Epigenetic and Genetic Control of Imprinting at the *Mez1* Locus in Maize

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ABSTRACT

Genomic imprinting is the mono-allelic expression of gene based on its parent-of-origin and is important for normal progeny development in plants. The goal of this research was to better classify the epigenetic modifications at the *Zea mays* (maize) imprinted gene *Mez1*, while also investigating the phenotypic consequence of a loss-of-imprinting. The *Mez1* gene in maize is imprinted in endosperm tissue, displaying expression solely from the maternal allele. A differentially methylated region (DMR) was identified in the 5'*cis*-proximal region of *Mez1* in endosperm tissue. In this DMR, the paternal allele displays significantly higher levels of both CpG and CpNpG DNA methylation relative to the corresponding region of the maternal allele. The chromatin modifications of the maternal and paternal alleles of *Mez1* and a second imprinted gene, *ZmFie1*, were studied using allele-specific chromatin immunoprecipitation (ChIP). HistoneH3 and HistoneH4 acetylation are maternally-enriched in endosperm tissue, while HistoneH3 Lysine27 tri-methylation (and to a lesser extent HistoneH3 Lysine27 di-methylation) show paternal allele enrichment. HistoneH3 Lysine9 di-methylation and HistoneH3 Lysine9 tri-methylation do not show parent-specific enrichment. These results suggest DNA methylation and histone modifications are involved in the epigenetic regulation of imprinting in plants.

Numerous studies have focused on understanding the mechanism of imprinting, however relatively little is known about the phenotypic consequence of expressing the normally silent allele of an imprinted gene. Several different alleles containing *Mu* transposon insertions into the 5'*cis*-proximal region of *Mez1* were characterized. Both

maternal and paternal inheritance of *mez1-mu* alleles can result in a loss-of-imprinting. This suggests that *Mu* transposon insertions at the *Mez1* locus can act by disrupting the production of a *trans*-acting factor or interfering with the *cis*-acting elements involved in imprinting. Interestingly, the *mez1-mu* insertions do not effect plant vegetative growth or seed development. These results suggest allelic communication is important between the two parental alleles of imprinted loci.

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Chapter 1
Literature Review

ORIGIN, BIOLOGICAL SIGNIFICANCE AND EVOLUTION OF IMPRINTING

Sexual reproduction in animals and plants involves the fusion of the haploid maternal and paternal gametes, resulting in the diploid offspring. At the DNA level, the primary sequence of the paternal and maternal genomes is nearly identical. However, their respective contributions to the transcriptome can be markedly different. For some loci, there are distinct differences in gene expression patterns based on the parent-of-origin. For instance, one parental allele is expressed while the other is explicitly and completely repressed. This regulation of expression based on the parent-of-origin is referred to as genomic imprinting and is observed in both mammals and plants (Morison *et al*, 2005; Feil and Berger, 2007). Genomic imprinting represents an example of germ-line specific epigenetic regulation. A striking characteristic of genomic imprinting is the differential regulation of identical alleles of the same gene, in the same nucleus. Imprinting is crucial for normal mouse development, yet evidence for imprinted gene expression in other model organisms such as *D. melanogaster* (fruit fly), *C. elegans* (nematode) and *D. rerio* (zebrafish) is questionable.

The term ‘imprinting’ was first used was to describe chromosomal inheritance patterns in the insect *Sciaridea diptera* (Crouse, 1971). Imprinting at a single locus was first demonstrated for the *R* gene in maize through a study looking at differences in phenotype associated with the mode of sexual transmission (Kermicle, 1970). The first gene to display a mono-allelic (expression from one parental allele and not the other) imprinted expression pattern, *Insulin Growth Factor2* (*Igf2*), was discovered in mice (De

Chiara *et al*, 1991). The imprinted expression of *Igf2* is crucial for normal mouse development. Years prior to the discovery of *Igf2* imprinting, it was known that genomic contributions from both parents are necessary for proper embryo development in mouse (reviewed in Reik and Walter, 2001). Artificially created mouse embryos generated from two female pronculei were unable to develop to term, while parthogenetic eggs that received a male pronucleus were viable (Surani *et al*, 1984). Additional experiments involving uniparental disomic mice (embryos that inherit specific chromosomes from one parent only) revealed that only certain regions of the genome require contribution of both parental genomes in order for normal development to occur (Cattanach and Kirk, 1985). These regions contain clusters of imprinted loci and the improper expression of these loci is responsible for the developmental abnormalities (Ohlsson *et al*, 1993 and references therein). Extensions of these studies revealed human diseases such as Prader-Willi, Angelman and Beckwith-Wiedemann syndromes are the consequence of improper regulation of certain imprinted loci (da Rocha and Ferguson-Smith, 2004).

The discovery of imprinting in *Arabidopsis*, like that of mammals, also resulted from studies of embryonic developmental abnormalities. A class of mutants (*FIS* – ***F****ertilization* ***I****ndependent* ***S****eed*) that displayed seed and/or endosperm development in the absence of fertilization was identified in *Arabidopsis thaliana* (Ohad *et al*, 1996; Chaudhury *et al*, 1997). When the *MEDEA* (*MEA*) mutant (also known as *FIS1*) was cloned, it was shown that the *MEA* gene is expressed solely from the maternal allele (Kinoshita *et al*, 1999; Vielle-Calzada *et al*, 1999). The inheritance of a mutant maternal *mea* (or other *fis* allele) results in improper endosperm/seed development and subsequent

seed atrophy and abortion (Ohad *et al*, 1996; Chaudhury *et al*, 1997; Grossniklaus *et al*¸1998). However, inheritance of a mutant *mea* allele from the paternal parent does not have any effect upon endosperm or seed development (Grossniklaus *et al*, 1998).

Many theories have been proposed to explain the biological importance of imprinting and the evolutionary forces that gave rise to this phenomenon. One of the most accepted theories concerning the evolution of imprinting was proposed to explain the evolution of the triploid endosperm tissue in maize (Haig and Westoby, 1989). Moore and Haig (1991) expanded on that initial work, discussing the negative consequences for a developing maize seed when the normal 2:1 ratio of maternal to paternal genomes is altered. The 2:1 ratio results from the double-fertilization event that occurs between a haploid sperm cell and the dikaryotic central cell, giving rise to the triploid endosperm. The theory proposed by Haig and Westoby is known as the genetic conflict hypothesis and is summarized below. [This theory is also referred to as the 'parental tug-of-war' theory and more recently as the 'kinship theory of genomic imprinting' (Haig, 2004).]

In polyandrous species (such as maize), the developing progeny within a brood will be equally related to the mother, but less related to each other. Due to the equal genetic interest in all her offspring, the mother desires to distribute her resources equally among them. On the other hand, the fathers' genetic interest is best served by enabling his offspring to acquire as many resources as possible, even at the expense of the mother and the other developing offspring. An offspring that can obtain more resources has a better chance of surviving, reproducing and carrying on the father's genetic lineage. From a gene expression standpoint, the mother would seek to down-regulate nutrient

acquisition genes and up-regulate fetal growth inhibitors. The father seeks to do the opposite, up-regulate nutrient acquisition genes in his offspring and down-regulate fetal growth inhibitors. As a result, imprinting has evolved as a complex 'tug-of-war' between male and female parents over controlling the expression of certain developmental genes (Haig and Westoby, 1989; Moore and Haig, 1991). Additional theories exist that suggest alternative explanations for the evolution of genomic imprinting (reviewed in Weisstein *et al*, 2002). A detailed explanation of those additional theories is beyond the scope of this review.

Analysis of *Igf2R* gene expression and imprinting status in evolutionary diverse mammals revealed imprinting evolved ~150 million years ago (MYA) (Killian *et al*, 2001). In plants, imprinting is observed in both monocot and dicot species. If imprinting evolved in the common ancestor of all monocots and dicots and was maintained independently after the monocot/dicot split, imprinting in plants evolved at least 140 MYA [the monocot-dicot divergence estimated from molecular sequence data and the fossil record is ~140 MYA (Sanderson *et al*, 2004)].

Disruption of imprinted expression patterns or mutation in certain imprinted genes results in developmental abnormalities in both mammals and plants (Kono *et al*, 2002 and references therein, Grossniklaus *et al*, 1998). Research towards understanding the mechanism of imprinting, how the cell differentiates between two nearly identical alleles and regulates their expression differently, has provided a wealth of knowledge in both mammals and plants. The objective of this research is to explore what genetic and epigenetic mechanisms are involved in regulating imprinting at the *Mez1* locus in *Zea mays* (maize).

IMPRINTING IN MAMMALS

Imprinting has been documented for at least 83 loci in mammals, 29 of which are imprinted in both mice and humans (Morison *et al*, 2005). As mentioned previously, the discovery of imprinting in mammals was made through studies of improper development of embryos with unequal contributions from each parent (reviewed in Reik and Walter, 2001). In addition to improper development in mice, several human diseases (Prader-Willi, Angelman and Beckwith-Wiedemann syndromes) are a consequence of imprinting gene defects (reviewed in Walter and Paulsen, 2003). The importance of imprinting in mammals is evident and understanding the mechanism of how these genes are imprinted may provide cures for the aforementioned diseases as well as providing insight to the complex process of mammalian development.

The general imprinting mechanism in mammals has four stages. Imprints need to be [1] established during development of germ cells, [2] maintained after fertilization and during development of the offspring, [3] read by the cellular machinery (which results in differential gene expression) and [4] erased in the germ cells of the new organism so the cycle can start again (Reik and Walter, 2001). In most cases, the molecular basis of the 'imprint' is differential DNA methylation of the two parental alleles (Edwards and Ferguson-Smith, 2007). The methylation of DNA is enzymatic process, carried out by three functional DNA methyltransferases in mammals, DNMT1, DNMT3a and DNMT3b (Bestor, 2000). These differentially methylated regions (DMRs) are a critical component of the mammalian imprinting mechanism (see below). A fundamental characteristic of this mechanism is that DNA methylation is regulated differently at imprinted loci than at

other genomic regions. Following fertilization, there is genome-wide demethylation and a wave of *de novo* methylation after implantation. Differentially methylated regions resist this demethylation and *de novo* methylation and imprinting marks remain true to their parental origin (reviewed in Reik and Walter, 2001).

Recent experiments have provided additional insight into the imprinting mechanism in mammals. Many of the imprinted genes in mammals are organized into clusters, each containing multiple imprinted genes (Edwards and Ferguson-Smith, 2007). Each cluster typically has three characteristic components: several protein-coding genes, a major *cis*-acting imprinting control region (ICR) and at least one non-coding RNA (Edwards and Ferguson-Smith, 2007). Other regulatory regions may play a role in the regulation of a particular cluster, however the expression of all the imprinted genes within a cluster is controlled by the single ICR. A critical component to the regulation of imprinting clusters is the differential methylation of the maternal and paternal ICRs. The ICR typically contains a DMR and the regulation of these clusters is dependent on which parental allele contains the methylated ICR.

The regulation of clusters occurs in one of two ways; ICRs can be methylated in the maternal germline (the most common) or ICRs can be methylated in the paternal germline. Although each cluster has unique components to its regulation, a few characteristics are conserved between clusters. The mechanism regulating clusters that inherit a methylated paternal ICR is not well defined. Clusters that inherit a methylated maternal ICR, such as the *Igf2/H19* cluster are better understood. The unmethylated paternal ICR in the *Igf2/H19* cluster acts as a promoter for a paternally-expressed non-coding RNA (ncRNA). This ncRNA is usually antisense to at least one gene in the

cluster. It is still unclear if the expression of the antisense ncRNA itself is responsible for the repression of all the silenced genes in the cluster. The mechanism for *Igf2/H19* is well understood, but appears to lack similarity with other imprinting clusters with a maternally methylated ICR (reviewed in Edwards and Ferguson-Smith, 2007).

In summary, studies in mammals have revealed three key characteristics of the imprinting mechanism. First, DNA methylation is an important epigenetic mark within imprinting control regions. As described above, the parental allele that contributes the methylated ICR dictates the manner in which the imprinted gene (cluster) is regulated. Second, DNA methylation within imprinting clusters is resistant to the genome-wide epigenetic resetting that occurs after fertilization. Third, antisense transcription of ncRNAs also appears to playing a role in regulating the expression of imprinted genes within a cluster. The discovery of imprinting in mammals originated with studies on embryo development and revealed a requirement for a genetic contribution from both parents.

IMPRINTING IN PLANTS

Similar to the discovery of imprinted genes in mammals, imprinting in plants was identified through studies of improper endosperm/embryo development. Early studies revealed certain mutations resulting in seed abortion require a functional maternal contribution and that the phenotype caused by these mutations could not be rescued by a wild-type paternal allele (Grossniklaus *et al*, 1998). Further experimentation identified a handful of genes in plants that displayed mono-allelic expression patterns. Current

research (and the goal of this project) is working toward understanding the underlying mechanisms that control imprinted gene expression in plants.

While imprinting in plants and mammals shares many characteristics, the remainder of this review will focus on components specific to imprinting in plants. Imprinting in plants has only been identified in angiosperms, where it is confined to the triploid endosperm tissue. The endosperm tissue is a nutritive food source for the developing embryo and is the result of a double fertilization event between a haploid sperm cell and the dikaryotic central cell. While imprinting may exist in embryo or somatic tissues, examples of this in plants have remained elusive. The location of imprinting in the endosperm is an important component of the imprinting mechanism in plants. The endosperm is a terminal tissue and makes no genetic contribution to the next generation. Therefore, imprinting marks in plants are not inherited by the progeny and do not need erasure in the progeny's primordial germ cells in order to re-initiate the imprinting sequence in the next generation. In this review, I will discuss the different classifications of plant imprinted genes, list some of the examples of imprinting and give a detailed overview of a model for how genes are imprinted in plants.

Currently, there are a handful of examples of imprinted genes in plants (Table 1). Imprinted genes in plants are categorized into two main groups, gene-specific or allele-specific imprinting. Examples in the gene-specific category display the characteristic parent-of-origin expression pattern in all alleles tested from multiple genotypic lines. This is in contrast to the allele-specific examples of plant-imprinted genes, in which only a few alleles display a parent-of-origin expression pattern. For example, an analysis of the maize *Dzr1* gene in three inbred lines (Mo17, BSSS53 and W64A) revealed imprinted

expression only when the Mo17 allele was inherited maternally. Crosses between BSSS53 and W64Aa or crosses with Mo17 as the paternal parent did not result in the imprinted expression of *Dzr1* (Chaudhuri and Messing, 1994). Four loci in maize demonstrate allele-specific imprinting: *R*, *Zein*, *α-Tubulin* and *Dzr1* (Table 1). Due to their allele-specific imprinting, these genes are different from gene-specific examples of imprinting and will be excluded from further discussion of plant imprinted genes.

Plant imprinted genes can be further divided into two subcategories, binary imprinting and differential imprinting (Dilkes and Comai, 2004). Binary imprinting is the strict mono-allelic expression of one of the parental alleles while the other is silent. This class of imprinted genes in plants is thought to represent only a small percentage of the imprinted loci (Dilkes and Comai, 2004). Differential imprinting is the bi-allelic expression of both parental alleles but the expression levels deviate from those expected based on genomic dosage. In addition, imprinted genes can also be classified according to how long the imprinted expression pattern persists during endosperm development. Some genes maintain their imprinted expression throughout endosperm development, while others show imprinted expression early during development and bi-allelic expression as endosperm development proceeds (Danilevskaya *et al*, 2003; Gutiérrez-Marcos *et al*, 2006).

The basis of knowledge regarding imprinting mechanisms in plants is mainly the result of research in *Arabidopsis thaliana*. *MEDEA* (*MEA*) was the first imprinted gene discovered in *Arabidopsis*, showing only expression from the maternal allele in the developing endosperm (Kinoshita *et al*, 1999; Vielle-Calzada *et al*, 1999). *MEA* encodes a SET domain *Polycomb* group (PcG) protein similar to the *Drosophila* gene *Enhancer of*

Zeste [E(z)] (Grossnikauls *et al*, 1998). *MEA* is one of three *Arabidopsis* class I SET-domain proteins (*CLF* and *SWN/EZA1* are the others), believed to methylate Histone3 lysine27 (Springer *et al*, 2003). Plant PcG proteins are involved in maintaining transcriptionally repressed states of their target genes during development by altering chromatin structure (Köhler and Grossniklaus, 2002). During plant reproduction, PcG proteins (*MEA* and others) play a role in controlling cell proliferation during embryogenesis (Grossniklaus *et al*, 1998). Initial studies thoroughly characterized the phenotype for mutations in *MEA*, while subsequent studies sought to identify the underlying mechanism of *MEA* imprinting.

Two additional genes, *FWA* and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*), are imprinted in *Arabidopsis*. As is the case for *MEA*, maternal-specific expression of *FWA* and *FIS2* is observed in the endosperm (Kinoshita *et al*, 2004; Jullien *et al*, 2006). One key difference between the imprinting of *MEA* and *FWA*/*FIS2* is the expression pattern throughout the plant life cycle. In contrast to the expression profile of *MEA*, *FWA* and *FIS2* transcripts are not found in any vegetative tissues. *FWA* expression is only detectable in the open flower, four day after pollination (DAP) seeds and six DAP endosperm + seed coat (Kinoshita *et al*, 2004). *FIS2* expression is only found in late silique tissue and in developing endosperm (Luo *et al*, 1999; Jullien *et al*, 2006).

Pheres1 (*PHE1*) is the fourth known imprinted gene in *Arabidopsis*. The imprinted expression pattern of *PHE1* is different from that of *MEA*, *FWA* and *FIS2*. *PHE1* is expressed solely from the paternal allele in 2-4 DAP gynoecia and silique tissue (Köhler *et al*, 2005). *PHE1* was identified as a downstream target of the MEA/FIE complex through analysis of microarray data from *mea* and *fie* mutants (Köhler *et al*,

2003). MEA and FIE are required to down-regulate *PHE1* expression during seed development and the MEA/FIE complex was shown to physically interact with the *PHE1* promoter (Köhler *et al*, 2003). *PHE1* encodes a MADS-domain type-1 class transcription factor which, when over-expressed (or when not down-regulated), results in the endosperm over-proliferation and seed abortion phenotype associated with *mea* and *fie* mutants (Köhler *et al*, 2003). Additionally, the repression of the maternal *PHE1* allele was shown to require *MEA* (Köhler *et al*, 2005). DNA methylation also appears to play a role in the expression of *PHE1*, albeit in a different context than that of other *Arabidopsis* imprinted genes. In the *mea* mutant background, *PHE1* expression is high, the endosperm over-proliferates and the seed aborts. However, in a *mea/ddm1* double mutant background where global methylation is reduced, *PHE1* expression is reduced and the seed abortion phenotype is rescued (Köhler *et al*, 2003).

