to the *Mez1* 3′ coding region and UTR (Mez1F33 and Mez1R33). Amplified DNA was ethanol precipitated, resuspended in 20 μl of water, and digested with 20 units of *Alu*I at 37° overnight. The enzyme was heat inactivated at 65° for 20 min and separated by electrophoresis in a TBE gel containing 2.5% Metaphor (FMC Bioproducts, Rockland, ME) and observed by ethidium bromide staining.

RACE analyses of transcription start sites: RNA was isolated from endosperm tissue from the B73, A632, and the *mez1-mu* genotypes. This RNA was used for RACE reactions using the FirstChoice RLM-RACE kit (Applied Biosystems) according to the manufacturer's protocol. The gene-specific primers employed were Mez1R40 (5′-TGGGACCAAGATTTCCACCAAA CATAAC-3′) and Mez1R31 (5′-CATCACCCGTGGAAACCC TAGC-3′). The resulting products were purified with a Qiagen PCR purification column and sequenced.

Analysis of allele-specific DNA methylation: DNA from individual heterozygous *mez1-mu* and wild-type sibling endosperms was digested with 10 units of the methyl-sensitive restriction enzyme *Bst*UI overnight at 60°. The digested DNA was ethanol precipitated and resuspended in 20 μl of water. A 30-μl PCR reaction was performed on 2 μl of DNA with primers corresponding to the *Mez1* 5′ DMR region (Mez1F55 and Mez1R52). The PCR reaction was ethanol precipitated, resuspended in 20 μl of water, and digested with 10 units of *Aci*I at 37° overnight. The entire digestion reaction was separated by electrophoresis in a TBE gel containing 2.5% Metaphor (FMC Bioproducts) and observed by ethidium bromide staining. For the bisulphite sequence reactions, 200 ng of genomic DNA was treated with the MethylEasy Xceed bisulfite conversion kit (Human Genetic Signatures, North Ryde, Australia) according to the manufacturer's instructions. Bisulfite-converted DNA was amplified by two rounds of PCR. The primary PCR was performed with the *Mez1* primers BSr2-F2M (5′-GATGGTTT GGTTTTGATGTGA-3′) and BSr2-R3 (5′-TCTTTTCTRTT CCCAAATAATTACCRTATAA-3′) (which specifically target the paternal allele in the genotypes studied) with 1 μl of template bisulfite DNA in a 25-μl reaction. The secondary PCR was carried out using the same primers and 0.5 μl of the primary PCR. No-template controls were performed for both rounds of PCR. PCR conditions were as follows: 94° for 15 min, 30 cycles of 94° for 1 min, 50° for 2 min, and 72° for 2 min, followed by 72° for 10 min. PCR products were purified with a Qiagen PCR purification column and sequenced.

***Hin*fI *Mu* activity assays:** DNA was isolated as described from segregating *mez1-mu* families. Approximately 250 ng of endosperm DNA was digested with 10 units of *Hin*fI or mock digested (50% glycerol, 10 mm Tris–HCl, 50 mm KCl, 1 mm dithiothreitol, 0.1 mm EDTA, 200 μg/ml BSA) at 37° overnight. The reaction was stopped by heat treatment at 80° for 20 min. One microliter was used directly in quantitative real-time PCR assays using a SYBR Green 2× PCR Master Mix (Applied Biosystems) in a 20-μl reaction according to the manufacturer's protocol. DNA from heterozygous *mez1-mu* and wild-type sibling samples were analyzed in duplicate with mu9242 (5′-AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC-3′) and Mez1R31 (5′-CATCACCCGYGGAAACCCTAGC-3′) primers.

Average Ct values were calculated for *Hin*fI and mock-digested samples. The percentage of methylation of the *Hin*fI site was determined for each *mez1-mu* allele according to the following formula: percentage of methylation $= 1/2^{[Ct(HinfI)-Ct(mock)]}$.