Five genes in maize have been identified that display mono-allelic gene expression characteristic of genomic imprinting. Three of the genes, *Nrp1, ZmFie1* and *Meg1*, show maternal-specific expression in the endosperm and no expression in any other tissue (Guo *et al*, 2003; Danilevskaya *et al*, 2003; Springer *et al*, 2002; Gutiérrez-Marcos *et al*, 2004). Based on the expression patterns, the mechanism controlling the imprinted expression of *Nrp1*, *ZmFie1* and *Meg1* appear to be similar to that of *Arabidopsis FWA* and *FIS2*. The fourth imprinted gene in maize, *ZmFie2*, displays a different pattern of imprinted expression. The expression of *ZmFie2* is not limited to endosperm tissue and is found in embryo tissue as well as several vegetative tissues (Springer *et al*, 2002; Danilevskaya *et al*, 2003). However, *ZmFie2* is only imprinted during early endosperm development and expression is bi-allelic in 10 DAP endosperm

tissue (Danilevskaya *et al*, 2003; Gutiérrez-Marcos *et al*, 2006). The final imprinted gene in maize, *Mez1*, shows a distinct pattern of expression relative to other maize imprinted genes. *Mez1* is expressed in the embryo and several vegetative tissues like *ZmFie2*, however it is constitutively imprinted throughout endosperm development (Haun *et al*, 2007). *Mez1* is an *[E(z)]* homolog related to the *Arabidopsis* gene *Curly Leaf* (*CLF*) and is phylogenetically similar to *MEA* (Springer *et al*, 2002). Sequence homology to *MEA* as well as *Drosophila melanogaster* E(Z) suggests MEZ1 is class I histone3 lysine27 methyltransferase (Springer *et al*, 2003).

IMPRINTING MECHANISM IN PLANTS

All examples to date suggest a single common mechanism for imprinting in plants. The model for the imprinting of *MEA* (and other imprinted genes in *Arabidopsis*) involves multiple epigenetic marks and several proteins (Figure 1). Substantial evidence suggests DNA methylation is a crucial component of the mechanism controlling the imprinted expression of *MEA* (Vielle-Calzada *et al*, 1999; Xiao *et al*, 2003). The first three stages for the general mechanism of imprinting in plants are the same as in mammals. The erasure stage is not necessary in plants because imprinting only occurs in endosperm, a tissue that makes no genetic contribution to the next generation. The following model is based on evidence from the imprinted gene *MEA* in *Arabidopsis*, however, many of the stages and components appear to be conserved for other imprinted genes.

Establishment: Prior to gametogenesis and during vegetative growth, DNA methylation is established in the promoter region of the imprinted gene (Figure 1 [A]). During gametogenesis, the male and female germ lines develop separately and genes such as *MEA* are regulated differently depending on the sex of the developing gametophyte. Studies show that the gene *DEMETER* (*DME*) is necessary for maternal activation of *MEA* in the central cell, the female progenitor of the endosperm (Xiao *et al*, 2003). *DME* encodes a protein containing a DNA glycosylase domain and is exclusively expressed in the central cell during female gametogenesis (Choi *et al*, 2002). *DME* transcription is abundant in immature flower buds and dramatically decreases in developing seeds after fertilization (Choi *et al*, 2002). Bisulfite DNA sequencing results showed the maternal *MEA* allele is hypomethylated in wild-type endosperm tissue but is hypermethylated in *dme* mutant endosperm (Gehring *et al*, 2006). This is the result of maternal-specific excision of 5-methylcytosines in wild-type tissues (Figure 1 [B]) (Choi *et al*, 2002; Choi *et al*, 2004; Gehring *et al*, 2006). In flowers inheriting a mutant *dme* allele, *MEA* expression is not detected. However, in a double mutant *met1 dme* background, *MEA* expression is restored (Xiao *et al*, 2003). This removal of methylcytosines is a necessary step in the activation of the maternal *MEA* allele. In the male gamete, *DME* expression is absent and the paternal allele maintains its methylated status and repressed transcription (Figure 1 [C]) (Choi *et al*, 2002). Inheritance of a hypomethylated paternal genome in a *met1* (and in other mutants that affect DNA methylation such as *ddm1*, *drm1 drm2 cmt3*, *ago4*, *rdr2* and *dcl3*) mutant background did not result in activation of the paternal *MEA* allele (Gehring *et al*, 2006). This suggests additional epigenetic marks and/or other yet-to-be identified proteins are necessary for

the silencing of the paternal *MEA* allele. Histone methylation was also shown to be enriched at the paternal allele of *MEA* in endosperm tissue (Figure 1 [E]) (Gehring *et al*, 2006). Little is known about when the histone methylation is established at *MEA*, however some evidence suggest the MEA protein itself is involved (see below). Interestingly *MEA* is methylated in vegetative and embryo tissue (Gehring *et al*, 2006), yet it is expressed from both parental alleles.

Maintenance: Prior to fertilization, the epigenetic states are maintained according to the parent-of-origin. Following fertilization and during early development, DNA methylation patterns are maintained in the paternal allele of the endosperm and both parental alleles of the embryo. The maternal allele remains hypomethylated and the expression of *DME* is repressed following fertilization and throughout seed development (Figure 1 [D]) (Choi *et al*, 2002).

Interpretation: After fertilization, the maintained epigenetic states of the two parental alleles are interpreted. The lack of DNA methylation at the maternal *MEA* allele results in expression, while methylation represses expression of the paternal *MEA* allele (Figure 1 [E]). Recent evidence suggests that *MEA* plays a role in its own imprinting. The MEA protein interacts with its own promoter (Baroux *et al*, 2006) as well as with other PcG proteins, suggesting that silencing of the paternal *MEA* allele is maintained in part by maternally-expressed MEA protein (Figure 1 [E]) (Jullien *et al*, 2006; Gehring *et al*, 2006). More specifically, MEA has been shown to interact with FIE, MSI1 and FIS2 to from a multi-protein complex (Spillane *et al*, 2000; Köhler *et al*, 2003; Wang *et al*, 2006). This complex localizes at the paternal *MEA* allele and maintains the suppressed expression state by maintaining histone methylation and a condensed chromatin structure.

Mutations in any of these four genes results in improper endosperm initiation/development and subsequent seed abortion (Luo *et al*, 2000; Köhler *et al*, 2003).

A consistent component of the imprinting mechanisms for *MEA* (as well as the other *Arabidopsis* imprinted genes *FWA* and *FIS2*), is the role DNA methylation plays in regulating the mono-allelic expression pattern. For both *FWA* and *FIS2*, a mutation in the methyltransferase gene *MET1* transmitted through the male parent results in reactivation of the silenced paternal allele (Kinoshita *et al*, 2004; Jullien *et al*, 2006). Further analysis revealed a requirement for *DME* in the female gametophyte is also required for the activation of *FWA* and *FIS2* (Kinoshita *et al*, 2004; Jullien *et al*, 2006). These results suggest the mechanism controlling the imprinted expression of *FWA* and *FIS2* is similar to that of *MEA* and reinforces the role of DNA methylation in the imprinting mechanism.

CONCLUSIONS

Imprinting is a form of epigenetic regulation in which two alleles with identical, or near-identical, sequence are differentially regulated within the same nucleus. Studies in *Arabidopsis* have revealed a mechanism involving DNA methylation and activation of the maternal allele. Though *MEA*, *FWA* and *FIS2* have different expression patterns, the mechanism controlling their imprinted expression in the endosperm appears to be similar. Work in *Arabidopsis* has been informative to this point; however, certain limitations make it difficult to answer some newly emerging questions. We propose using maize (specifically the *Mez1* gene) to study imprinting as a new model for plant imprinting for several reasons. Maize has a large endosperm, which allows for assays such as chromatin

immunoprecipitation (ChIP) that are difficult to perform in *Arabidopsis*. The availability of transposon insertion lines at the *Mez1* locus also make it possible to study the affect on imprinting when *cis*-proximal promoter regions are displaced by several thousand base pairs. Lastly, preliminary analysis of *Mez1* imprinting suggests it may be regulated by a mechanism distinct from that of imprinted genes in *Arabidopsis*. Studies of *Mez1* could potentially fill in the gaps of the *MEA* mechanism, or may shed light on an alternative mechanism for the regulation of imprinting in plants. To that end, this project has three specific questions: 1) What is the role of DNA methylation in regulating *Mez1* imprinting? 2) What histone modifications are enriched in a parent-specific manner at *Mez1?* 3) What is the effect of *Mutator* transposon insertions into the 5'*cis*-proximal sequences on *Mez1* imprinted expression? Using the maize *Mez1* locus, our studies will focus on defining the *cis*-acting sequences and epigenetic modifications that regulate the imprinted expression of the *Mez1* gene.

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Gene	Species	Parental Expression	Epigenetic Control	Function?	Reference(s)
MEA	Arabidopsis	Maternal	5' & 3' parental DMR, PcG	H3K27 methyltransferase	Kinoshita *et al* , 1999; Vielle-Calzada *et al* , 1999, Gehring *et al* , 2006
FWA	Arabidopsis	Maternal	Maternal Demethylation		Kinoshita *et al* , 2004
PHE1	Arabidopsis	Paternal			Kohler *et al* , 2005
FIS2	Arabidopsis	Maternal		Transcription Factor	Jullien *et al* , 2006
Mez1	Maize	Maternal	5' DMR	H3K27 methyltransferase	Haun *et al* , 2007
Fie1	Maize	Maternal	5' DMR	PcG Protein	Gutierrez-Marcos *et al* , 2006
Fie2	Maize	Maternal	5' DMR	PcG Protein	Gutierrez-Marcos *et al* , 2006
Meg1	Maize	Maternal		Defensin-like gene	Gutierrez-Marcos *et al* , 2004
Nrp1	Maize	Maternal		Transcription Factor	Guo *et al* , 2003
Zein*	Maize	Maternal	Maternal Demethylation	Zein storage protein	Lund *et al* , 1995a
α-Tubulin*	Maize	Maternal	Maternal Demethylation	alpha tubulin	Lund et al, 1995b
dzr1*	Maize	Maternal		zein transcription factor	Chaudhuri and Messing, 1994
R*	Maize	Maternal		MYB transcription factor	Kermicle *et al* , 1970

Table 1. Plant imprinted genes. DMR – Differentially Methylated Region, '*' - allele specific imprinting

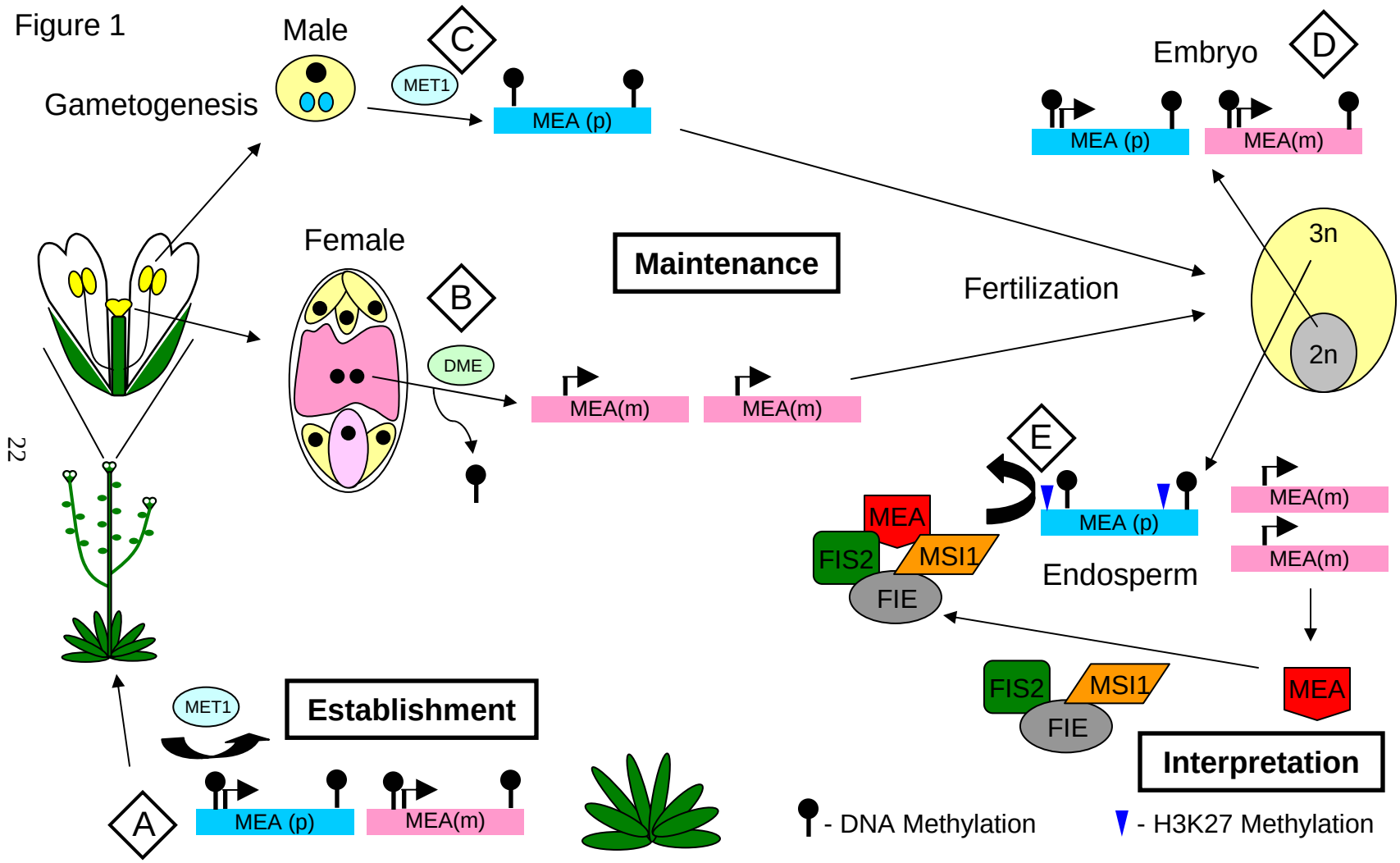

Figure 1. Model of *MEA* imprinting mechanism in *Arabidopsis*. See text for details.

Chapter 2
Genomic imprinting and DNA methylation of maize *Enhancer of zeste* (*Mez*) homologs

INTRODUCTION

The contribution of the maternal and paternal genomes to the transcriptome can be different even though their primary DNA sequence is often nearly identical. Genes that show mono-allelic transcription are referred to as imprinted, if differential expression depends upon the parent-of-origin. Genomic imprinting represents a unique example of differential gene regulation of identical alleles in the same nucleus. Numerous studies have documented the existence of genes that display an imprinted pattern of gene expression in both mammals and plants (Morison *et al*, 2005; Gehring *et al*, 2004; Grossniklaus, 2005). In mammals, mechanisms exist that control genomic imprinting such that both paternal-specific and maternal-specific loci are expressed (Wrzeska and Rejduch, 2004) and normal embryonic development strictly requires contributions from both genomes.

At the transcriptional level there is currently evidence for gene-specific imprinting of four maize genes, *ZmFie1* (Danilevskaya *et al*, 2003), *ZmFie2* (Gutiérrez-Marcos *et al*, 2006), *Meg1* (Gutiérrez-Marcos *et al*, 2004) and *Nrp1* (Guo *et al*, 2003). There are two maize orthologs of the *Arabidopsis FIE* gene, *ZmFie1* and *ZmFie2* (Springer *et al*, 2002; Gutierrez-Marcos *et al*, 2006). *ZmFie1* is imprinted in the endosperm in a manner similar to that of the *Arabidopsis MEA* in that paternal transcripts cannot be detected throughout seed development (Danilevskaya *et al*, 2003). The other maize ortholog, *ZmFie2*, displays a delayed activation of the paternal allele in the endosperm such that expression

is imprinted in early endosperm tissue but displays bi-allelic expression at later time points (Danilevskaya *et al*, 2003). Similar to *ZmFie2*, there is also evidence for delayed paternal activation of the gene *Meg1* (Gutierrez-Marcos *et al*, 2004). The expression of the maize transcription factor *Nrp1*, from the *No-apical-meristem* gene family, is restricted to the endosperm tissue and only transcripts derived from the maternal allele are detected (Guo *et al*, 2003). Differentially methylated regions (DMRs) have been identified for *ZmFie1* and *ZmFie2*, which correlate with maternal expression of these two genes in maize endosperm (Gutierrez-Marcos *et al*, 2006).

MEA is homologous to the *Drosophila Polycomb* group (PcG) gene *Enhancer of Zeste [E(z)]* (Grossniklaus *et al*, 1998). Plant PcG proteins are thought to function as part of a multiprotein complexes involved in maintaining transcriptional repression of certain genes during development (Goodrich *et al*, 1997). *MEA* is one of three *Arabidopsis* genes that are related to the *Drosophila E(z)* gene, along with *CURLY LEAF (CLF)* and *SWN* (*EZA1*) (Grossniklaus *et al*, 1998, Goodrich *et al*, 1997; Preuss 1999). The maize genome also encodes three homologs of the *Drosophila E(z)* gene; *Mez1*, *Mez2* and *Mez3* (Springer *et al*, 2003). The goal of this study was to determine if the maize *E(z)*-like genes; *Mez1*, *Mez2* and *Mez3* display an imprinted pattern of gene expression in the endosperm.

RESULTS

Imprinted expression of *Mez1* in the endosperm. The possibility of imprinted expression patterns for the maize *E(z)* homologs was tested using an allele-specific RT-PCR assay (Figure 1). CAPS markers were identified that allowed the allele-specific

analysis of *Mez1*, *Mez2* and *Mez3* expression in different maize genotypes (Table 2). The allele-specific expression pattern of *Mez1* and *Mez2* could be investigated in tissues generated from the B73 and Mo17 genotypes. Embryo and endosperm tissues were isolated from ears derived from B73 self pollinations, Mo17 self pollinations, B73 (♀) x Mo17 (♂) pollinations and Mo17 (♀) x B73 (♂) pollinations. Total RNA was extracted from endosperm and embryo tissue isolated 13 days after pollination (DAP). The primers used for the allele-specific RT-PCR expression analysis flank introns to clearly distinguish the presence of amplifiable genomic DNA from cDNA. Each amplified PCR product was digested with the restriction enzyme *AluI* to identify the parental nature of the transcripts.

The presence and intensity of both maternal and paternal *Mez1* transcripts in the embryo of heterozygous F_1 plants resulting from reciprocal crosses indicated that *Mez1* expression was bi-allelic in the embryo (Figure 1b). Similarly, the presence of both maternal and paternal *Mez2* transcripts was seen in heterozygous embryo tissue (Figure 1c). The expression pattern of *Mez2* in the endosperm was very similar to the pattern observed in the embryo. These findings indicated that *Mez2* does not display allele-specific expression patterns in these two genotypes and was not imprinted in these tissues. In contrast, only the maternal allele of *Mez1* was detected in the endosperm tissue of heterozygous plants from reciprocal crosses (Figure 1b). This result indicates that *Mez1* is regulated by genomic imprinting in maize endosperm. Analysis of tissue derived from other inbreds suggests that *Mez3* does not display an imprinted pattern of expression (Table 1).

Unlike many of the other imprinted genes detected to date in maize (e.g. *R* and *B*), the presence of a reciprocal pattern of expression in both of the F_1 heterozygotes suggested that *Mez1* imprinting is common to both inbred alleles, not to a specific allele of only one inbred line. We proceeded to test for imprinting of *Mez1, Mez2* and *Mez3* in a set of five different reciprocal crosses (Table 1). In some cases, specific genes were non-polymorphic and the imprinting status could not be assessed. *Mez1* was consistently imprinted in the endosperm tissue of all crosses analyzed while neither *Mez2* nor *Mez3* were found to display imprinted expression. These data suggest that the imprinting of *Mez1* is not an allele-specific imprinting phenomenon but is an example of gene-specific imprinting common to all alleles tested.

Confirmation of imprinting using Sequenom allele-specific expression assays. A secondary technique was used to monitor allele-specific expression in endosperm tissues derived from B73 x Mo17 crosses. Sequenom technology uses mass-spectrometry to distinguish the primer extension products of two alleles and calculate the relative proportion of the two alleles in a sample (Jurinke *et al*, 2005). Allele-specific assays based on SNPs were developed for *ZmFie1, Mez1, Zmet3* and *Mez2*. Previous studies have documented imprinting for *ZmFie1* (Danilevskaya *et al*, 2003), while there is no previous evidence (or expectation) for *Zmet3* to display an imprinted pattern of expression (Cao *et al*, 2000). For *Mez1*, three separate assays that test the relative frequency of distinct SNPs were developed. The fraction of transcripts derived from the B73 allele were determined for B73 x Mo17 and Mo17 x B73 cDNA as well as mixes of cDNA derived from B73 and Mo17 inbred lines (Figure 2a and 2b). The results from the Sequenom analysis were fully consistent with maternal specific expression of *Mez1* and

ZmFie1 in both 13 DAP (days after pollination) and 19 DAP endosperm tissue of maize. As expected, *Mez2* and *Zmet3*, display bi-allelic, non-imprinted patterns of gene expression. Both of these genes display expression patterns consistent with the maternal:paternal dosage ratios.

***Mez1* imprinting is maintained throughout endosperm development.** Results from Vielle-Calzada *et al* (2000) suggest that the entire paternal genome of *Arabidopsis thaliana* is transcriptionally inactive during early endosperm and embryo development. Different genes showed different times of reactivation during endosperm and embryo development. A similar observation has recently been made in maize (Grimanelli *et al*, 2005). Moreover, both *ZmFie2* and *Meg1* exhibit delayed reactivation of the paternal allele (Danilevskaya *et al*, 2003; Gutierrez-Marcos *et al*, 2004). Expression from the paternal allele of *ZmFie2* is delayed 5-10 DAP relative to the maternal allele (Danilevskaya *et al*, 2003). It is possible that the parent-of-origin effects on *Mez1* expression we have observed in 13 DAP endosperm are due to delayed activation of the paternal genome, not gene-specific imprinting regulation of the *Mez1* locus. In order to further explore the possibility that *Mez1* expression may be due to a delayed activation of the paternal genome, we determined the allele-specific expression of *Mez1* at multiple time points during endosperm development (Figure 3a and 3b). Only transcripts corresponding to the maternal allele of *Mez1* were detected in all endosperm tissue tested, from 8 DAP until 27 DAP, suggesting that *Mez1* shows imprinted expression throughout seed development.

Allele-specific expression of *Mez1* is not caused by allele-specific DNA degradation. There is a formal possibility that allele-specific degradation (Yerk *et al*, 1993), or

sequence elimination of the paternal allele, not allele specific transcription, could cause the presence of only maternal *Mez1* transcripts. To test this possibility DNA was isolated from endosperm tissue and intron primers were used to amplify the genomic sequence surrounding the CAPS marker. Using PCR followed by restriction digestion we were able to detect similar levels of DNA from both parental alleles throughout endosperm development (Figure 3c). These results confirm the presence of both parental genomes throughout maize endosperm development, indicating the silencing of the *Mez1* paternal allele is not the result of paternal allele degradation.

Sequence analysis of the *Mez1* promoter. For the imprinted *FWA* gene, a SINE transposable element located in FWA has been identified as an important *cis*-acting determinant of imprinting regulation (Lippman *et al*, 2004). In contrast, transposons and repeats found at the *MEA* locus, which show differential methyaltion patterns (Gehring *et al*, 2006), are not required for imprinting (Spillane *et al*, 2004). To search for *cis*-acting regulatory sequences mediating imprinting at the *Mez1* locus, we obtained sequence upstream of *Mez1* through analysis of a BAC clone (b0165E14) containing the *Mez1* gene. The *Mez1* genomic sequence (AY422167) includes ~2.8kb of upstream sequence. No known retrotransposons or other known repetitive sequences were identified in this region. A simple sequence repeat SSR (containing 23 TA repeats) was found at bp -1662 to -1617.

Danilevskaya *et al* (2003) suggested that the presence of two CpG islands could potentially be involved in "marking" a gene for imprinting in plants based on the observation of two CpG islands in the *ZmFie1* coding sequence. However, using the same methods as Danilevskaya *et al* (2003), only a single CpG island, near the start of

transcription, was detected in the imprinted *Mez1* sequence (Figure 1a). No other CpG islands were detected in this sequence.

DNA methylation analysis of the *Mez1* 5' upstream region. Bisulfite sequencing was used to determine the methylation status of the *Mez1* 5' proximal sequence. DNA was isolated from B73 x Mo17 F_1 hybrid endosperms and treated with sodium bisulfite which results in the conversion of unmethylated cytosines to uracil but does not affect methylated cytosines. A set of primers were used to amplify regions from the $(TA)_{23}$ SSR through to the first exon of *Mez1* (Figure 4a). In addition, primers that target the region 5' of the $(TA)_{23}$ SSR and exon 9 near the middle of the *Mez1* gene were also used. In most cases the same primers could be used to amplify the B73 and Mo17 allele, however for some regions we needed to design B73 and Mo17 specific primers due to sequence polymorphisms. The resulting PCR products for each of these regions were cloned and sequenced. For most of the regions, we were able to determine whether each of the individual clones was maternally or paternally derived based upon B73/Mo17 polymorphisms. The relative level of maternal and paternal methylation is shown in Figure 4b and 4c. There was very little methylation present in the sequences near the transcription start site of *Mez1*. However, a 556bp differentially methylated region (DMR) wass located from -677 to -1232bp relative to the transcription start site. This region displayed very high levels of methylation in the CpG and CpNpG context, on the paternal allele of *Mez1*, but low levels of methylation on the maternally inherited *Mez1* alleles (Figure 4). In contrast, asymmetric cytosine residues showed very little methylation for either maternal or paternal *Mez1* alleles. There was no evidence for

differential methylation in the region 5' of the $(TA)_{23}$ SSR or in exon 9 of the *Mez1* gene (data not shown). These regions both displayed virtually no methylation.

A methylation-sensitive PCR assay, that determines the methylation status of a methyl-sensitive *BstUI* site, was developed to confirm this differentially methylated domain. Mock and *BstUI* digests were performed using genomic DNA from inbred and reciprocal F_1 hybrid endosperm tissue. PCR amplification was performed using the mock and *BstUI* digested DNA to assess whether this site was methylated (amplification following *BstUI* digest indicates that this site was methylated and protected from digestion). In order to determine whether methylation was present on one or both of the two alleles, the amplified DNA (representing the methylated fraction) was digested with *HaeIII* which differentiates the B73 and Mo17 alleles (Figure 5a). The analysis of the reciprocal F_1 hybrid samples indicates that the paternal *Mez1* allele is protected from cleavage by *BstUI* and is therefore methylated (Figure 5a and 5b). The ability to amplify both alleles following *BstUI* digestion from DNA isolated from F_1 hybrid immature ear tissue indicates bi-allelic methylation of this site in vegetative tissues (Figure 5). We also tested for methylation at a second *BstUI* site that lacked methylation according to our bisulfite analysis. The failure to amplify a product following *BstUI* digests of genomic DNA confirms that lack of methylation at this site (data not shown).

Phylogenetic relationships of imprinted and non-imprinted *E(z)*-like genes. Maize and *Arabidopsis* both contain three *E(z)*-like homologs (Springer *et al*, 2003). While alignments and phylogenetic analyses based on the protein sequences of these genes suggest that *MEA* is highly divergent, there is evidence that *MEA* is a relatively recent duplicate of *SWN* (*EZA1*). This evidence is based upon the location of these two genes in

collinear regions of the *Arabidopsis* genome and phylogenetic analyses based upon synonymous sequence changes (C. Spillane, S. Laouielle, K. Wolfe and U. Grossniklaus, unpublished data). An alignment was performed beginning with the protein sequence and then selecting a conserved region for performing DNA alignments for the maize, *Arabidopsis*, rice, *Sorghum* and poplar *E(z)*-like genes. The DNA alignment was then analyzed using MEGA to obtain a bootstrapped neighbor-joining tree based on synonymous or non-synonymous changes (Figure 6). Interestingly, the two genes with evidence for imprinting, *MEA* and *Mez1*, do not show close relationships with one another. Instead, both genes are more closely related to genes that do not show an imprinted pattern of gene expression.

DISCUSSION

Genomic imprinting is the differential expression of an allele dependent on which parent it was inherited from. In plants, the verified instances of imprinting have predominantly been documented in the triploid endosperm tissue. In *Arabidopsis*, the best studied example of genomic imprinting is the *MEA* gene. *MEA* encodes an *E(z)*-like SET domain protein. SET domain proteins are putative histone methyltransferases and on the basis of sequence similarity and chromatin immunoprecipitation (ChIP) assays, MEA most likely encodes a HistoneH3 lysine27 methyltransferase (Springer *et al*, 2003; Gehring *et al*, 2006, Makarevich *et al*, 2006). The *Arabidopsis* genome contains two closely related *E(z)*-like genes, *CLF* and *SWN* (*EZA1*) (Goodrich *et al*, 1997; Preuss *et al*, 1999). No genetic evidence for genomic imprinting has been observed for *CLF* and *SWN* (C. Spillane and U. Grossniklaus, unpublished). We have previously characterized three maize *E(z)*-like genes, *Mez1*, *Mez2* and *Mez3* (Springer *et al*, 2002). Here, we demonstrate that the *Mez1* gene is imprinted in maize endosperm whereas the *Mez2* and *Mez3* genes exhibit bi-allelic expression. We further demonstrate that *Mez1* is imprinted in multiple genetic backgrounds, and therefore likely represents gene-specific, rather than allele-specific imprinting.

cis-acting sequences that control imprinting

The identification of additional examples of imprinting in plants allows further testing of the emerging models of imprinting regulation. Recent studies have documented a role for DNA methylation in the control of imprinted gene expression patterns (Xiao *et al,* 2003; Kinoshita *et al*, 2004; Gutierrez-Marcos *et al*, 2006; Jullien *et al,* 2006; Vielle-Calzada *et al*, 1999; Luo *et al*, 2000). It has been proposed that allele-

specific loss of DNA methylation, induced by DME, plays a critical role in imprinting (Gehring *et al*, 2006). We have documented the presence of bi-allelic methylation of a region of the *Mez1* 5' proximal sequence in vegetative tissues but mono-allelic methylation patterns in the endosperm tissue. These findings are similar to those described for *MEA* in *Arabidopsis* at late stages of seed development (Gehring *et al,* 2006).

An important unresolved question regarding imprinted gene expression revolves around understanding the *cis*-acting sequences that determine imprinted expression. We were able to compare the sequence of the *Mez1* promoter, and specifically the DMR, with the sequence of promoters of other imprinted genes in plants. In our analysis, we did not detect any primary sequence motifs common to the promoters of the known imprinted genes in *Arabidopsis* and maize. In addition, we did not detect the double CG-island noticed by Danilevskaya *et al*, (2003) at the *ZmFie1* locus. However, it is possible that our search window of 2766 bp was too limited and needs to include more distal sequences. In animals, some of the imprinting regulatory elements can occur at loci >100kb distal from the transcribed region (Ferguson-Smith and Surani, 2001) and the regulation of paramutation in maize can involve sequence over 100kb away from the gene itself (Stam *et al*, 2002). Further experimentation is necessary to map the *cis*-acting sequences responsible for the imprinted expression of *Mez1*.

The majority of examples of imprinting in plants share the common features of maternal-specific expression in endosperm tissue. These imprinted genes can be divided into two classes based on whether they are also expressed in other plant tissues. *FWA*, *ZmFie1* and *Nrp1* are all imprinted in the endosperm and not expressed at detectable

levels in other plant tissues (Kinoshita *et al*, 1999; Guo *et al*, 2003; Danilevskaya *et al*, 2003). However, the *MEA* and *Mez1* genes are expressed in vegetative tissues including the embryo as well as in endosperm tissue. Assuming a plant imprinting model of DNA methylation as the default state and allele-specific demethylation in the endosperm would suggest that these genes might be methylated and expressed in vegetative tissues. Indeed, there is evidence for bi-allelic methylation and expression of *Mez1* in immature ear tissue. It will be interesting to monitor the developmental time-course of DNA methylation patterns for these genes and how this methylation affects their expression. Future experiments will elucidate the role of DNA methylation and histone modifications in controlling imprinting.

EXPERIMENTAL PROCEDURES

Plant materials. Maize inbred lines were grown using standard conditions. Leaf tissue was harvested for DNA collections. Reciprocal crosses and self pollinations were performed. The ears were harvested 7, 10, 13, 17, 20, 23 and 27 DAP. Endosperm and embryo tissue was collected by dissection. Tissue derived from multiple kernels from the same ear was pooled prior to performing RNA or DNA extractions.

Identification of SNPs for CAPS analysis. In order to monitor imprinting it was necessary to identify polymorphisms within the coding regions of the *Mez1, Mez2* and *Mez3* genes. A region corresponding to the 3' coding region and UTR from *Mez1* and *Mez3* was amplified and sequenced from a set of 24 maize inbred lines. DNA was extracted from leaf tissue using the Qiagen Plant DNeasy plant mini kit (Valencia, CA) according to the manufacturer's instructions. PCR reactions were performed in a 30 μl total volume containing approximately 50 ng of DNA, 5 pmol of each primer, 0.65 units of HotStarTaq polymerase (Qiagen, Valencia, CA), 3 μl of 10X reaction buffer, and 0.2 μl of 25mM dNTPs. Primers used were mez1F33 and mez1R33 for *Mez1*; mez2_SF and mez2_SR for *Mez2*; and mez3_SF and mez3_SR for *Mez3.* All primer sequences are provided in Table 3. Conditions of the polymerase-chain-reaction were as follows: 94^{o} for 15', 35 cycles of 94 ^{o} for 30", 58^{o} for 30", 72^{o} for 1', followed by 72^{o} for 10'.

For each of the three genes, we identified sequence polymorphisms that allowed for the development of a Cleaved Amplified Polymorphism (CAPs) assay. Sequence information from B73 and Mo17 identified a CAPS marker in *Mez2*. This CAPS marker was used to classify the remaining inbred lines (Table 2). These CAPS assays can be

employed to detect the presence of both alleles in genomic DNA or cDNA samples. The *Mez1* and *Mez2* CAPS assays rely upon a polymorphism in an *Alu*I restriction site while the *Mez3* CAPS assays utilizes a polymorphisms in a *Hae*III site.

CAPS analysis of allele-specific expression patterns. RNA was isolated from endosperm and embryo tissue using the Qiagen RNeasy plant mini kit according to the manufacturer's instructions. Contaminating DNA was removed by digestions with RQ1 DNase (Promega, Madison, WI). 5 μg of total RNA was mixed with 0.5 μg oligo dT (Promega) and heated to 70 ^{o} for 10 minutes followed by 1 minute on ice. First strand cDNA synthesis was performed by adding 6 μl 5X reaction buffer, 0.5 μl RNasin, 3 μl 5mM dNTPs, and 1 μl M-MLV reverse transcriptase (Promega). This reaction was incubated at 42 ^{o} for 50 minutes followed by 70 ^{o} for 15 minutes. The resulting cDNA was purified by phenol: chloroform extraction and ethanol precipitation. The cDNA was resuspended in 20 μl ddH_2O. PCR reactions were performed as above and the amplified DNA was ethanol precipitated and resuspended in 20 μl H_2O. Restriction digestions were performed by mixing 10 μl DNA, 2 μl 10X reaction buffer, 0.2 μl BSA, 2 μl *Alu*I or *Hae*III and 5.8 μl H_2O and incubating at 37 ^{o} overnight. The digested products were separated by electrophoresis in a 2.5% Metaphor (FMC Bioproducts, Rockland, ME) TBE gel and observed by ethidium bromide staining.

Analysis of genomic DNA CAPS markers. Genomic DNA was extraction from endosperm tissue using the Qiagen Plant DNeasy plant mini kit (Valencia, CA) according to the manufacturer's instructions. PCR amplification was performed as above using the

primers mez1F20 and mez1R9 (Table 3). The amplified DNA was processed in the same manner as the amplified cDNA products.

Mass spectrometry based analysis of allele-specific expression: RNAs from 13 and 19 DAP endosperm tissues were treated with DNAse prior to allele-specific expression analyses. cDNAs were synthesized from all three biological replicates of Mo17xB73 and B73xMo17 hybrid RNAs. Mixed cDNAs were also synthesized from 2:1, 1:1 and 1:2 mixes of the biological replicates of Mo17 and B73 inbred RNAs. The cDNAs were reverse transcribed using Superscript III reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

PCR-based assays for allele-specific expression analyses based on SNPs were designed in collaboration with Sequenom (San Diego, CA). See table 3 for PCR and extension primers for these assays. PCR and extension PCR reactions on cDNA and DNA templates were performed using the manufacturer's specifications (Sequenom, San Diego, CA). Mass spectrometry quantification of allele ratios was performed at the University of Minnesota Genotyping Facility. Multiple measurements of the ratio of the two alleles were performed for each of the three biological replicates of mixed RNA and F_1 RNAs.

Bisulfite Sequencing. Endosperm tissue was dissected from B73×Mo17 and Mo17×B73 18 DAP kernels. DNA was isolated using the Qiagen DNeasy kit according to manufacturer's protocol. 2 µg of DNA was digested with 10 units *Bam*HI and *Hind*III in a volume of 100 µl at 37°C overnight. Digested DNA was extracted with

phenol:chloroform, precipitated with 100% ethanol supplemented with 2 µl tRNA and resuspended in 20 µl H_2O. DNA was denatured at 97°C for 1 minute, snap-cooled on ice for 2 minutes and incubated with 1 µl 6.3M NaOH (freshly prepared) for 30 minutes at 39°C. 208 µl of bisulfite solution (40.5 g sodium bisulfite in 80 mls H_2O with slow stirring, pH to 5.1 with freshly prepared 10M NaOH , add 3.3 ml 20mM hydroquinone, volume to 100 ml, protect from light) was added to DNA at 39°C. Samples were incubated for 5 cycles of 55°C for 3 hours, 95°C for 5 minutes. Samples were cleaned-up using Qiagen PCR Purification kit according to manufacturer's protocol (eluted in 100 µl EB). Added 5 µl 6.3M NaOH and incubated at 37°C for 15 minutes. DNA was precipitated with 0.1 vol 10M NH_4OAc, 3 volumes 100% ethanol and 2 µl tRNA (resuspended in 100 µl Qiagen EB). 2 µl of bisulfite-treated DNA was used per PCR reaction. PCR components were 4 µl dNTPs, 5 µl 10X ExTaq buffer, 1 µl reverse primer, 38 µl H_2O. Primers used were mez1BSr7-F5 and mez1BSr7-R9M, mez1BSr7-F5 and mez1BSr7-R9B, mez1BSr6-F3M and mez1BSr6-R8, mez1BSr6-F3B and mez1BSr6-R8, mez1BSr5-F2M and mez1BSr5-R3, mez1BSr5-F2B and mez1BSr5-R3, mez1BSr4-F7 and mez1BSr4-R10, mez1BSr3-F6 and mez1BSr3-R9M, mez1BSr3-F6 and mez1BSr3-R9B, mez1BSr2-F4M and mez1BSr2-R8, mez1BSr2-F4B and mez1BSr2-R8 (Table 3). PCR conditions were as follows: 95°C for 5 minutes, added 1 µl ExTaq (Takara), ran 5 cycles of 95°C for 20", 60°C for 3', 72°C for 3'. Added 1 µl forward primer, ran 10 cycles of 95°C for 20", 60°C for 1.5', 72°C for 2'. Ran 30 cycles of 95°C for 20", 50°C for 1.5', 72°C for 2', followed by 72°C for 10'. PCR products were gel excised from a low-melting point agarose gel and extracted using the Qiagen Gel Extraction kit according to manufacturer's protocol (resuspended in 30 µl EB). 2 µl

of PCR product was cloned using TA TOPO Cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated, insert size verified by PCR using M13F and M13R primers and sequenced.