RESULTS

Characterization of the *Mu* transposon insertion alleles at the *Mez1* locus: Three *Mutator* (*Mu*) transposon insertion alleles for the *Mez1* locus were identified during a screen of Pioneer Hi-Bred's trait utility system for the corn mutant collection (Bensen *et al.* 1995). These three insertion alleles were backcrossed into the B73 and Mo17 genetic backgrounds for a minimum of three generations (most of the experiments utilized lines backcrossed five times). Further classification of these lines revealed the location and nature of the inserts (Figure 1). The *Mu* insertions (designated *mez1-m1*, *mez1-m2*, and *mez1-m4*) are all located in the 5′-*cis* proximal region of *Mez1*. All three *Mu* insertions are located between the *Mez1* transcription start site (on the basis of RACE and full-length cDNA clones) and the previously identified DMR (Haun *et al.* 2007). The *-m1*, *-m2*, and *-m4* insertions are 84, 197, and 205 bp, respectively, upstream of the wild-type *Mez1* translation initiation codon. The *Mutator* terminal inverted repeat sequences were used to predict the type of *Mu* element that was inserted into each allele, and this was confirmed by PCR assays. However, we have not confirmed that these represent full-length intact elements.

On the basis of the family pedigrees and sequencing of several regions of the *Mez1* locus we were able to infer the likely progenitor haplotype for each of the *Mu* insertion alleles. The *mez1-m1* allele is an insertion of a *MuDR* element into a B73 *Mez1* haplotype. The *Mez1* coding sequence linked to the *mez1-m1* insertion contains B73-like SNPs, and imprinting of the *Mez1* gene can be assayed when this allele is crossed to Mo17. The *mez1-m1* allele has been backcrossed to B73 for at least four generations, so a plant that is heterozygous for the *mez1-m1* insertion will be homozygous for B73-like SNPs in the DMR and coding regions of the *Mez1* locus. The *mez1-m2* and *mez1-m4* alleles are insertions of a *Mu7* and a *Mu4* element, respectively, into an A632 *Mez1* haplotype. The A632 *Mez1* allele has coding-sequence single nucleotide polymorphisms similar to the Mo17 *Mez1* allele. Therefore, the *mez1-m2* and *mez1-m4* alleles can be crossed to B73 to assay imprinting. The *mez1-m2* and *mez1-m4* alleles have been backcrossed to Mo17 for at least three generations, resulting in plants that are heterozygous for the *mez1-m2* or *mez1-m4* insertions and homozygous for Mo17-like SNPs in the coding region and DMR.

The epigenetic state of the *Mu* insertion was determined for each allele by assaying the methylation status of a *Hin*fI site in the TIR. Previous research has demonstrated a strong correlation between *Mu* activity and the methylation of this site (Chandler and Walbot 1986; Chomet *et al.* 1991; Brown and Sundaresan 1992; Martienssen and Baron 1994). No methylation was detected at the *Hin*fI sites present in the *mez1-m1* and *mez1-m4* insertions in DNA isolated from leaf or endosperm tissue isolated from multiple plants (supplemental Table 1). However, moderate levels of methylation were detected in all leaf and endosperm samples tested for *mez1-m2* (supplemental Table 1). This suggests that the *Mu* element in *mez1-m2* has been epigenetically silenced while the *Mu* elements in *mez1-m1* and *mez1-m4* are in an active state.

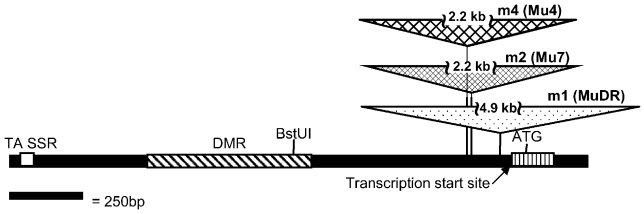

Figure 1.—*Mu* transposon insertions at *Mez1*. Approximately 2000 bp of the *Mez1* 5′-*cis* proximal region is indicated by the thick solid line. The first exon, DMR, and TA simple sequence repeat (SSR) are shown as rectangles. The transcription start site is indicated by an arrow. The location of each *Mu* transposon is shown by triangles. The location of the methyl-sensitive *Bst*UI restriction site used for the methylation assay is also indicated.