Bisulfite Verification: Genomic DNA was extracted as described above from B73, Mo17, B73×Mo17, Mo17×B73 endosperm tissue and B73×Mo17 and Mo17×B73 immature ear tissue. 500 ng of DNA of each tissue was digested with 10U *Bst*UI or 1 µl glycerol (mock digest) in a total volume of 40 µl at 60°C for four hours. The reaction was cleaned up using a Qiagen PCR Purification kit according to manufacturer's protocol (eluted with 50 µl EB). 4 µl of DNA was then PCR amplified as described above using mez1F55 and mez1R52 primers (Table 3). PCR products were ethanol precipitated and resuspended in 30 µl H_2O. DNA was then digested with 20 units *Hae*III at 37°C for four hours. The digested products were separated by electrophoresis in a 2.5% Metaphor TBE gel and observed by ethidium bromide staining.

Sequence analysis. The amino acid and nucleic sequences for *Mez1, Mez2, Mez2, Sdg711, Sdg718, Medea, Clf, Eza1* and *E(z)* were aligned using ClustalX version1.83 (Thompson *et al*., 1997). These alignments were edited and the phylogenetic relationships were analyzed using MEGA (Kumar *et al*., 2001). The neighbor joining method was used and a bootstrap analysis with 500 replicates was performed. The *Mez1, MEDEA, ZmFie1* and *Sdg711* promoter sequences were tested for the presence of CNSs using the methods described by Kaplinsky *et al* (2002).

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TISSUE	CROSS				
	B73 X Mo17	**A619 X W64a**	**B84 X B57**	**Oh43 X IL14H**	**B57 X B79**
Mez1 endosperm	Imprinted	Imprinted	Imprinted	Imprinted	Not polymorphic
Mez2 endosperm	Biallelic	Biallelic		Not polymorphic	
Mez3 endosperm	Not polymorphic	Not polymorphic	Biallelic	Biallelic	Biallelic

Table 1. Imprinting of *Mez1*, *Mez2* and *Mez3* in maize hybrids.

Inbred Line	*Mez1* (*AluI*)[a]	*Mez2* (*AluI*)[b]	*Mez3* (*HaeIII*)[c]
A619	N	NA	NA
B57	N	N	Y
B73	Y	Y	Y
B75	N	N	Y
B77	Y	N	Y
B79	N	Y	N
B84	Y	Y	N
H100	Y	N	Y
H95	N	Y	Y
HP301	Y	Y	Y
Il14H	Y	Y	Y
Ki3	Y	Y	Y
Ky21	Y	N	Y
M37W	Y	Y	Y
Mo17	N	N	Y
MS71	N	N	Y
N7A	N	Y	Y
NC358	Y	Y	Y
Oh43	N	Y	N
Oh7B	Y	Y	Y
P39	Y	N	Y
Pa91	Y	Y	Y
W64a	Y	NA	NA
Va26	N	Y	N

Table 2. Summary of CAPS markers for *Mez1*, *Mez2* and *Mez3* in 24 maize inbred lines. For each gene, the presence of the restriction enzyme recognition site is noted. Crossing an inbred line that contains the restriction site by an inbred line that does not contain the site will allow the two alleles to be distinguished in genomic DNA and cDNA.

Primer	Sequence (5' → 3')
mez1F33	CACAAAGAAGCAGATAAGCGTGGAAAG
mez1R33	TTGGGGCCTATGAATGACAGGTTC
mez2_SF	GGGTTGGTATATATGCGAAGGA
mez2_SR	TCGCAGCCATTATTTGATACTC
mez3_SF	GGTTGGTATCTATGCGAAGGAG
mez3_SR	GGCAAATAAATTCCATCCCTCT
mez1F20	CCGTCTGCCATATGCTGACTTGTACC
mez1R9	CCTCTGTTGTTTTGGGCGAGCTTC
mez1-F1	ATGGAAGCAGCGGCGGC
mez1-R1	GCAAATTAGAGCTTCACCACA
mez1-F2	GATGACAGAAGACCAATCTGT
mez1-R2	GGATGTACTTCCGAATCACTG
mez1-F3	GAAGACTAAATCTCAACAAAGTG
mez1-R3	CTTGTGCGCAACTCAGTACC
mez1-F4	GAGCTCTTAGTGGTGTTGATT
mez1-R4	TCAGCCTCTTGTGTTTTGGG
mez2/3-F1	ATGGCTTGCTCCTCGAAGC
mez2/3-R1	CAGGAGTGCCTCTGCAGC
mez2/3-F2	GATCCCTCCGTACACCACT
mez2/3-R2	GAGTGCTTCTCATTTTTCTCA
mez2/3-F3	CTCTTCTGCCGCAGATGCT
mez2/3-R3	GACCATCATCATGCATCTCTT
mez2/3-F4	CTCCTGGCAAAAGGCAGAA
mez2/3-R4	GACCAGATAGTAAATTTCTGG
M13F	GGTTTTCCCAGTCACGAC
M13R	TCACACAGGAAACAGCTATGAC
mez1BSr7-F5	ATAAGGGTTTGGTGAATGTTTTATTTT
mez1BSr7-R9B	CATATTAAAACRCATTAACACTCARCTATAA
mez1BSr7-R9M	CATATTAAAACRCATTAACACTCACCTATAA
mez1BSr6-F3M	GTATTGTGTTAAGGTTTYGAATGTATAGTTAGT
mez1BSr6-F3B	ATTGTGTYGAGGTTTYGAATGTATAGTT
mez1BSr6-R8	TCTCAAACARACCCATAATTCCTTAAAA
mez1BSr5-F2M	GATGGTTTGGTTTTGATGTGA
mez1BSr5-F2B	TTGGTTTTGATTTTTGATGGGA
mez1BSr5-R3	TCTTTTCTRTTCCCAAATAATTACCRTATAA
mez1BSr4-F7	GGTAATTATTTGGGAAYAGAAAAGAYGAGT
mez1BSr4-R10	CCTACCRRTTACCTRTAATCATCTCRTAA
mez1BSr3-F6	AGATYAGTGATTATAGATAAGTTAAAGTTTAGTGTGAT
mez1BSr3-R9M	ATCACCCRCRRAAACCCTAA
mez1BSr3-R9B	CCATCACCCRTAAAAACCCTAA
mez1BSr2-F4M	TAGGGTTTTTGYGGGTGATGGAAG
mez1BSr2-F4B	TAGGGTTTTTAYGGGTGATGGAAG
mez1BSr2-R8	ATTACCCCCTCRACCTAAAACAAA
mez1F55	GGGGGCTAAAACTTGATAATAAACA
Mez1R52	CGGTCTCTGTGCATTTGTCCTT

Table 3. Primers used for PCR, bisulfite sequencing and Sequenom allele-specific expression assays.

Figure 1

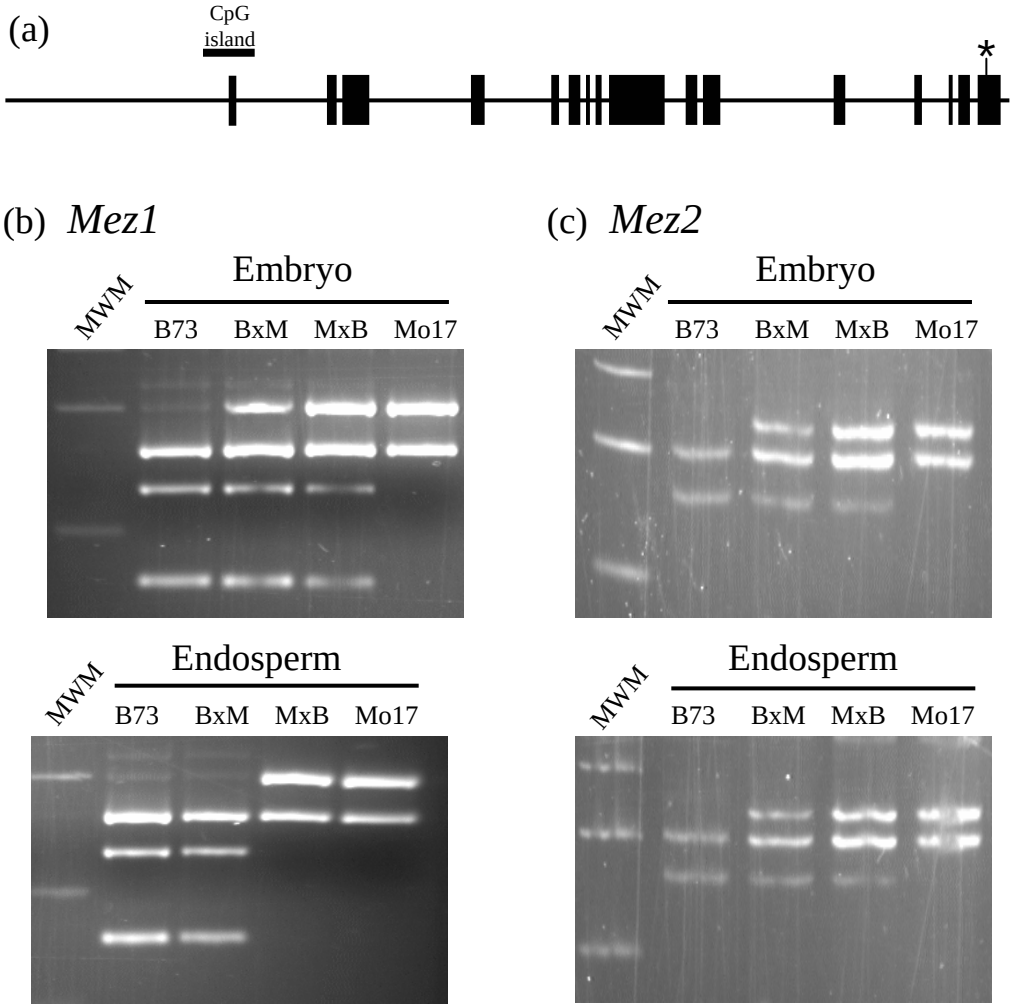
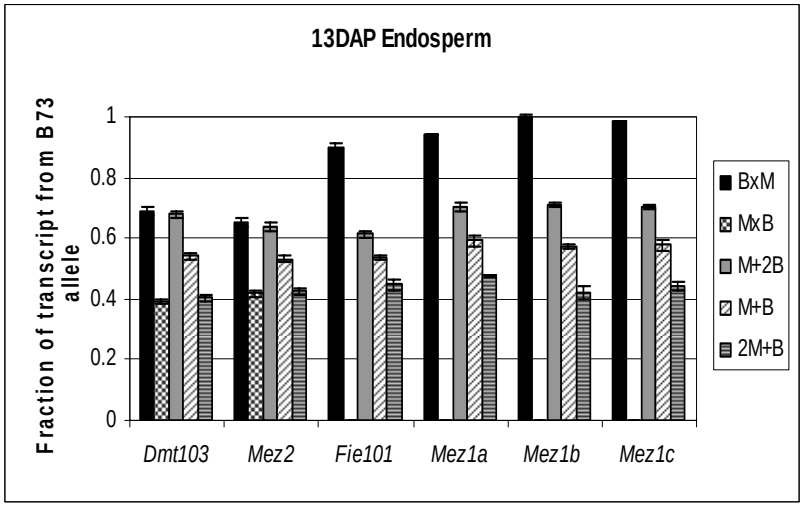


Figure 1. (a) *Mez1* genomic structure. The black boxes indicate the position of exons. Above the sequence the position of a CpG island is indicated. The '*' indicates the *Alu I* CAPS marker in the last exon. (b,c) Allele-specific analysis of *Mez1* and *Mez2* expression patterns. (b) The allele specific expression of *Mez1* was monitored in the embryo and endosperm tissue derived from four genotypes; B73, B73 (female) × Mo17 (male), Mo17 (female) × B73 (male) and Mo17. The expression in the embryo is bi-allelic while the endosperm shows mono-allelic expression of the maternal allele. The first lane in each image is the molecular weight marker (MWM). (c) *Mez2* allele-specific expression was monitored in the same samples. *Mez2* is expression was bi-allelic in both embryo and endosperm tissues.

Figure 2

(a)



(b)

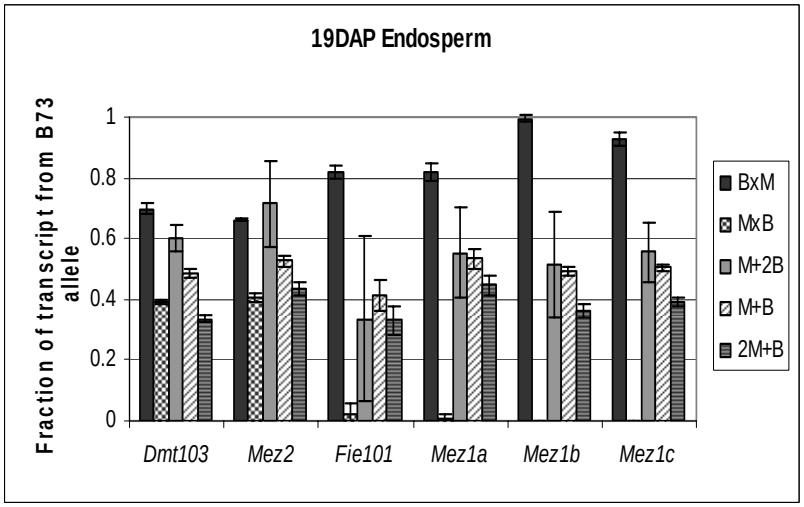


Figure 2. Analysis of allele-specific gene expression using primer extension. Sequenom assays (MALDI-TOF detection of SNP primer extension products) were designed for a B73/Mo17 SNPs in *Zmet3*, *Mez2*, *ZmFie1* and three different SNPs in *Mez1*. (a) Allele-specific expression was performed using cDNA extracted from 13DAP endosperm tissue from B73×Mo17 and Mo17×B73 crosses. In addition, we mixed cDNA extracted from B73 and Mo17 in a 1:1 (B+M), 1:2 (B+2M) and 2:1 (2B+M) ratio as controls to demonstrate linear detection of both alleles in endosperm tissue. (b) The same set of cDNA samples were also analyzed in 19DAP endosperm tissue.

Figure 3

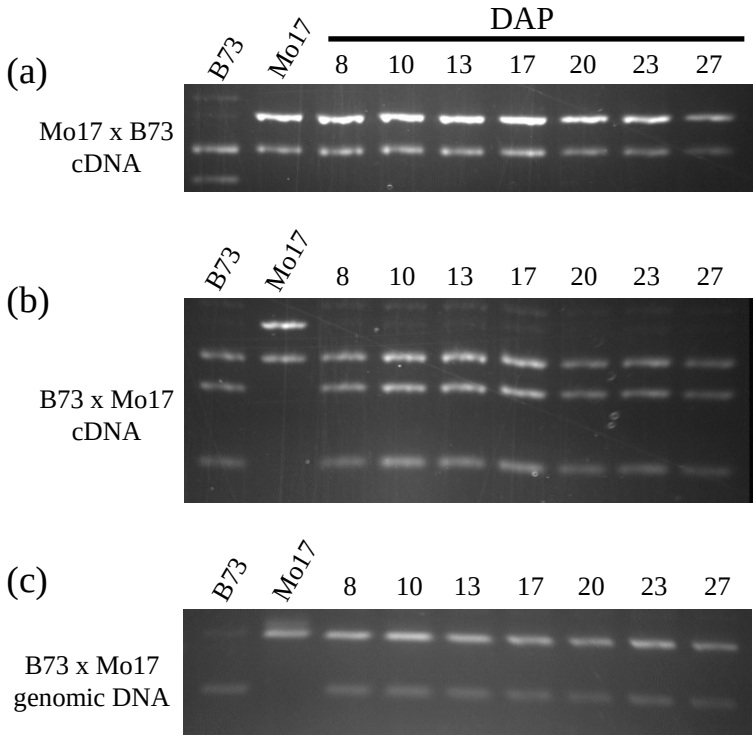


Figure 3. Allele-specific expression of *Mez1* during endosperm development. Endosperm tissue was harvested at different times during development and used for allele-specific analysis of *Mez1*. The first lane is derived from B73 homozygous endosperms at 13 DAP while the second lane is derived from Mo17 homozygous endosperms at 13 DAP. These lanes simply illustrate the expected patterns produced by mono-allelic expression of the B73 or Mo17 allele. (a) Analysis of *Mez1* allele-specific expression in tissue harvested from a cross of Mo17 (female) × B73 (male) at 8, 10, 13, 17, 20, 23 and 27 days after pollination (DAP). (b) Tissue was harvested at the same time-points from a cross of B73 as the female by Mo17 as the male. (c) A different set of primers were used to amplify genomic DNA isolated at 8, 10, 13, 17, 20, 23 and 27 DAP to detect whether both alleles are present in the genomic DNA at all time points.

Figure 4

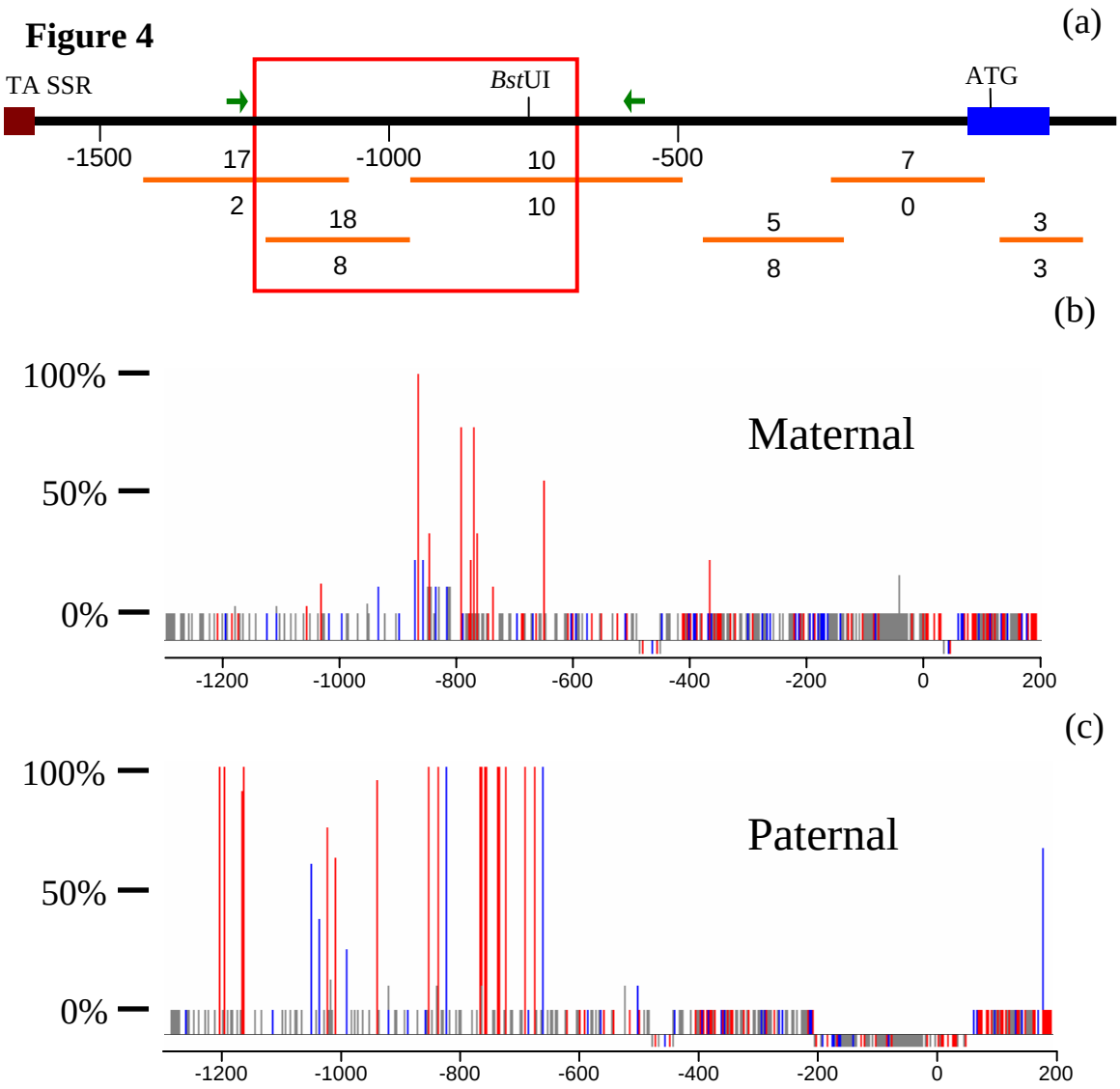


Figure 4. Methylation status of the *Mez1* promoter. DNA was isolated from B73 (female) × Mo17 (male) endosperm tissue, treated with sodium metabisulfite and PCR-amplified. The PCR products were cloned and sequenced to determine the methylation patterns of the forward strand of DNA.. In most cases, B73/Mo17 sequence polymorphisms allowed for the unambiguous determination of parent-of-origin for each bisulfite clone. (a) Diagram of the *Mez1* promoter. The regions amplified by PCR and cloned are shown in orange, with the number of maternal and paternal clones analyzed shown above and below the line, respectively. The differentially methylated region (DMR) is outlined in red. The primers used for verification are shown as green arrows as well as the location of the *BstUI* site. (b, c) The percent methylation at each CpG (red), CpNpG (blue) and CpNpN (gray) is plotted for the maternal and paternal alleles. Cytosines for which no data was obtained are shown below the line.

Figure 5

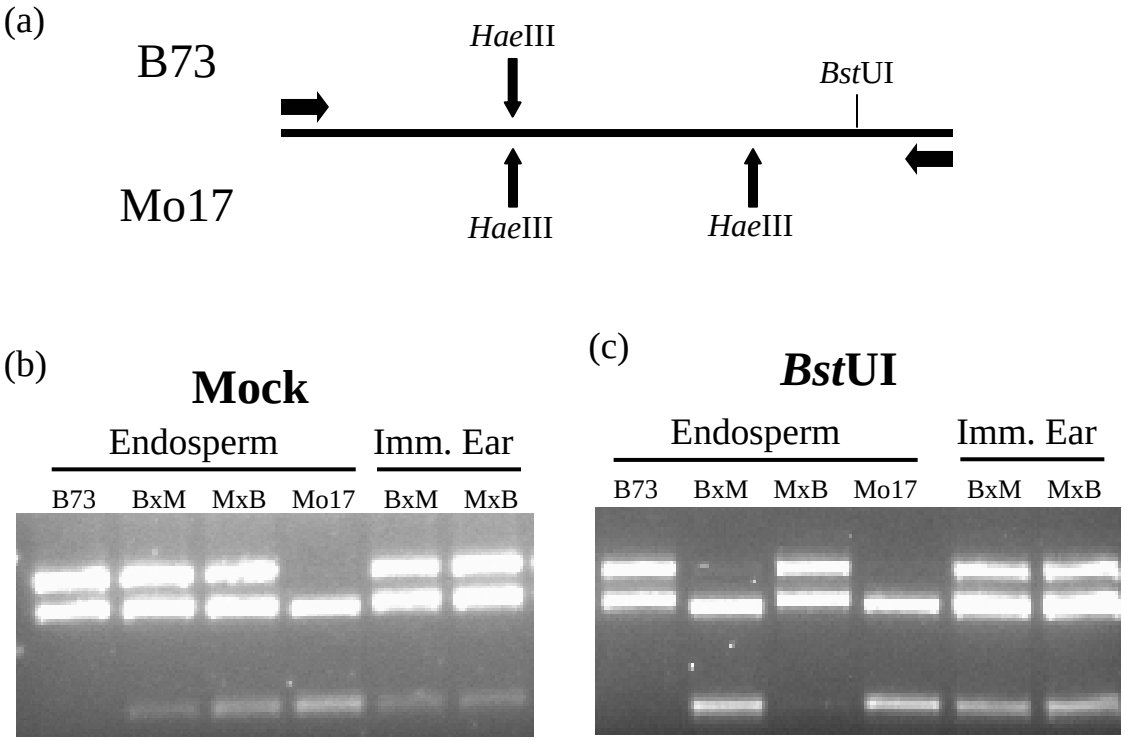


Figure 5. Confirmation of differential methylation in maternal and paternal alleles by methyl-sensitive PCR. In order to confirm the presence of differential methylation of the maternal and paternal alleles in the endosperm we employed a combination of methyl-sensitive PCR and CAPS analysis. (a) Primers that flank a *BstUI* site that is predicted to be methylated on the paternal allele and demethylated on the maternal alleles were used to perform PCR on mock digested and *BstUI* digested genomic DNA that was derived from homozygous and reciprocal heterozygous endosperm DNA samples. Following PCR amplification, the DNA was digested with *HaeIII* which has an additional site in the Mo17 allele that is absent in the B73 allele. (b) The mock digest shows that both alleles can be amplified and detected using this system. (c) Following digestion with *BstUI* we were able to determine that in the reciprocal hybrids only the paternal allele was protected from digestion and amplified. This result confirms the paternal specific methylation of this site. In addition, the same assays were performed using DNA isolated from immature ear tissue and provides evidence for bi-allelic methylation in vegetative tissues.

Figure 6

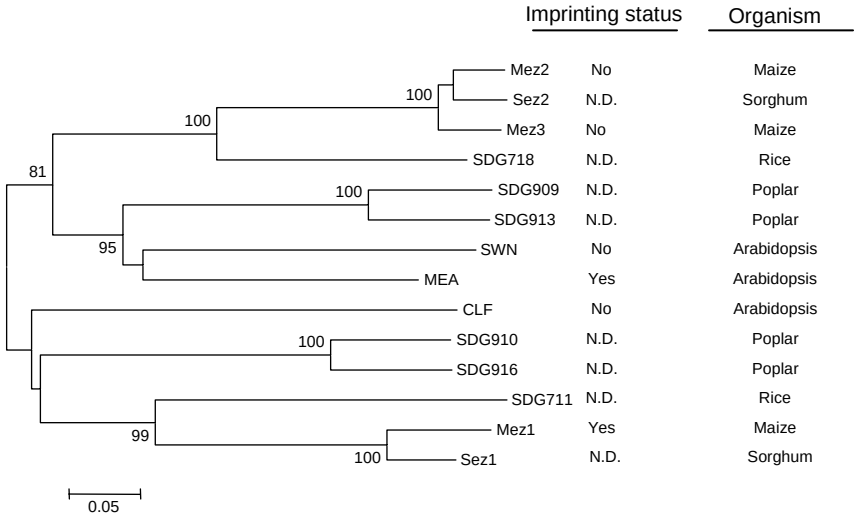


Figure 6. Relationships among plant *enhancer of zeste*-like genes. A ~684bp nucleic acid sequence corresponding to bp 2085 to 2768 of the *Mez1* (AF443596) coding sequence was aligned with the following sequences using ClustalX: *Drosophila Enhancer of zeste* (U00180), *MEDEA* (AF060485), *CLF* (Y10580), *EZA1* (AF100163), *Mez2* (AF443597), *Mez3* (AF443598), *Sdg711* (AP003044), *Sdg718* (AF407010). The alignment was analyzed using neighbor-joining methods to analyze synonymous changes using MEGA version2.1. Bootstrap analysis was performed using 500 replicates and values for all nodes supported at greater than 80% are shown.

Chapter 3
Analysis of allele-specific chromatin modifications at imprinted maize loci

INTRODUCTION

Genomic imprinting is the differential regulation of gene expression based on the parental inheritance. At imprinted loci, the primary DNA sequence of each parental allele is nearly identical, yet only one parental allele is expressed. Imprinting is a unique and rare form of gene regulation that is essential to the normal development of progeny in both mammals and plants (Kono *et al*, 2002; Grossniklaus *et al*, 1998). Numerous genes in both plant and animals have been identified as showing a mono-allelic (imprinted) pattern of gene expression (Grossniklaus, 2005; Feil and Berger, 2007; Morison *et al*, 2005). Studies on mammalian imprinted genes have revealed a variety of molecular mechanisms for both maternally and paternally imprinted genes (Edwards and Ferguson-Smith, 2007). Several diseases in humans, such as Prader-Willi and Angelman syndromes, have been linked to a disruption of imprinted gene regulation (da Rocha and Ferguson-Smith, 2004).

Relative to the number of imprinted genes in animals, exploration for imprinted genes in plants has revealed only a few examples of imprinted loci (Feil and Berger, 2007). Studies on the maternal-effect phenotypes of FIS-class (**F**ertilization **I**ndependent **S**eed) mutant alleles helped identify the imprinted gene *MEDEA* (*MEA*) and others in the model plant *Arabidopsis thaliana* (Grossniklaus *et al*, 1998; Kinoshita *et al*, 1999). Through sequence homology, several genes in *Zea mays* have also been identified as imprinted (Danilevskaya *et al*, 2003; Gutiérrez-Marcos *et al*, 2003; Haun *et al*, 2007). To

date, all examples of imprinting in plants have been identified in the triploid endosperm tissue. Endosperm is a unique tissue found in seed-producing flowering plants and is necessary for supplying nourishment to the developing embryo (Berger F, 2003). Maternal inheritance of a mutant *mea* in *Arabidopsis* results in endosperm over-proliferation and seed abortion (Köhler *et al*, 2003). Recent studies on several imprinted genes in *Arabidopsis* have provided several clues into the mechanism of imprinting in plants.

The imprinting mechanism in plants can be divided into three phases: establishment, maintenance and interpretation. Based on work in *Arabidopsis*, a general model has been formulated. Prior to gametogenesis, a DNA methyltransferase enzyme (such as *MET1* in *Arabidopsis*) establishes a DNA methylation 'imprint' at important regulatory regions of imprinted loci (Jullien *et al*, 2006, Vielle-Calzada *et al*, 1999). In the developing male gametophyte DNA methylation is maintained (Choi *et al*, 2002), while in the female gametophyte DNA methylation is removed by the DNA glycosylase *DEMETER* (Choi *et al*, 2002; Xiao *et al*, 2003; Choi *et al*, 2004; Gehring *et al*, 2006). Following fertilization, interpretation of the hypomethylated state of the maternal alleles results in expression, while the hypermethylated paternal allele remains silent (Xiao *et al*, 2003). Recent studies show the *MEA* protein is involved in controlling its own imprinted expression (Baroux *et al*, 2006; Gehring *et al*, 2006). Using chromatin immunoprecipitation (ChIP) on *Arabidopsis* floral tissue, the MEA protein was found to localize to its own promoter (Baroux *et al*, 2006). Additionally, chromatin immunoprecipitation studies in silique tissue suggested histone H3 lysine27 dimethylation was enriched at the paternal allele of *MEA* (Gehring *et al*, 2006). These

results are consistent with the predicted function of MEA, which encodes a SET domain *Polycomb* group (PcG) protein similar to the *Drosophila* gene *Enhancer of Zeste [E(z)]* (Grossnikauls *et al*, 1998). MEA, a class-I SET domain protein, is believed to methylate histone H3 lysine27 residues (Springer *et al*, 2003). These results from *Arabidopsis* and other evidence suggest DNA methylation is not acting alone to regulate imprinted gene expression.

Numerous studies in both plants and mammals have identified the importance of histone modifications in the regulation of development and imprinting (Li, 2002; Baroux *et al*, 2007). Histone tails protruding from the core histones of eukaryotic chromatin can be post-translationally modified with chemical moieties including acetylation, methylation, phosphorylation, ubiquitylation and others (Peterson and Laniel, 2004). Site-specific modifications of histones along with DNA methylation have been shown to correlate with specific chromatin states and DNA-mediated processes (reviewed in Grewal and Moazed, 2003; Peterson and Laniel, 2004). In the regulation of imprinting, studies in mouse showed allele-specific histone modifications at the imprinting control region of the mammalian imprinted gene *IGF2R* (Yang *et al*, 2003; Thanh *et al*, 2004)

The role of histone modifications in regulating imprinted genes in plants is poorly understood. The small size and non-persistent nature of the *Arabidopsis* endosperm tissue makes procedures, such as ChIP, for studying such marks extremely difficult. Furthermore, chromatin immunoprecipitation protocols are designed primarily for mammalian systems, adding to the logistical difficulties of performing ChIP in plants. To date, there is no data on the histone modification patterns present at imprinted genes in plant endosperm tissue. This study utilized a plant-specific nuclei preparation

technique to study the histone modifications of the imprinted genes *Mez1* and *ZmFie1* in the endosperm tissue of *Zea mays*.

RESULTS

Confirmation of Chromatin Immunoprecipitation Protocol for Maize Endosperm.

Chromatin immunoprecipitation (ChIP) is a popular technique for studying histone modification localization in numerous species. However, the use of ChIP to monitor histone modifications has only recently been applied to the large, complex genome of maize (Haring *et al*, 2007; *Casati et al*, 2008). In addition, there are no published protocols for performing ChIP on starchy maize endosperm tissue. We developed a novel ChIP protocol for maize endosperm based upon protocols from several other groups (Nagaki *et al*, 2003; Upstate Biotech/Millipore, Billerica, MA) and refined this protocol via experimentation. This ChIP protocol was applied to endosperm and seedling tissue of maize. Approximately 4 g of endosperm tissue was dissected from 14 day after pollination (DAP) kernels derived from the cross of Mo17 × B73 (M×B). Cross-linked chromatin was extracted from the pool of M×B endosperm tissue and incubated with the antibodies described below. Chromatin was sonicated prior to antibody incubation, resulting in an average fragment size of approximately 500bp (see experimental procedure, data not shown). This resulted in a resolution of approximately 1000bp for localizing a particular histone modification to specific genomic location.

Several approaches were used to verify that the ChIPs were actually enriching for the proper chromatin. Initially, regions of the *GAPC* and *Actin* genes as well as the retrotransposon *Copia* were used to test for enrichment of activating and silencing

modifications, respectively. *Actin* and *GAPC* are 'housekeeping' genes that show constitutive expression in most tissues. The chromatin structure near the 5'*cis*-proximal region of these genes is expected to be enriched for HistoneH3 acetylation, a modification representative of gene expression. On the other hand, the *Copia* retrotransposon element is expected to be enriched for the silencing histone modification H3K9 dimethylation (H3K9me2) (Haring *et al*, 2007). An antibody to the invariant core HistoneH4 domain (provided by Judith Berman) was also used as a positive control and to compare nucleasome occupancy. Quantitative Real-Time PCR was used to determine the amount of immunoprecipitated chromatin relative to the amount of chromatin input (Haring *et al*, 2007). Chromatin immunoprecipitations with an antibody to HistoneH4 showed enrichment relative to chromatin immunoprecipitations performed with a pre-immuno IgG control for *GAPC*, *Actin* and *Copia*. (Figure 1). Both the *GAPC* and *Actin* genes exhibited significant enrichment for H3 acetylation compared to the IgG control in their 5'*cis*-proximal regions (Figure 1a & 1b). The silent *Copia* elements showed evidence for enrichment of H3K9me2 as well as some, albeit lower, enrichment for H3 acetylation (Figure 1c). These results suggest that our chromatin immunoprecipitation were enriching for the appropriate chromatin types.

Paternal-allele enrichment of *ZmFie1* with a 5-methylcytosine antibody.

In this experiment, the levels of histone modifications present at the maternal and paternal alleles of imprinted loci were compared. This requires the development of assays that will allow quantification of the amount of maternal and paternal alleles in immunoprecipitated chromatin. A series of quantitative single nucleotide polymorphism (SNP) assays were developed using the MassArray technology (Jurinke et al, 2005). This

platform utilizes mass spectrometry to compare the relative abundance of single base extension products. Prior to screening for histone modifications at imprinted loci in maize, we attempted to verify that we could successfully monitor allele-specific enrichment for a known chromatin modification. A previously identified region within the *ZmFie1* that is paternally enriched for DNA methylation was monitored (Hermon *et al*, 2007). *ZmFie1* was chosen in lieu of *Mez1* due to lack of quantitative SNP assays available within the differentially methylation region (DMR) of *Mez1*. Sonicated and denatured chromatin from M×B endosperm tissue was incubated with a 5-methylcytosine antibody and the immunoprecipitated DNA was subjected to quantitative SNP analyses. The percentage of chromatin derived from the paternal allele was calculated for the 5-methylcytosine and input samples for four SNPs in *ZmFie1*. One of these SNPs is 5' of the DMR; two are within the DMR and the fourth is within the coding region. In the input DNA sample (no antibody), we detected 33% paternal alleles as expected due to the 1:2 ratio of paternal:maternal DNA in endosperm (Figure 2). The two SNPs within the DMR of *ZmFie1* showed evidence for enrichment of the paternal allele in three different chromatin immunoprecipitations (Figure 2). However, the SNPs that are 5' and 3' of the DMR did not show any paternal enrichment in chromatin that was precipitated with a 5-methylcytosine antibody. These results indicate that a combination of our ChIP and quantitative SNP assays can be used to investigate allele-specific histone modifications at imprinted loci.

Parental allele-specific enrichment of histone modifications at *Mez1* and *ZmFie1*.

The combination of ChIP and quantitative SNP assays were used to investigate the allele-specific histone modifications present at the maize imprinted genes *Mez1* and

ZmFie1. Chromatin immunoprecipitation reactions were performed using a series of eight different antibodies as well as an IgG control. The antibodies that were used are specific to H4, H3 acetylation, H4 acetylation, H3K4me2, H3K9me2, H3K9me3, H3K27me2, and H3K27me3. Each antibody was used to perform at least two immunoprecipitations (technical replications) on three different samples of chromatin (biological replications). The technical replications were performed by doing multiple chromatin immunoprecipitations from a pool of chromatin using the same antibody. The biological replications were performed on different dates using different sources of biological material. The immunoprecipitated chromatin was then used to perform quantitative SNP assays in order to identify regions of imprinted genes with maternally and/or paternally enriched histone modifications (Table 1).