***mez1-mu* insertions do not affect reproductive or vegetative development and *Mez1* expression levels in seedlings:** Plants that were heterozygous for each *mez1-mu* allele were self-pollinated to generate seeds segregating 1:2:1 for the *mez1-mu* insertion. The resultant ears were morphologically normal (supplemental Figure 1A). To determine if the *mez1-mu* insertions had an effect on overall seed weight, 100 seeds from the ears described above were individually weighed. A normal distribution of individual seed weights for all three *mez1-mu* alleles was obtained (supplemental Figure 2, A–C). The 12 heaviest and lightest seeds from each self-pollination were germinated and genotyped to verify that certain genotypic classes were not enriched. For each *mez1-mu* allele, the resultant seedlings segregated ~1:2:1 for *mez1-mu* in both the heavy and light seeds (supplemental Figure 2D).

Mez1 is imprinted in endosperm tissue, but expression is bi-allelic in all other tissues tested (Springer *et al.* 2002; Haun *et al.* 2007). Plants heterozygous and homozygous for the *mez1-mu* insertion alleles showed no abnormal growth phenotype or reduced germination relative to their wild-type siblings (supplemental Figure 1, B–D). The expression level of *Mez1* was analyzed in tissue from seedlings segregating for the *mez1-mu* insertion for all three alleles. Quantitative real-time PCR results did not provide evidence for altered expression levels of *Mez1* in seedlings homozygous for *mez1-mu* relative to wild-type or heterozygous siblings (supplemental Figure 1E). The three *Mu* transposons

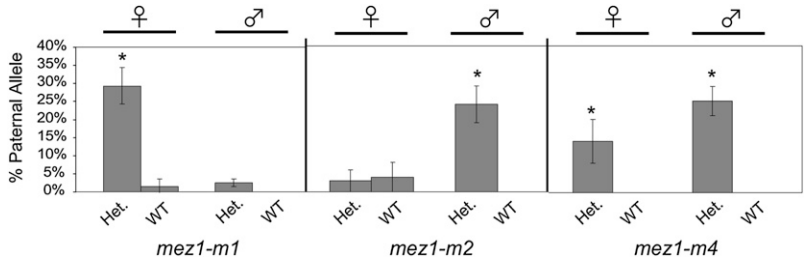

FIGURE 2.—*mez1-mu* insertions result in paternal *Mez1* transcripts in individual endosperms. A quantitative SNP assay (MassARRAY from Sequenom) was used to determine the proportion of *Mez1* transcripts that are derived from the paternal allele (*y*-axis). Each of the three *mez1-mu* alleles was transmitted from the maternal and paternal parent (indicated above the graphs). Individual endosperms were then genotyped for the presence (Het.) or absence (WT) of the *mez1-mu* allele. The proportion of transcripts derived from the paternal allele was determined for four endosperm samples for each heterozygous genotype and for two samples for each wild-type genotype. The asterisk indicates all genotypes that exhibit statistically significant expression from the paternal *Mez1* allele.

insertion alleles do not exhibit altered expression levels of *Mez1* in seedling tissues.