Initially, the proportion of the maternal and paternal allele detected in input chromatin and chromatin precipitated with an antibody that recognizes histone H4 was compared (Figure 3). As expected, ~1/3 of the input chromatin was derived from the paternal allele. In general, there was not consistent evidence for maternal or paternal H4 differences (Figure 3). One region of the *Mez1* promoter (immediately 5' of the transcription start site) exhibited a minor enrichment for the paternal allele. The general lack of maternal or paternal enrichment in H4-precipitated chromatin suggests that both alleles have similar levels of histone occupancy.

The allele-specific enrichment for the seven histone modifications was scanned throughout the *Mez1* and *ZmFie1* loci. There were three histone modifications with strong evidence for parent-of-origin specific enrichment for *Mez1* and *ZmFie1*. Histone H3K27 trimethylation (H3K27me3) was paternally enriched in the 5'*cis*-proximal

regions of both imprinted genes (Figure 4). The paternal enrichment can be observed by comparing the proportion of the paternal allele obtained from different immunoprecipications with the H3K27me3 antibody with the proportion of the paternal allele detected in input chromatin. The paternal alleles accounted for roughly 33% of the input chromatin. However, in the 5' proximal regions of *Mez1* and *ZmFie1*, 40-70% of the chromatin precipitated using H3K27me3 was derived from the paternal allele. In addition, we noted a low level of paternal enrichment for H3K27me3 throughout the coding region. This observation is due to the fact that nearly all quantitative SNP measurements of H3K27me3 enriched chromatin exhibited values greater than 0.33. In contrast, histone H3 and histone H4 acetylation were enriched in the 5' coding region of the maternal allele for both *Mez1* and *ZmFie1* (Figures 5 & 6). There was less evidence for allele-specific differences in the level of the other four histone modifications (Figures 7 and 8). Histone H3K4 dimethylation (H3K4me2) showed a low level of maternal bias throughout the coding region of *Mez1* and *ZmFie1* (Figures 7d and 8d). Histone H3K27 dimethylation (H3K27me2) exhibited a low level of paternal bias throughout the coding regions of *Mez1* and *ZmFie1* (Figures 7a and 8a). There was no evidence for enrichment of histone H3K9 dimethylation (H3K9me2) or histone H3K9 trimethylation (H3K9me3) (Figure 7 and 8).

The immunoprecipitated chromatin was also used to determine whether allele-specific chromatin modifications were detected at a third imprinted gene, *Nrp1*. A SNP that is located 938bp 3' of the transcription start site was used to detect maternal or paternal enrichment (Figure 9). *Nrp1* exhibited patterns of maternal and paternal histone modifications that were similar to *Mez1* and *ZmFie1*. Chromatin precipitated with H3

acetylation or H4 acetylation antibodies was consistently enriched for the maternal allele. In contrast, chromatin precipitated with H3K27me2 or H3K27me3 was enriched for the paternal allele. In general, the three imprinted loci all exhibited similar patterns of allele-specific histone modifications (Table 1).

Dynamics of allele-specific histone modifications at imprinted loci.

We focused on the regions and modifications that were identified in our scan of *Mez1* and *ZmFie1* to determine whether these modifications were consistent in both B73 x Mo17 and Mo17xB73 reciprocal hybrids. In addition, the level of allele-specific enrichment was tested in seedling tissue where these genes are not imprinted. The two reciprocal hybrids show very similar patterns of maternal enrichment for histone acetylation and paternal enrichment for H3K27me3 in the 5' proximal regions of *Mez1* and *ZmFie1* (Figure 10). This suggests that these modifications are associated with the parental origin of the allele and are not specific to the B73 or Mo17 allele. There was no evidence for maternal or paternal enrichment of these histone modifications in seedling tissue (data not shown). The seedling data exhibited a higher level of variation in the allele-specific data. This may be due to the fact that very little chromatin was precipitated and the quantitative SNP assays are known to exhibit higher levels of variation when used with low levels of starting material.

Quantitative PCR analyses were used to monitor enrichment at three different regions of the *Mez1* locus (Figure 11). The quantitative real-time PCR analysis can indicate the presence of absence of a modification but does not allow partitioning of the modification as maternal or paternal. As expected, the three regions showed evidence for the presence of H4. This confirmed that the immunoprecipitation successfully enriched

for chromatin. In the region that is 5' of the DMR we observed significant enrichment for H3K27me3 but no evidence for H3 or H4 acetylation (Figure 11a). The second region, very near the transcription start site, exhibited evidence for H3K27me3 and histone acetylation (Figure 11b). The quantitative SNP analyses within this same region suggested that the H3K27me3 is present on the paternal allele while the H3 and H4 hyperacetylation occurs on the maternal allele. The region within the *Mez1* coding sequence does not show enrichment for H3K27me3 or histone acetylation (Figure 11c). There was no evidence for maternal or paternal enrichment for this region in the quantitative SNP analyses.

DISCUSSION

Imprinted gene expression is a unique epigenetic phenomenon wherein a cell is able to differentiate between two nearly identical parental alleles in the same nucleus and regulate their expression differently. DNA methylation and histone modifications represent two known mechanisms by which epigenetic regulation can occur. A series of studies have documented differential DNA methylation of the maternal and paternal alleles of imprinted plant genes (reviewed in Huh *et al*, 2007). However, there is very little data on histone modifications at imprinted genes. There is one published report that indicates that H3K27me3 is enriched at the paternal allele of *Mea* in *Arabidopsis* floral tissue (Gehring *et al*, 2006). In this study we were able to classify certain histone modifications as either paternally enriched or maternally enriched in the regulatory and coding regions of three maize imprinted genes, *Mez1*, *ZmFie1* and *Nrp1*. The three genes exhibited very similar patterns of histone modification enrichment, suggesting a role for

histone modifications in imprinted gene regulation in plants. We will discuss our results in the context of describing the chromatin state for the paternal and maternal alleles of imprinting genes. The generic view of chromatin at an imprinted maize gene is illustrated in Figure 12.

Histone modifications present at the silenced paternal allele of imprinted loci.

Many studies have identified higher levels of DNA methylation at the silent paternal allele of imprinted genes (reviewed in Huh *et al*, 2007). Indeed, our lab has found a region of the *Mez1* locus that exhibits paternal specific DNA methylation (Haun *et al*, 2007). Similarly, two studies have identified paternal specific DNA methylation at *ZmFie1* (Gutierrez-Marcos *et al*, 2006; Hermon *et al*, 2007). In this study we were able to identify a second epigenetic mark present on the paternal allele of imprinted loci. H3K27me3 is significantly paternally enriched in the 5'*cis*-proximal regions of *Mez1* and *ZmFie1*. In both cases, the region of paternal enrichment is primarily observed in the *cis*-regulatory regions that are immediately 5' of the transcription start site. For *ZmFie1*, this region of H3K27me3 enrichment appears to terminate near the transcription start site and extend at least 2 kb upstream. Our ability to better define the region of enrichment at *Mez1* is obscured by the lack of suitable SNP assays from -500bp to -3000bp. This region includes the previously identified differentially methylated region (DMR) and would be good candidate for the localization of additional silencing marks (such as H3K27me3). Indeed, real-time PCR results indicated enrichment of H3K27me3 in a region 5' to the DMR. Taken together, these results suggest the region for paternal enrichment of H3K27me3 at *Mez1* terminates approximately 1kb into the coding region and extends 5' at least 2.5kb. A SNP assay at position -3243bp shows no paternal

enrichment for H3K27me3 (data not shown), indicating the boundary for paternal enrichment of H3K27me3 is between -1500bp and -3243 bp. We observed minor levels of paternal enrichment for H3K27me2 and H3K27me3 in the coding regions of *Mez1* and *ZmFie1*. However, real-time PCR analyses do not provide evidence for significant levels of this modification in these regions.

The presence of a region of H3K27me3 was predicted in *Arabidopsis*. Genetic studies suggested that a functional maternal allele of *MEA* was important for silencing of the paternal *MEA* allele (Jullien *et al*, 2006). The MEA protein was shown to be present at the chromatin of the paternal *MEA* allele (Baroux *et al*, 2006). Based upon homology with *Drosophila* and mammalian proteins with known biochemical activity (Müller *et al*, 2002; Nakayama *et al*, 2001), *MEA* and *Mez1* are both expected to encode H3K27 histone methyltransferase. In this study, we were able to find evidence for enriched H3K27me3 near the promoter of several imprinted maize genes. This suggests that the maternally produced MEZ1 protein may be involved in methylating histones at the paternal alleles of *Mez1, ZmFie1* and *Nrp1*.

Interestingly, there is no evidence for paternal enrichment of H3K9me2 or H3K9me3. Those modifications are typically associated with gene repression (Peterson and Laniel, 2004), most notably repression of repetitive elements (Jackson *et al*, 2004). We did identify enrichment for H3K9me2 in the *Copia* retrotransposon sequence (Figure 1). This data suggests that the histone modifications associated with the dynamic process of imprinting are specific to H3K27me3. In contrast, H3K9 methylation is used to form constitutive heterochromatin for the silencing of repetitive elements, such as *Copia*.

Histone modifications present at the active maternal allele of imprinted loci.

The scan of histone modifications also included several modifications, such as H3 acetylation, H4 acetylation and H3K4me2 that are generally found at actively transcribed regions (Peterson and Laniel, 2004). The three imprinted maize genes all exhibited maternal enrichments for H3 and H4 acetylation in the coding regions. For *Mez1*, the region of maternal enrichment for histone acetylation begins around the transcription start site and extends for several thousand base pairs into the coding region. For *ZmFie1*, a lack of suitable SNP assays in the coding region does not allow for a thorough analysis of the region for maternal-specific histone acetylation enrichment. However, there is strong evidence the enriched histone acetylation region begins near the transcription start site. The coding region of *Nrp1* also shows maternal enrichment of histone acetylation. It is interesting that the maternal enrichment is much stronger near the 5' end of the transcript than at the 3' end of the transcripts. This could reflect a greater requirement of histone acetylation for the efficient initiation of transcription and a lower requirement for elongation / termination. The other activating modification, H3K4me2, exhibited a slight bias towards the maternal allele in the coding regions of *Mez1* and *ZmFie1* but not in *Nrp1*. The level of maternal enrichment for H3K4me2 is less pronounced than that observed for H3 and H4 acetylation.

This study provides a survey of the chromatin modifications that are present at imprinted loci in plants. The maternal and paternal alleles differ in their levels of histone H3K27 methylation, histone H3 and H4 acetylation and DNA methylation. It is likely that these modifications are important in establishing and maintaining the imprinted state of these loci. Indeed, a reverse-genetic screen of chromatin modifying enzymes has identified several histone deacetyltransferases and histone methyltransferases that are

required for proper imprinting of *Mez1* (data not shown). This suggests that the histone methylation and acetylation are actually required components of the imprinting mechanisms in maize. The combination of the biochemical tools developed in this study and genetics lines with impaired imprinting is likely to shed light and the interplay of chromatin modifications and imprinting.

Experimental Procedures

Plant Material. Maize inbred lines were grown at the Minnesota Agricultural experiment station (Saint Paul, MN) during the summer of 2007. Reciprocal crosses between the inbred lines B73 and Mo17 were performed to generate hybrid endosperm tissue. Ears were harvested at 14 and 24 days after pollination (DAP). Endosperm tissue (1-2 g, approximately 30-40 endosperms) from individual Mo17×B73 and B73×Mo17 ears was collected by dissection. Tissue derived from multiple kernels from the same ear was pooled prior to crosslinking (see ChIP procedure below). Etiolated seedling tissue was generated from Mo17×B73 and B73×Mo17 seeds grown in the dark at 30°C for 10 days. Above-ground tissue was harvested and washed with water prior to performing ChIPs.

Chromatin Immunoprecipitation (ChIP). Crosslinking: Fresh endosperm and etiolated seedling tissue was completely submerged in 40 ml of fresh prepared 1% (v/v) formaldehyde. A vacuum was applied for 2 minutes, released and reapplied for an additional 8 minutes. 1 volume (40 ml) of 0.25M glycine was added to quench any unreacted formaldehyde. A vacuum was applied for 1 minute, released and reapplied for an additional 4 minutes. The formaldehyde/glycine solution was discarded and tissue was washed five times with water. Tissue was frozen in liquid nitrogen and ground to a fine powder. *Nuclei Extraction*: All buffers were made fresh and kept on ice unless otherwise indicated. PMSF or plant protease inhibitors were added to appropriate solutions immediately prior to use. Powderized tissue was suspended in 10 ml nuclei extraction buffer [10mM Tris-HCl pH 7.5, 3mM $CaCl_2$, 2mM $MgCl_2$, 0.1mM PMSF (Sigma, P7626), 1 tablet complete mini per 50 ml buffer (Roche, #11836153001), 1%

(v/v) plant protease inhibitors (Sigma, P9599), 0.5% (v/v) Tween 40 (Sigma, P1504)] and gently inverted to completely resuspend frozen tissue. The solution was filtered through Miracloth (Calbiochem, # 475855) into a 50 ml tube on ice. Samples were spun at 600xg in a swing-bucket type centrifuge for 10 minutes at 4°C. The supernatant was discarded and pellet was resuspended in 5 ml nuclei extraction buffer containing 25% (w/v) sucrose. With a cut tip, resuspended nuclei were pipeted on top of 5 ml nuclei extraction buffer containing 50% (w/v) sucrose in a fresh 50 ml tube. The solution was pipeted carefully down the wall of the tube to layer the 25% sucrose solution on top of the 50% sucrose solution. Samples were spun at 1500xg in a swing-bucket type centrifuge for 10 minutes at 4°C. The supernatant was removed with a pipet and nuclei in pellet was mixed with 1.5 ml SDS lysis buffer [50mM Tris-HCl pH 8.0, 10mM EDTA, 1% (w/v) SDS, 0.1mM PMSF, 1% (v/v) plant protease inhibitors] and gently resuspended. Lysate was stored on ice for 20 minutes to facilitate complete nuclei lysis. 750 µl was aliquoted into two fresh 1.5 ml tubes and kept on ice prior to sonication. *Sonication*. Samples were sonicated with nine 10 second pulses (the exact number of 10s pulses is species and tissue-specific) on a Heat Systems-Ultrasonics sonicator set at 30% duty cycle, power setting 2. Samples were kept on ice during sonication and for 1 minute between each 10 second pulse. Samples were spun at 12,000xg at 4°C for 10 minutes and the supernatant containing sonicated chromatin was transferred to a fresh 1.5 ml tube. *Assessing Sonication Efficiency*: A 48 µl aliquot of sonicated chromatin was removed and mixed with 48 µl water and 4 µl 5M NaCl. Samples were incubated at 65°C overnight. Samples were then incubated with 1 µl RNAse A at 37°C for 30 minutes. DNA was extracted with 1 volume phenol:chloroform followed by ethanol precipitation

supplemented with 2 µl glycogen (Roche, 10901393001) and 10 µl 3M sodium acetate. DNA in samples were quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop; Wilmington, DE) and analyzed on a 2% agarose gel. *Immunoprecipitation*: For each immunoprecipitation, 100 µl of sheared, crosslinked chromatin was added to a fresh 1.5 ml tube (this resulted in ~7 µg of DNA per immunoprecipitation for endosperm tissue and ~4 µg of DNA per immunoprecipitation for etiolated seedling tissue). To each sample 900 µl of ChIP dilution buffer [16.7mM Tris-HCl pH 8.0, 167mM NaCl, 1.2mM EDTA, 0.01% (w/v) SDS, 1.1% (v/v) Triton X-100 (Sigma, #234729)] with 10 µl plant protease inhibitors. Chromatin was precleared by adding 60 µl protein A agarose (Upstate, #16-157) and incubated at 4°C for 1 hour on a rotating platform. Protein A agarose was pelleted by centrifugation at 4000xg for l minute. 10% of the supernatant was removed as the 'input' sample and stored at 4°C. The remaining supernatant was transferred to fresh 1.5 ml tube and the appropriate antibody was added (Table 2). Chromatin/antibodies were incubated overnight at 4°C on a rotating platform. To immunoprecipitate bound chromatin, 60 µl protein A agarose was added to each sample and incubated for 1 hour at 4°C on a rotating platform. Protein A agarose was pelleted by centrifugation at 4000xg for l minute and the supernatant was removed without disturbing pellet. Protein A agarose/antibody/chromatin immunocomplexes were washed in the following buffers for 5 minutes at 4°C on a rotating platform followed by centrifugation at 4000xg for l minute and careful removal of supernatant. One wash in low salt immune complex wash buffer [20mM Tris-HCl pH 8.0, 150mM NaCl, 2mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X-100]. One wash in high salt immune complex wash buffer [20mM Tris-HCl pH 8.0, 500mM NaCl, 2mM EDTA, 0.1% (w/v)

SDS, 1% (v/v) Triton X-100]. One wash in LiCl immune complex wash buffer [20mM Tris-HCl pH 8.0, 500mM NaCl, 2mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X-100]. Two washes in TE wash buffer [10mM Tris-HCl, 1mM EDTA, pH 8.0]. Following the final wash, DNA was eluted from the protein A agarose beads with ChIP elution buffer [100mM $NaHCO_3$, 1% (w/v) SDS]. 100 µl ChIP elution buffer was added to pelleted protein A agarose and incubate at room temperature for 15 minutes on a rotating platform. Samples were centrifuged at 4000xg for 1 minute and careful removal of supernatant to fresh 1.5 ml tube. The elution step was repeated a second time and the eluted samples were combined for a final volume of ~200 µl. *Precipitating ChIP DNA*: Eluted DNA was cleaned-up with 1 volume (200 µl) phenol:chloroform, followed by ethanol precipitation supplemented with 2 µl glycogen and 20 µl 3M sodium acetate. Pellet was resuspended in 100µl 10mM Tris.

Real-Time PCR Analysis. Prior to performing ChIP allele-specific SNP assays, real-time PCR was performed to verify enrichment of DNA in antibody samples compared to no-antibody (IgG) controls (Figure 1). Real-time PCR was performed using Applied Biosystems' SYBR Green PCR Master Mix (Foster City, CA, USA) in a 20 µl reaction according to manufacturer's protocol. 2 µl of immunoprecipitated DNA was amplified using an Applied Biosystems 7900HT Real-Time PCR System. A 2-fold dilution series (undiluted, 1:2, 1:4, 1:8 and 1:16 dilutions) calibration line was made from crosslinked, sonicated chromatin. DNA for the calibration line was purified using phenol:chloroform followed by ethanol precipitation supplemented with 1 µl glycogen (see above). Primers were designed to amplify the second exon of the maize *actin1* gene (Genbank #J01238),

the reverse-transcriptase coding region of the TY1 class retrotransposon *copia* (Genbank #J01238), and the 5' *cis*-proximal promoter region of maize the *GAPDH* gene (Genbank # X15596). All primer sequences are provided in Table 3. For each experiment, no-template controls, no-antibody controls and input samples were analyzed for every primer set. Cycle threshold (Ct) values were obtained for each sample and a linear regression line was fit to the calibration samples ($\log_{10}$ template (ng) vs Ct). Using the equation from the calibration line, the amount of starting template DNA for each input and antibody sample was calculated. A percentage of input (%IP) was then calculated for each antibody sample to determine relative enrichment. Melting curve analysis was also performed to verify only single amplicons were formed during the assay for all primers sets tested (data not shown). Real-time PCR experiments were also performed using the technique described above on immunoprecipitated DNA to measure overall enrichment at certain genetic loci (Figure 11). A percent of input (%IP) was calculated as described above. Relative enrichment for each antibody was calculated by dividing the %IP for IgG from every sample. Primers for the regions of *Mez1* analyzed are provided in table 3.

Quantitative SNP Assays. Immunoprecipitated DNA from the ChIP procedure above was the template for a PCR-based allele-specific enrichment analysis based on SNPs. PCR and extension primers (see Table 3) for these assays were designed in collaboration with Sequenom (San Diego, CA) based on known SNP polymorphisms within the imprinted genes. PCR reactions on ChIP and 'input' DNA samples were performed using the manufacturer's specifications (Sequenom, San Diego, CA). Mass spectrometry quantification of parental allele ratios was performed at the University of Minnesota

Genotyping Facility. Five control DNA samples were processed along with ChIP samples. 4:1 (and 1:4) control samples were mixed from individual B73 and Mo17 leaf DNA samples. BxM and MxB hybrid endosperm DNA was used as the 2:1 (and 1:2) control samples and BxM hybrid leaf DNA was used as the 1:1 control. Control samples were replicated three times for each SNP assay.

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Modification	Mez1	ZmFie1	NRP1
H4	**Not enriched**	**Not enriched**	**Not enriched**
H3 Acetylation	**Maternally enriched in 5' coding region**	**Maternally enriched in 5' coding region**	**Maternally enriched in 5' coding region**
H4 Acetylation	**Maternally enriched in 5' coding region**	**Maternally enriched in 5' coding region**	**Maternally enriched in 5' coding region**
H3K4me2	**Low-level maternal enrichment in coding region**	**Low-level maternal enrichment in coding region**	**Not enriched**
H3K9me2	**Not enriched**	**Not enriched**	**Not enriched**
H3K9me3	**Not enriched**	**Not enriched**	**Not enriched**
H3K27me2	**Low-level paternal enrichment in coding region**	**Low-level paternal enrichment in coding region**	**Low-level paternal enrichment in coding region**
H3K27me3	**Paternally enriched in promoter region**	**Paternally enriched in promoter region**	**Paternally enriched in promoter region**

Table 1. Summary of parent-specific histone modifications at three imprinted genes of maize.

Antibody	Source	Volume
IgG	Upstate	5-10 µl
H4	Judith Berman	10 µl
H3 Acetyl	Upstate #06-599	10 µl
H4 Acetyl	Upstate #06-866	10 µl
K3K4me2	Upstate #07-030	10 µl
H3K9me2	Upstate #07-212	10 µl
H3K9me3	Abcam #ab8898	10 µl
H3K27me2	Upstate #07-452	6-10 µl
H3K27me3	Upstate #07-449	10 µl
5-methylcytosine	Calbiochem #NA81	7.5-15 µl
5-methylcytosine	Fitzgerald #20-CS90	7.5-15 µl

Table 2. Antibodies used for ChIP protocol, showing source and volume used per immunoprecipitation.

Primer	Sequence (5' → 3')
Mez1 F80	TAGGCCCCTTCGTAATGCAAGAT
Mez1 R75	AGACAGCTTTTCTCGCTTGATCTCAT
Mez1 F69	TGTATTCGATATTTTATGCTGACGGAGGAA
Mez1 R30	TCGGTACGTGTGAGCGTTATTATGG
Mez1 RTF2	GTGAAGATGAAGCCATTGAAGATGAGGAG
Mez1 R43	ACCATCCACAACAATGCAAAAGAAAAAG
GAPC F3	GTGTAACAGTCAGTCGGCTTTAAAAGGA
GAPC R1	CTAGGAGGACGACGGGTGGA
Actin F2	GATGATGCGCCAAGAGCTG
Actin R2	GCCTCATCACCTACGTAGGCAT
Copia F1	CGATGTGAAGACAGCATTCCT
Copia R1	CTCAAGTGACATCCCATGTGT
Mez_1ChIP11_F*	ACGTTGGATGAAGCATGTGATCAGCACCAG
9008D_ChIP43_F*	ACGTTGGATGTAGTTTGGCGCGCGGCAAAG
9002A_ChIP53_F*	ACGTTGGATGGTCAGCTTGTGGCGAAAGAG
Mez_1ChIP20_F*	ACGTTGGATGTACGATTACCGCTATGAGCC
Mez_2ChIP46_F*	ACGTTGGATGTCTTTGATCCTTCGGGTCTC
Fie1_ChIP31_F*	ACGTTGGATGAAGCTAATCAACCAGCACGG
Mez_1ChIP4_F*	ACGTTGGATGTGGCTTATCTATGGTCACTG
Mez_1ChIP12_F*	ACGTTGGATGCTCAGCGATTCGTCTATTGG
Mez_1ChIP19_F*	ACGTTGGATGTCGTGAGAACTCATCGTTCC
Fie2_ChIP37_F*	ACGTTGGATGGCAGAAATGATGAGCGAAGG
9002C_ChIP50_F*	ACGTTGGATGGTTAGTTTTGTGGCTGCAGG
9008A_ChIP44_F*	ACGTTGGATGGCCATCGTCTCTTCTAATAC
Fie1_ChIP26_F*	ACGTTGGATGACACAAAATGTGCATCGCCG
Mez_1ChIP21_F*	ACGTTGGATGGCTGCCAAATTTGTCCTAAC
Mez_1ChIP23_F*	ACGTTGGATGTGTCGCATGATGGGACATCG
9008A_ChIP45_F*	ACGTTGGATGATTCCACAGAGTCTACCTCC
Mez_1ChIP3_F*	ACGTTGGATGCTCCGTCAGCATAAAATATCG
Fie1_ChIP28_F*	ACGTTGGATGGGCATGTAAGTGAAACCCAA
Fie2_ChIP34_F*	ACGTTGGATGTTGTCTTCGGTTCCCAAAGC
Mez_1ChIP14_F*	ACGTTGGATGGTCTTACAAATGGGACACGC
Mez_1ChIP18_F*	ACGTTGGATGGACGGCAAAGAAAGATTGAC
Mez_1ChIP17_F*	ACGTTGGATGGTAAAGCTGTAGTTGTAGAC
Mez_1ChIP13_F*	ACGTTGGATGATAAGGGTCACAGTGCATCC
Mez_1ChIP1_F*	ACGTTGGATGTTAGTTGACTATGCATTCG
Mez_1ChIP2_F*	ACGTTGGATGTGACATAGTGTATGTCATT
Mez_1ChIP24_F*	ACGTTGGATGTTTTTTTCTACTTCTAGCC
Mez_1ChIP7_F*	ACGTTGGATGGTTCTGCATTCTGATATGAG
Nrp1_ChIP39_F*	ACGTTGGATGTCCTCGATGGTGTCACAGAA
Fie2_ChIP35_F*	ACGTTGGATGTGGATCCGACAACAAAAGGC
Fie1_ChIP30_F*	ACGTTGGATGTACCATGTAAGAGTGAACGC
Mez_1ChIP25_F*	ACGTTGGATGAGAGAGTAGGTTCACGAGAG
9002C_ChIP49_F*	ACGTTGGATGTCCTTGAAGAGGCGCGAGTA
Fie2_ChIP33_F*	ACGTTGGATGTACTACGACGTCTTCGCCAC
9009O_ChIP55_F*	ACGTTGGATGTCAGGAGCCGCCTGGCCG
Mez_1ChIP5_F*	ACGTTGGATGAAGCGACTTCGCGTGTGAG
9009O_ChIP56_F*	ACGTTGGATGCCTGTCTGGTTCCAATAGAG
9008E_ChIP41_F*	ACGTTGGATGGCCACAACCATAGTCAATCG
Mez_1ChIP8_F*	ACGTTGGATGATGCACCCTGCATAGTCTTG
9008E_ChIP40_F*	ACGTTGGATGGGATAGAGGCGATGGTGTC
Fie2_ChIP36_F*	ACGTTGGATGTGATGAGCGAAGGCTTCAAC

Primer	Sequence (5' → 3')
Fie1_ChIP27_F*	ACGTTGGATGAATGGTGGGATGTCCGGTTC
Fie1_ChIP29_F*	ACGTTGGATGGTCCATAGTAGATGTGTCGG
Mez_1ChIP16_F*	ACGTTGGATGCAGGGCAAGCCAAAGAAATG
Mez_1ChIP6_F*	ACGTTGGATGTTCTTGTCTTAGGTCGAGGG
Mez_1ChIP10_F*	ACGTTGGATGTATACCCCGTTTGGATGTAG
Nrp1_ChIP38_F*	ACGTTGGATGACCACCGGGATCAAGAAGAC
Mez_1ChIP9_F*	ACGTTGGATGCCATCAAATATCATAGCACAC
Mez_1ChIP15_F*	ACGTTGGATGGAGATCACTGTTGCAAGACG
Mez_1ChIP11_R*	ACGTTGGATGTTGCTTGGGTACATGTTTTG
9008D_ChIP43_R*	ACGTTGGATGAGATCCCTGCTGCGTGGCAT
9002A_ChIP53_R*	ACGTTGGATGCCGACTGATCACTGATGATG
Mez_1ChIP20_R*	ACGTTGGATGTTGCCCATCATCCTTTGCTC
Mez_2ChIP46_R*	ACGTTGGATGTATTGAGGCTAGCGAGGAAC
Fie1_ChIP31_R*	ACGTTGGATGCGACGCCTGAGCGAGAGGA
Mez_1ChIP4_R*	ACGTTGGATGCGAAGAACCTACTTGAACCC
Mez_1ChIP12_R*	ACGTTGGATGAAATCTCCACAACACTCCCC
Mez_1ChIP19_R*	ACGTTGGATGAGGTGGTCAGCCAAGTATAG
Fie2_ChIP37_R*	ACGTTGGATGAGCTAAGAGAGCTTTGTTGG
9002C_ChIP50_R*	ACGTTGGATGACCTGTTCGCCACGTGAAAG
9008A_ChIP44_R*	ACGTTGGATGGGCCTCCTCAGGTTTCATTT
Fie1_ChIP26_R*	ACGTTGGATGTAGTTGAGGCAGTGGATGTG
Mez_1ChIP21_R*	ACGTTGGATGTCAATGGGCGTGCAAAGAAG
Mez_1ChIP23_R*	ACGTTGGATGCATGAACCAAACAGGCCTTC
9008A_ChIP45_R*	ACGTTGGATGGGTAGAAACAGTAGGTGGTG
Mez_1ChIP3_R*	ACGTTGGATGCGTTTGACAGCTTCAAGGAC
Fie1_ChIP28_R*	ACGTTGGATGAGATTGGAGAAGGCTGAAAG
Fie2_ChIP34_R*	ACGTTGGATGTTCTCAGTGCTTAATGAGGG
Mez_1ChIP14_R*	ACGTTGGATGGCTAATATGCTGATGCAAAGG
Mez_1ChIP18_R*	ACGTTGGATGGAGGAACCTTTTGACCAAAAC
Mez_1ChIP17_R*	ACGTTGGATGCATATACCGTACTTCAAGGC
Mez_1ChIP13_R*	ACGTTGGATGGAAAACAACAGTGCCTCTGG
Mez_1ChIP1_R*	ACGTTGGATGGGGGCTAAAACTTGATAAT
Mez_1ChIP2_R*	ACGTTGGATGAACAAGGATCAACTAGAAC
Mez_1ChIP24_R*	ACGTTGGATGACGTTGAGTTTGTGCAATAC
Mez_1ChIP7_R*	ACGTTGGATGCAGCACAATAAGGAACACTG
Nrp1_ChIP39_R*	ACGTTGGATGTCACAGACATCATCGACGAG
Fie2_ChIP35_R*	ACGTTGGATGTGCCTTATGTGCCTCTTGTG
Fie1_ChIP30_R*	ACGTTGGATGTGTATACGTGGTTTGCCTAC
Mez_1ChIP25_R*	ACGTTGGATGTTAAGGGTGTTATGTCAAC
9002C_ChIP49_R*	ACGTTGGATGTTCGGGCTCGACGCCTTCT
Fie2_ChIP33_R*	ACGTTGGATGACTCCCCAAACCTCAGTAAG
9009O_ChIP55_R*	ACGTTGGATGACCACCTCTTCGGCGGCTC
Mez_1ChIP5_R*	ACGTTGGATGATGCGGACGACGCCACTAC
9009O_ChIP56_R*	ACGTTGGATGCTTTACGGAAAGCTGCTCTG
9008E_ChIP41_R*	ACGTTGGATGGCACGAATTGAAATGGTTTG
Mez_1ChIP8_R*	ACGTTGGATGGAGAAAGGACTTTCGTCTTG
9008E_ChIP40_R*	ACGTTGGATGGAGTAGTCCGTCCACACCTT
Fie2_ChIP36_R*	ACGTTGGATGGATTCTGCAGAGCTTTGTTG
Fie1_ChIP27_R*	ACGTTGGATGAAACCTGGCTCATCATCGAC
Fie1_ChIP29_R*	ACGTTGGATGAACCTCAACTCAGCAAGTCC
Mez_1ChIP16_R*	ACGTTGGATGCTCTATTCTCTCTTGCCCAC
Mez_1ChIP6_R*	ACGTTGGATGCAAATTACTCCGAACCGACC

Primer	Sequence (5' ⟶ 3')
Mez_1ChIP10_R*	ACGTTGGATGTCCATGTGCCCAAACAACAG
Nrp1_ChIP38_R*	ACGTTGGATGGGATGGCCGGAATGTTGTTC
Mez_1ChIP9_R*	ACGTTGGATGGTTGTGTTTGTATTGCTGCC
Mez_1ChIP15_R*	ACGTTGGATGTGCTTCATGATCAATGGTAG
Mez_1ChIP11_UEP*	CAGCACCAGCAGCAG
9008D_ChIP43_UEP*	GGAGTGGACTGGAGC
9002A_ChIP53_UEP*	CGAAAGAGCCACTTGC
Mez_1ChIP20_UEP*	ATGAGCCTGACAGAGC
Mez_2ChIP46_UEP*	cGTCTCCTAGCCCAAGC
Fie1_ChIP31_UEP*	agGTGAACCGGCGGGCG
Mez_1ChIP4_UEP*	GTCACTGATCTGTTGGAC
Mez_1ChIP12_UEP*	TCTTTCTGATTCCACCTTT
Mez_1ChIP19_UEP*	TCCTTTTCAACCTGAACAA
Fie2_ChIP37_UEP*	CAACGGTTGAGTTCTTATC
9002C_ChIP50_UEP*	ggggTGCAGGACGACCCAT
9008A_ChIP44_UEP*	tcTACTCTCAAGGTTTCTCAC
Fie1_ChIP26_UEP*	ACCATATAGAACCACTTATCA
Mez_1ChIP21_UEP*	gggCAAATCAGATTCAGCCTC
Mez_1ChIP23_UEP*	gtagGGGACATCGAAGCCGCA
9008A_ChIP45_UEP*	tcattAGCCTTCCTCCAATTTCT
Mez_1ChIP3_UEP*	TTATAACCTCGAGATAAAACAAT
Fie1_ChIP28_UEP*	tcAAGTGAAACCCAAACAAGAAG
Fie2_ChIP34_UEP*	ccTTCCCAAAGCACAATTTCATTG
Mez_1ChIP14_UEP*	gggtAAATACTGTGGACGAGTTCC
Mez_1ChIP18_UEP*	AAAGATTGACCAACTTTATCATTAT
Mez_1ChIP17_UEP*	gAGTTGTAGACTTGTATTGCAATTT
Mez_1ChIP13_UEP*	tTGCATCCAACCTTAATATATCCTTT
Mez_1ChIP1_UEP*	ttttGTTGACTATGCATTCGAGACCT
Mez_1ChIP2_UEP*	TTGAATCTGAATTTTTGTTAATACCA
Mez_1ChIP24_UEP*	ttttgTCTACTTCTAGCCTATCACATA
Mez_1ChIP7_UEP*	tagcGAAAATAACGTTTAAGAACATGA
Nrp1_ChIP39_UEP*	agccgCGATGGTGTCACAGAAGGACGA
Fie2_ChIP35_UEP*	acCTTGTTGACTATTATACAGAACTCTA
Fie1_ChIP30_UEP*	ttttaGATTCAATCCAAAAGCTTAAACT
Mez_1ChIP25_UEP*	GATATGAGAGAATTTTATAGGATAAAAC
9002C_ChIP49_UEP*	GCGCGAGTACACGGT
Fie2_ChIP33_UEP*	CATCGACTGCTCTCTC
9009O_ChIP55_UEP*	GGGACTTGCGCGGCGA
Mez_1ChIP5_UEP*	taGGGGCTAGGGTTTCC
9009O_ChIP56_UEP*	ccAAAGCTCGTCTACGGT
9008E_ChIP41_UEP*	TTGATTGTCCTTTGAACG
Mez_1ChIP8_UEP*	GGTTGACAAAGATGGTGG
9008E_ChIP40_UEP*	cccaGTGGGGCGGGCAGGG
Fie2_ChIP36_UEP*	TCAACGGTTGAGTTCTTATC
Fie1_ChIP27_UEP*	TGGACCCTCCTTTTTCTTGTC
Fie1_ChIP29_UEP*	gGTGTCGGTTTTGGTACTCTA
Mez_1ChIP16_UEP*	ccccAAGAAATGGCACATGATT
Mez_1ChIP6_UEP*	ccctcAGGTCGAGGGGGCAATG
Mez_1ChIP10_UEP*	ATAGAATTGAGAATTGGAGTTG
Nrp1_ChIP38_UEP*	cataaCAGGTGGCCATGGCCGGT
Mez_1ChIP9_UEP*	CATAGCACACTATTTCATAAATAT
Mez_1ChIP15_UEP*	gtGGCACAATATAATAGAATGGTG

Table 3. Primers used for CAPS imprinting assays, genotyping *mez1-mu* insertion alleles, DMR analyses, *Mez1* real-time PCR Taqman (*) assays and Sequenom (**) assays.