Loss of imprinting is associated with *Mu* insertion alleles of *Mez1*: Although the expression levels of *Mez1* were not affected in seedling tissue, we proceeded to assess whether the *Mu* insertion alleles affected imprinting of *mez1* expression in endosperm. Quantitative SNP assays were used to test for paternal *Mez1* transcripts in pooled endosperm RNA from 67 different crosses involving the *mez1-mu* alleles (supplemental Table 1). When certain *mez1-mu* alleles were transmitted through the maternal or paternal allele, we observed expression of the paternal allele, suggesting that the *Mu* insertion alleles were affecting normal imprinting. We proceeded to analyze individual endosperms that were segregating for the presence of a maternal or paternal *mez1-mu* insertion allele using cleaved amplified polymorphic sequences (supplemental Figure 3) and quantitative SNP analyses (Figure 2). Individual endosperms were isolated and genotyped from two different reciprocal crosses for each of the three *mez1-mu* insertion alleles for a total of 12 ears. From each cross, four heterozygous endosperms and two homozygous wild-type sibling endosperms were identified by molecular genotyping and were used to perform CAPS and quantitative SNP analyses. It should be noted that although we will use the term "heterozygote," the endosperm is triploid and therefore an endosperm with a maternally inherited *mez1-mu* allele carries two copies of the *mez1-mu* allele (Aaa) while an endosperm with a paternally inherited *mez1-mu* allele carries a single copy (AAa).

Allele-specific expression analyses of endosperms segregating for the *mez1-m1* allele reveal evidence for expression of the normally silenced paternal allele when *mez1-m1* is inherited from the maternal parent (Figure 2). There is little or no expression of the paternal allele in wild-type siblings or when the *mez1-m1* allele is inherited from the paternal parent. Inheritance of the *mez1-m2* allele from the paternal parent results in production of *Mez1* transcripts from the paternal allele (Figure 2). Paternal *Mez1* transcripts are detected when the *mez1-m4* allele is inherited from the maternal or paternal parent. These results are in agreement with the screen of pooled endosperms from a large number of crosses (supplemental Table 1). Approximately 15–30% of the *Mez1* transcripts are derived from the paternal allele in endosperms with a loss of imprinting (Figure 2). In some cases (such as maternal transmission of *mez1-m4*), this was significantly lower than the expected 33% and may reflect a partial activation of the paternal *Mez1* allele.

Loss of imprinting at *ZmFie1* upon maternal transmission of *mez1-m1*: The finding that the wild-type paternal allele lost the imprinted expression when a *mez1-m1* or *mez1-m4* allele was maternally inherited was somewhat surprising. One potential model to explain this result is that maternally produced *MEZ1* is required for silencing of the paternal allele similar to the imprinting at *MEA* in Arabidopsis and that *mez1-m1* and *mez1-m4* interfere with the proper timing or level of production of MEZ1, and therefore the paternal allele is not properly silenced. This model might predict that imprinting at other loci would be impaired in endosperms with maternally inherited *mez1-m1* or *mez1-m4*. A quantitative SNP assay was also used to test for loss of imprinting at a second imprinted gene, *ZmFie1*, in crosses of *mez1-m1*. The ability to detect transcripts from the paternal *ZmFie1* allele in each of the endosperms that inherits a maternal *mez-m1* allele provides evidence for a partial loss of imprinting at *ZmFie1* (Figure 3). In contrast, no transcripts were detected from the paternally inherited *ZmFie1* allele in wild-type siblings (Figure 3). Due to a lack of polymorphic markers, we were unable to test for a loss of imprinting at *ZmFie1* in crosses of the *mez1-m4* allele.

Paternal loss of imprinting does not affect allele-specific DNA methylation: The loss of imprinted expression observed upon paternal transmission of *mez1-m2* or *mez1-m4* suggested that the *Mu* insertion may be interfering with the ability to silence the paternal allele. The methylation status of the DMR region of *Mez1* in endosperms segregating for paternally inherited *mez1-m2* and *mez1-m4* was assessed by digesting genomic DNA with a methyl-sensitive restriction enzyme, PCR amplifying the region, and then performing a CAPS analysis. The insertion of a *Mu* transposon did not affect

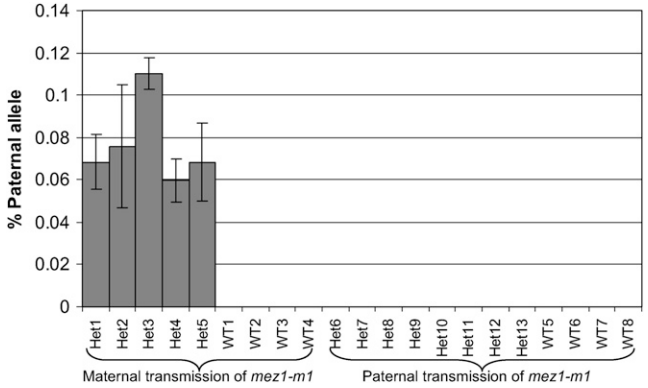

FIGURE 3.—Maternal transmission of *mez1-m1* affects imprinting at *ZmFie1*. The imprinting of *ZmFie1* was assayed in crosses with maternally (left) or paternally (right) transmitted *mez1-m1*. The proportion of *ZmFie1* transcripts derived from the paternal allele (*y*-axis) is shown for 18 individual endosperms.

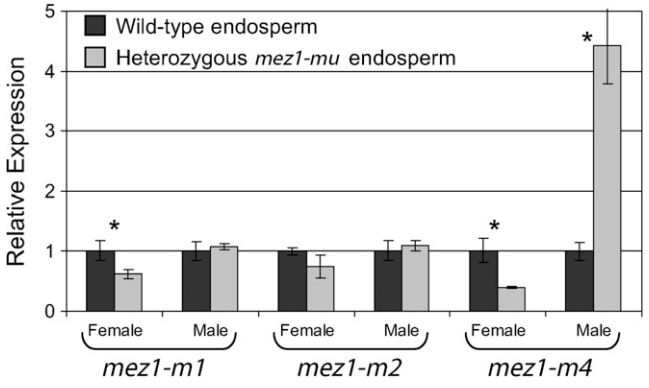

FIGURE 4.—Quantification of the total level of *Mez1* transcripts in *mez1-mu* lines. Real-time PCR comparison of *Mez1* transcript levels in sibling wild-type (solid bars) and heterozygous (shaded bars) *mez1-mu* endosperm tissue. The transcript levels were measured following maternal or paternal inheritance of the *mez1-mu* allele (indicated on the *x*-axis). Ct values were normalized with the *GAPC1* gene (see MATERIALS AND METHODS), and expression was calculated for heterozygous *mez1-mu* endosperms relative to wild-type siblings. The relative expression of *Mez1* was averaged from four heterozygous *mez1-mu* and four wild-type endosperms. Significant differences ($P < 0.05$) in the comparison of wild-type and heterozygous endosperm tissue are indicated by an asterisk.

the methylation status of either parental allele within the DMR (supplemental Figure 3). Despite the fact that the paternal allele was activated, we still observed hypermethylation of the paternal *mez1-m2* or *mez1-m4* allele relative to the wild-type maternal allele within the DMR. These results were further confirmed using bisulfite sequencing of the paternal allele in individual endosperms with paternally inherited *mez1-m2* and *mez1-m4* alleles (data not shown). Sequencing of these PCR products revealed high levels of paternal DNA methylation in a pattern that was very similar to what is observed in wild-type *Mez1* alleles (HAUN *et al.* 2007).

Altered *Mez1* expression levels and transcript start sites in endosperms with a loss of imprinting: The total level of *Mez1* transcripts was assessed in individual endosperms segregating for the three *mez1-mu* insertion alleles using quantitative real-time PCR (Figure 4). Both endosperms that exhibit a maternal loss of imprinting (maternal transmission of *mez1-m1* and *mez1-m4*) exhibit reduced total expression levels of *Mez1* (Figure 4). This is consistent with the hypothesis that the maternal loss of imprinting is due to reduced production of the MEZ1 protein that may be necessary for maintaining silencing of the paternal allele. The *mez1-m2* insertion did not affect total *Mez1* expression levels when maternally or paternally transmitted (Figure 4). Interestingly, maternal transmission of *mez1-m4* resulted in reduced levels of *Mez1* expression while paternal transmission resulted in higher levels of *Mez1* expression (Figure 4). These findings suggest that the endosperms with a maternally induced loss of imprinting exhibit reduced *Mez1* expression while the endosperms with a paternally induced loss of imprinting exhibit equivalent or higher expression levels for *Mez1*.

Transposon insertions within or near the promoter can also result in novel transcripts (BARKAN and MARTIENSSEN 1991; CUI *et al.* 2003). In some cases these transcripts initiate within the *Mu* element (BARKAN and MARTIENSSEN 1991) and in other cases the transcripts initiate outside of the *Mu* element (CUI *et al.* 2003). 5′-RACE PCR was performed on endosperm RNA isolated from wild-type B73 and A632, as well as from *mez1-m1*, *mez1-m2*, and *mez1-m4* genotypes. Surprisingly, we noted that the B73 and A632 *Mez1* transcripts use slightly different initiation sites (Figure 5). All transcripts observed in *mez1-m1* RACE reactions used the same transcription start site as the wild-type B73 allele (Figure 1). The majority of *mez1-m2* transcripts initiated at a site 8 bp 5′ of the A632 wild-type site (Figure 5). There were also some *mez1-m2* transcripts that initiated at a site ~100 bp 5′ of the A632 transcription start site (TSS). The most prevalent *mez1-m4* TSS was located ~120 bp 5′ of the A632 TSS, but we did also observe some transcripts initiating at the major TSS of *mez1-m2*. We did not detect any chimeric transcripts that included any *Mu* sequence, suggesting that we did not observe transcripts that initiate within the *Mu* elements. None of the altered transcripts includes an additional ATG sequence, which suggests that these transcripts are not likely to encode novel proteins. However, it is possible that these altered transcripts may display altered mRNA stability or translation initiation rates.

DISCUSSION

Imprinting is a unique form of gene regulation that results in the expression of a gene from only one of the parental alleles. Many recent studies of imprinted genes

```
                                                    -m4
TAGGCAGCCA GCCCGCTGCC AGCCTCACCC CACACTCCCA
-m2 -m4                          -m2_alt
CAGGCCACAA GGTCACGAGC CGCTCATTTC AAAACAAAAA

CGCCTCTCTC ACCCTCCTCC GCAGCCCCGC TTCACCTCTC

                                        -m1  -m2
TCCCTCCCCC TCTTCTCCCC CCACCCCTCC CTCCCCCACC

      *A632  *B73 / –m1
AAATCCTCGT TCATTTTTTT CCAAGCGACT TCGCGTGTGA

GGGCGGGAGA GCGCGCGGGG CTAGGGTTTC CCGGGTGATG
```

Figure 5.—The proximal promoter and 5′-UTR sequence for *Mez1* is shown. The Met translation initiation codon is shown in boldface type at the 3′-end of the sequence. The insertion sites for the *mez1-m1*, *mez1-m2*, and *mez1-m4* alleles are indicated by inverted triangles. The transcription start sites for B73, A632, and the *mez1-mu* alleles as determined by RACE are indicated by asterisks. Note that in some cases the minor alternate sites are also indicated by _alt. The underlined region is present in the B73 haplotype but not in the A632 haplotype.

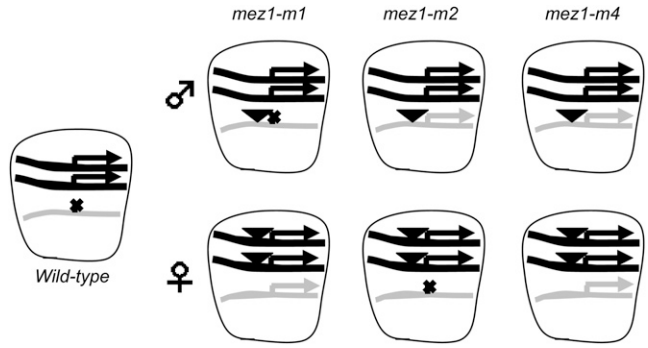

Figure 6.—Diagram illustrating different affects of *mez1-m1*, *mez1-m2*, and *mez1-m4* insertions on *Mez1* imprinting in endosperm tissue. Wild-type endosperm displays mono-allelic expression of the maternal allele of *Mez1*. Diagram of endosperm tissue is shown with the expression state of the maternal (solid lines) and paternal (shaded lines) alleles of *Mez1*. Expression is indicated by an arrow, and lack of expression is indicated by a an "X." Solid triangles indicate the presence of a *Mu* transposon insertion. The allelic expression states are shown for endosperms with a maternally or paternally inherited *mez1-m1*, *mez1-m2*, or *mez1-m4* allele.

in plants have begun to unravel the complex mechanism that allows a cell to distinguish between the two nearly identical parental alleles. In this study we have utilized several *Mutator* insertion alleles of the imprinted *Mez1* gene to further probe the mechanisms of imprinting. These alleles can result in a loss-of-imprinting phenotype. These findings allow us to discuss the biological role of imprinting and to add new information to the plant-imprinting mechanism.

***mez1-mu* alleles provide insights into the imprinting mechanism of plants:** Studies in both maize and Arabidopsis have provided insight into the mechanism that regulates imprinting in plants (reviewed in Huh *et al.* 2007). Recent work has identified key components of that mechanism, including DNA methylation (Vielle-Calzada *et al.* 1999; Xiao *et al.* 2003; Haun *et al.* 2007), histone methylation (Gehring *et al.* 2006; Jullien *et al.* 2006b), and self-imprinting auto-regulation (Baroux *et al.* 2006). The results presented here allow us to further speculate on the mechanism of imprinted expression in plants.

Paternal inheritance of mez1-mu results in activation of paternal Mez1: When the *mez1-m2* or *mez1-m4* alleles are inherited from the paternal parent, we observe a loss of imprinting such that this paternal allele is expressed (Figure 6). The ubiquitous bi-allelic expression of *Mez1* in vegetative tissue (Springer *et al.* 2002) and the presence of a paternal hypermethylated region (Haun *et al.* 2007) suggest that the *Mez1* imprinting mechanism may involve targeted paternal-allele silencing. The presence of a *Mu* element within the *Mez1* promoter may interfere with the ability to recruit silencing factors or with the ability of silencing to spread throughout the critical regulatory regions. However, the observation of differential methylation in the DMR region despite the loss of imprinting suggests that the *Mu* insertion does not affect the ability to establish a DMR and therefore a DNA methylation imprint. The presence of the *Mu* element may block the ability of the chromatin modifications in 5′ regulatory regions to act upon the proximal promoter sequences. An alternative possibility is that the *Mu* element provides promoter activities (Barkan and Martienssen 1991). The finding that the *Mez1* transcripts produced by *mez1-m2* and *mez1-m4* alleles use a novel transcription start site suggests that the *Mu* element provides promoter activities. It is quite possible that the *Mu* promoter activities are not subject to imprinting and therefore these alleles exhibit paternal expression in endosperm tissue. Notably, the *mez1-m1* allele, which does not utilize a novel transcription start site, was properly imprinted when paternally inherited.

Maternal inheritance of mez1-mu results in activation of the paternal allele: Maternal inheritance of *mez1-m1* and *mez1-m4* results in endosperms in which the wild-type paternal allele is not silenced (Figure 5). This finding suggests that the maternal allele provides a *trans*-acting factor that is required for proper silencing of the paternal allele. Recent studies have suggested the MEA protein produced by the maternal allele is involved in establishing and maintaining the silencing of the paternal allele (Baroux *et al.* 2006; Gehring *et al.* 2006; Jullien *et al.* 2006b). If a similar mechanism is involved in regulating *Mez1* imprinting, it is possible that disrupting the expression of the maternal *Mez1* allele could result in a loss of targeted paternal allele silencing. Indeed, for the two alleles that exhibit a loss of imprinting upon maternal transmission, *mez1-m1* and *mez1-m4*, we observed reduced *Mez1* expression levels in these endosperms relative to wild-type siblings. The *mez1-mu* insertions likely reduce the amount of maternal *Mez1* expression or alter temporal and spatial expression patterns, and this reduction disrupts the proper silencing of the paternal allele. The observation that imprint-

ing of both *Mez1* and *ZmFie1* is disrupted when *mez1-m1* is maternally inherited implies that the maternally encoded *Mez1* is required for imprinting at multiple loci.

***mez1-m1*, *mez1-m2*, and *mez1-m4* result in a different pattern of paternal Mez1 activation:** It was somewhat unexpected that these three *mez1-mu* insertion alleles each affect imprinting in slightly different ways despite the fact that all three insertions are clustered within a very small region. Our characterization of the alleles may provide clues about why each has slightly different effects upon imprinting. The use of slightly different transcription start sites may provide an explanation for why the *mez1-m2* and *mez1-m4* alleles exhibit a paternal loss of imprinting but the *mez1-m1* allele does not (see above). Both *mez1-m1* and *mez1-m4* alleles result in a loss of imprinting upon maternal inheritance. Both alleles contain insertions of active *Mu* elements while the *mez1-m2* allele contains an inactive *Mu*. It is possible that these insertion sites condition suppressible *Mu* insertions (Barkan and Martienssen 1991) in which the maternally inherited loss-of-imprinting phenotype requires an active *Mu* element.

Bi-allelic expression of *Mez1* in endosperm tissue has no phenotypic consequence: Many theories, including the "kinship theory of genomic imprinting" (Haig 2004), have been proposed to explain why imprinting has evolved in plants. However, very little is known about why certain plant genes are subjected to imprinting. One of the most important questions regarding the biological importance of imprinting—what is the consequence of a loss of imprinting?—has yet to be addressed. In mice, a study of the imprinted locus *Dlk1-Gtl2* used insertional mutagenesis to generate an allele with a loss of imprinting (Steshina *et al.* 2006). Paternal inheritance of the 15-kb integration allele produced a loss of imprinting of *Dlk1-Gtl2*. This altered the expression levels of *Dlk1* and *Gtl2*, resulting in decreased survival and dwarfism of the offspring (Steshina *et al.* 2006). We were able to study the phenotypes associated with transposon insertion alleles that display bi-allelic expression to determine if the presence or absence of imprinting at *Mez1* affects seed size.

All three *mez1-mu* alleles in this study resulted in a loss-of-imprinted expression of *Mez1* in endosperm tissue. However, the *mez1-mu* alleles had no effect on germination or seedling growth, nor was the weight of heterozygous or homozygous *mez1-mu* seeds different from that of wild-type seeds. Mature *mez1-mu* plants appeared phenotypically normal and developed normal flowers with no decrease in fertility (data not shown). The only observed effect of these *mez1-mu* insertions was the disrupted expression of *Mez1* imprinting in endosperm tissue. Developing seeds on an ear segregating 1:2:1 for the *mez1-m4* allele showed no obvious segregating abnormalities. These results suggest that the silencing of paternal *Mez1* is not necessary for normal endosperm and seed development. The endosperms with maternally impaired imprinting had reduced levels of *Mez1* transcripts but do not exhibit altered phenotypes. In contrast, when paternal transmission of the *mez1-m4* allele disrupts imprinting, there are higher levels of *Mez1* transcripts. The observation of altered *Mez1* expression levels without alterations to kernel size suggests that the level of *Mez1* transcripts is not critical for proper growth and development of the endosperm. It is possible that imprinting of the multiple Polycomb group genes *Mez1* and *ZmFie1* provides redundancy such that the loss of imprinting at one locus has minimal phenotypic consequences.

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