Figure 1

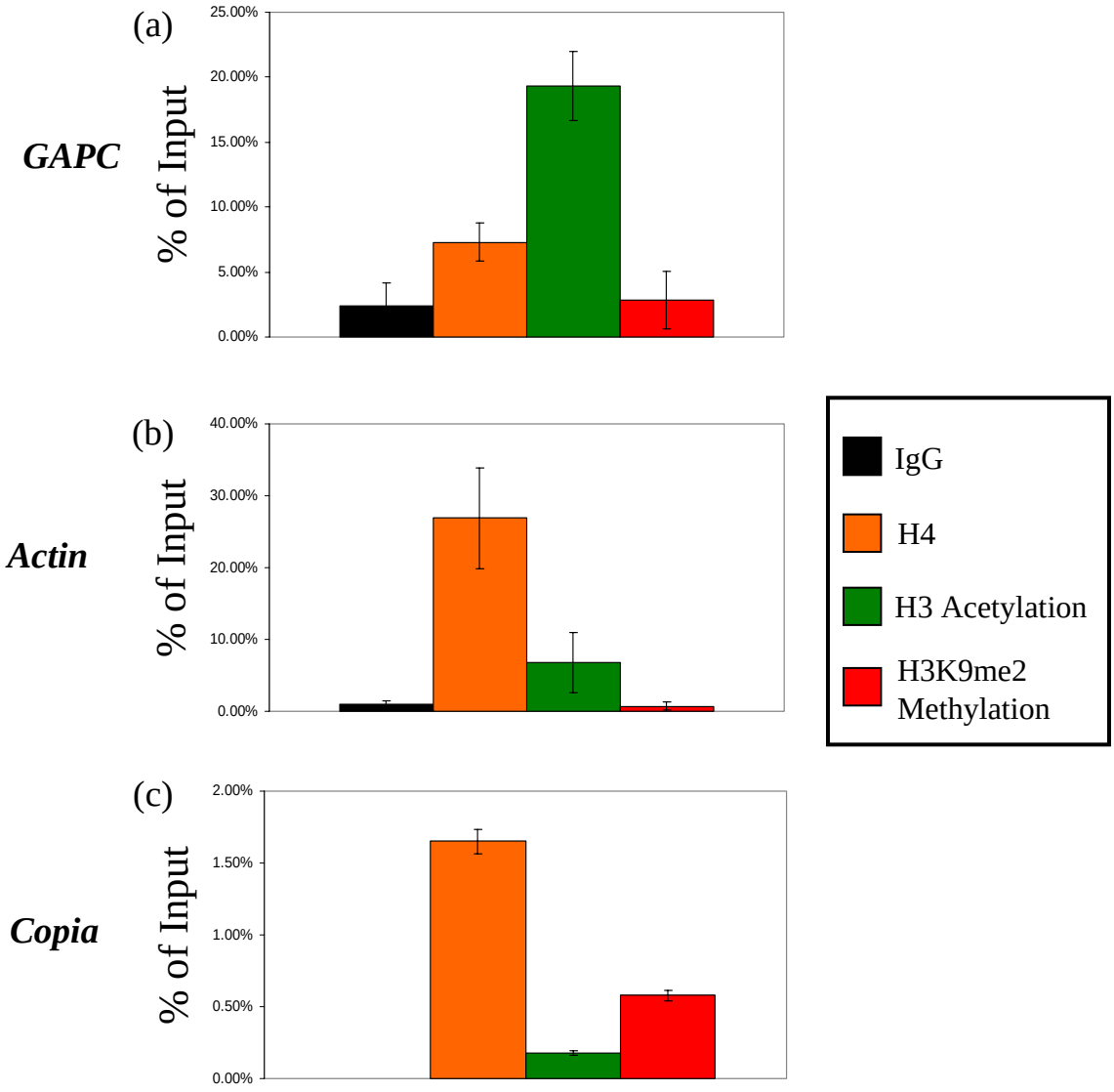

Figure 1. Verification of ChIP. ChIP was performed on 14 DAP endosperm tissue with IgG and antibodies specific to H4, H3 acetylation or H3K9me2. Quantitative real-time PCR was performed with primers specific to *Actin*, *GAPC* or *Copia* to quantify the concentration of DNA recovered in each immunoprecipitation and in the input chromatin. The amount of DNA from each immunoprecipitation was calculated from a standard curve and compared to the calculated concentration of input control. Enrichment for a particular histone modification will result in a % of input significantly higher than IgG of a non-enriched histone modification. (a,b) GAPC and Actin show a significant amount of enrichment for H4 and H3 acetylation. (c) Copia shows a significant amount of H4 and H3K9me2 enrichment.

Figure 2

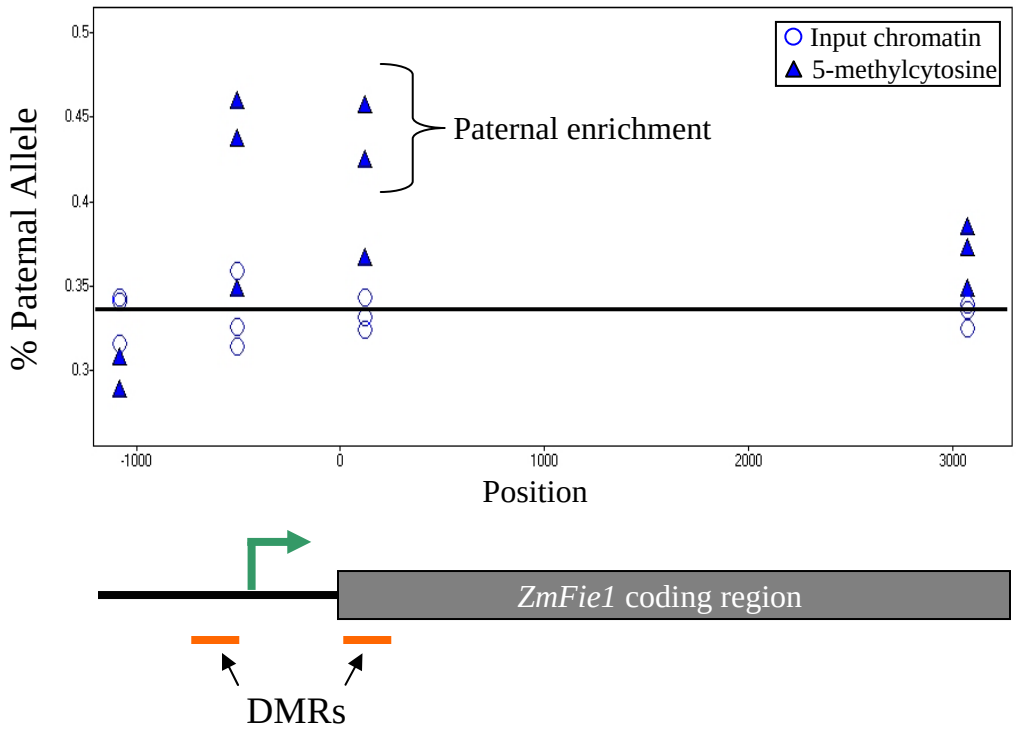

Figure 2. Paternal enrichment of 5-methylcytosine in the promoter of *ZmFie1*. Chromatin from 14 DAP endosperm tissue was immunoprecipitated with an antibody against 5-methylcytosine. Four different quantitative SNP assays were performed on the immunoprecipitated chromatin to determine the percent of the paternal allele (y-axis) in input chromatin and in chromatin precipitated with the 5-methylcytosine antibody. The position of the four SNPs in the *ZmFie1* gene is indicated along the x-axis. A schematic of the *ZmFie1* gene is shown with the position of the transcription start site (green arrow), coding region (grey box) and DMR (orange lines indicate regions determined by Hermon et al.,2007) shown. The horizontal black line indicates the expected value of 0.33 if there is no enrichment for maternal or paternal alleles. The open circles represent averaged, normalized input values for each SNP assay and are all near the expected value. The blue triangles represent the percent of the paternal allele detected in the chromatin precipitated with the 5-methylcytosine antibody. Multiple biological replicates are shown for each SNP assay.

Figure 3

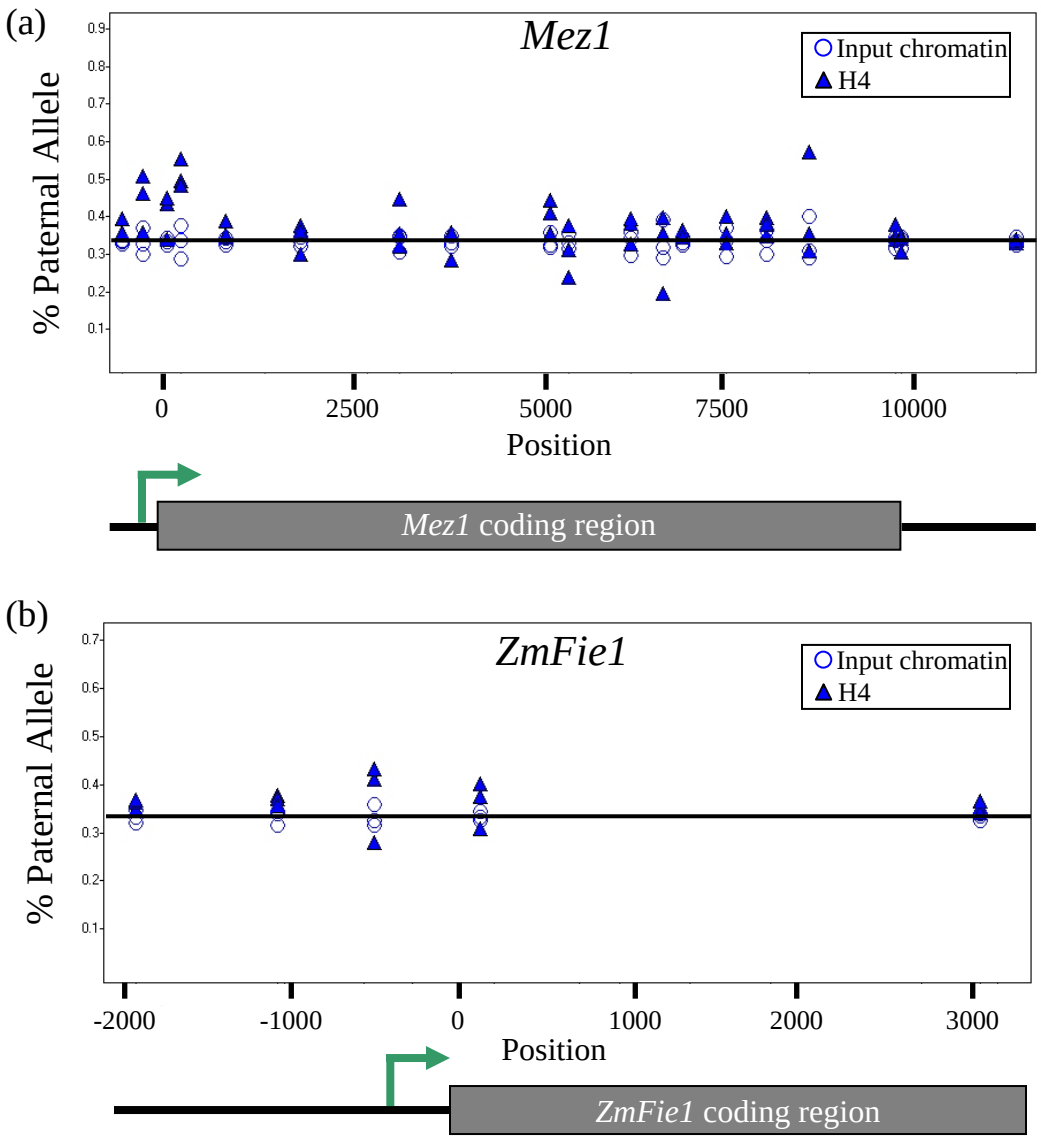

Figure 3. Analysis of nucleosome occupancy on the maternal and paternal alleles of *Mez1* and *ZmFie1*. A series of quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with a histone H4 antibody (blue triangles). The position of each SNP within the *Mez1* (A) or *ZmFie1* (B) transcript is indicated along the x-axis. The location of the transcription start site is indicated by the green arrow. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 4

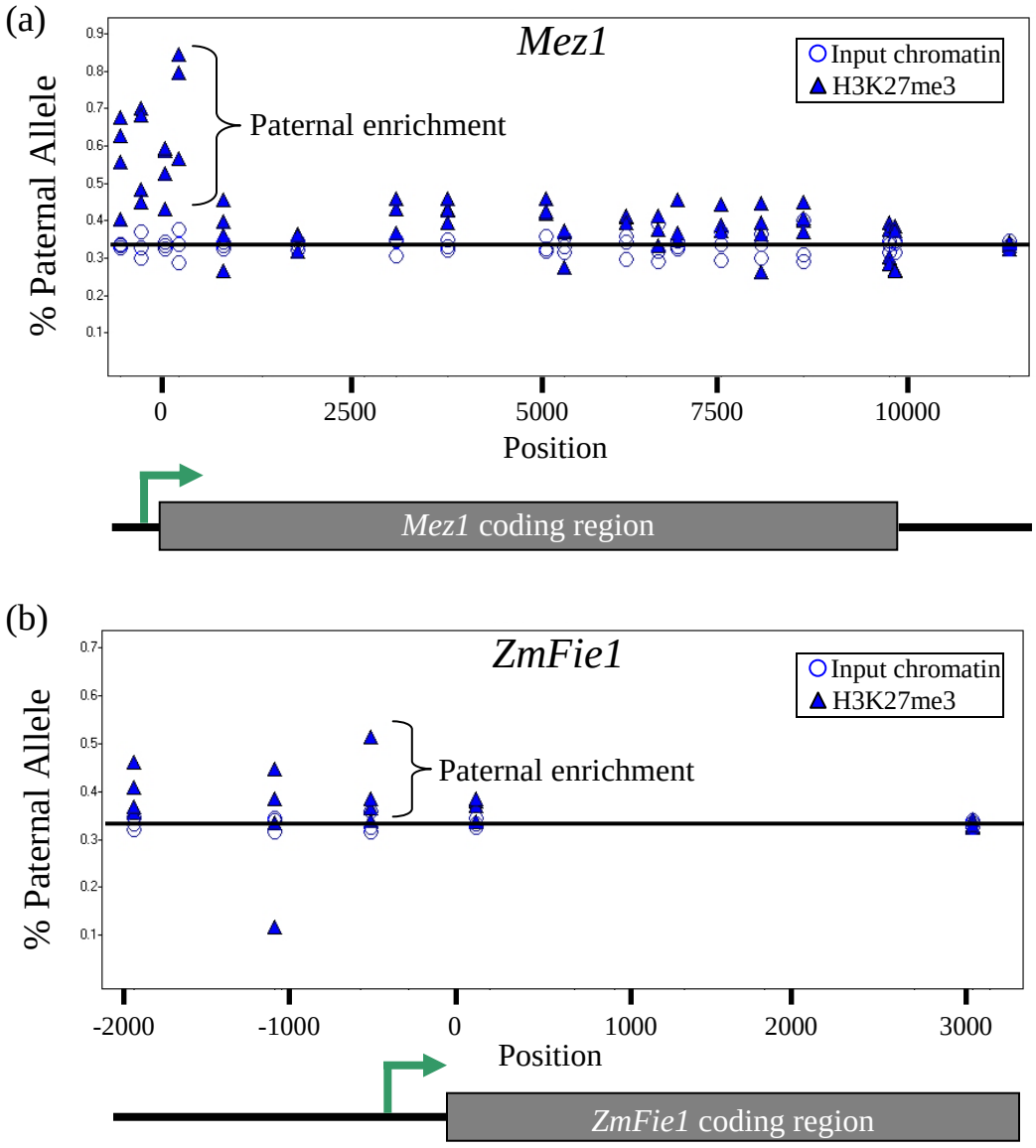

Figure 4. Paternal enrichment for H3K27me3 in the promoter of *Mez1* and *ZmFie1*. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using an antibody that recognizes H3K27me3. The quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with a H3K27me3 antibody (blue triangles). The position of each SNP within the *Mez1* (A) or *ZmFie1* (B) transcript is indicated along the x-axis. The location of the transcription start site is indicated by the green arrow. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 5

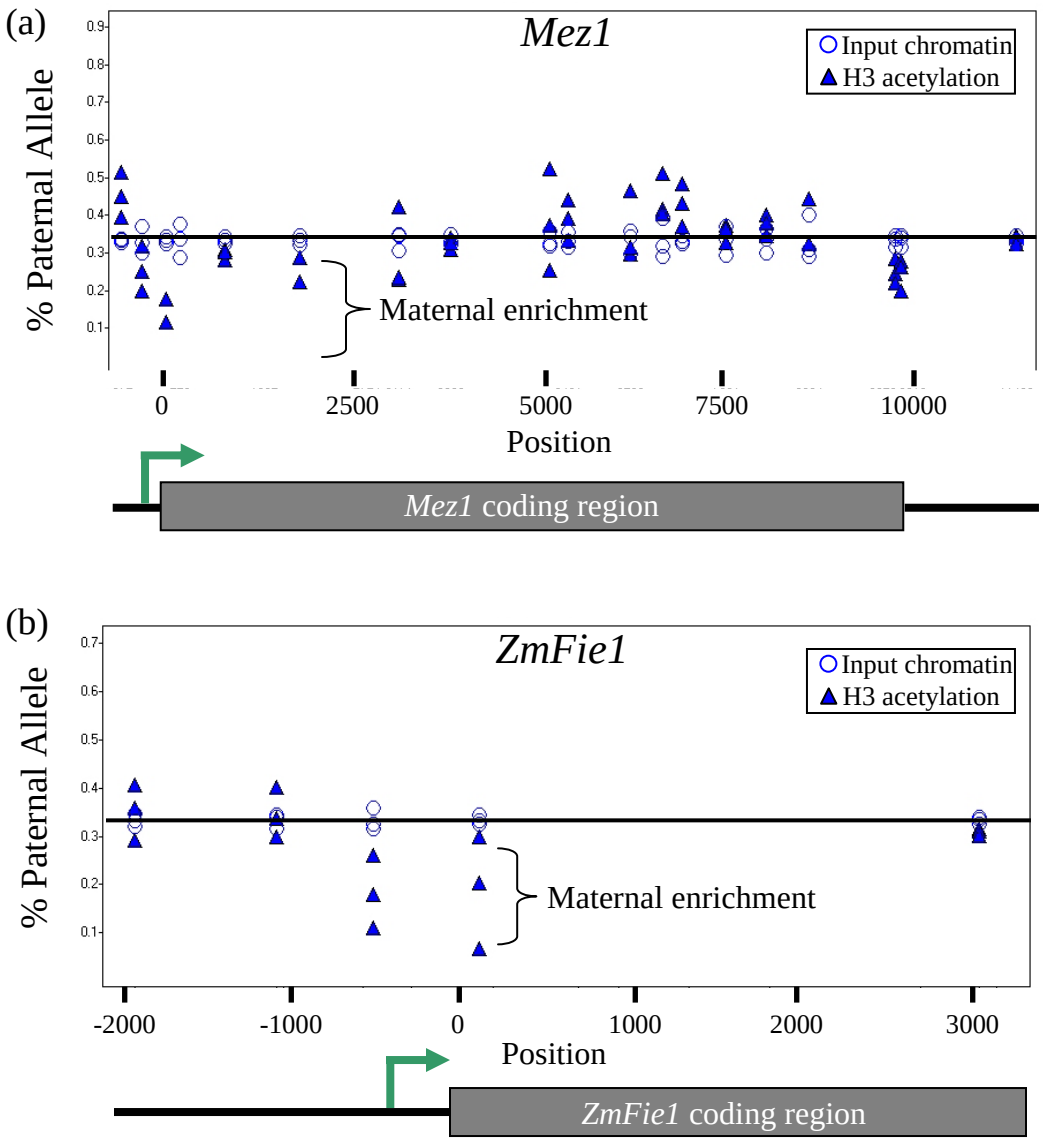

Figure 5. Maternal enrichment for H3 acetylation in the promoter of *Mez1* and *ZmFie1*. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using an antibody that recognizes H3K27me3. The quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with a H3 acetylation antibody (blue triangles). The position of each SNP within the *Mez1* (A) or *ZmFie1* (B) transcript is indicated along the x-axis. The location of the transcription start site is indicated by the green arrow. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 6

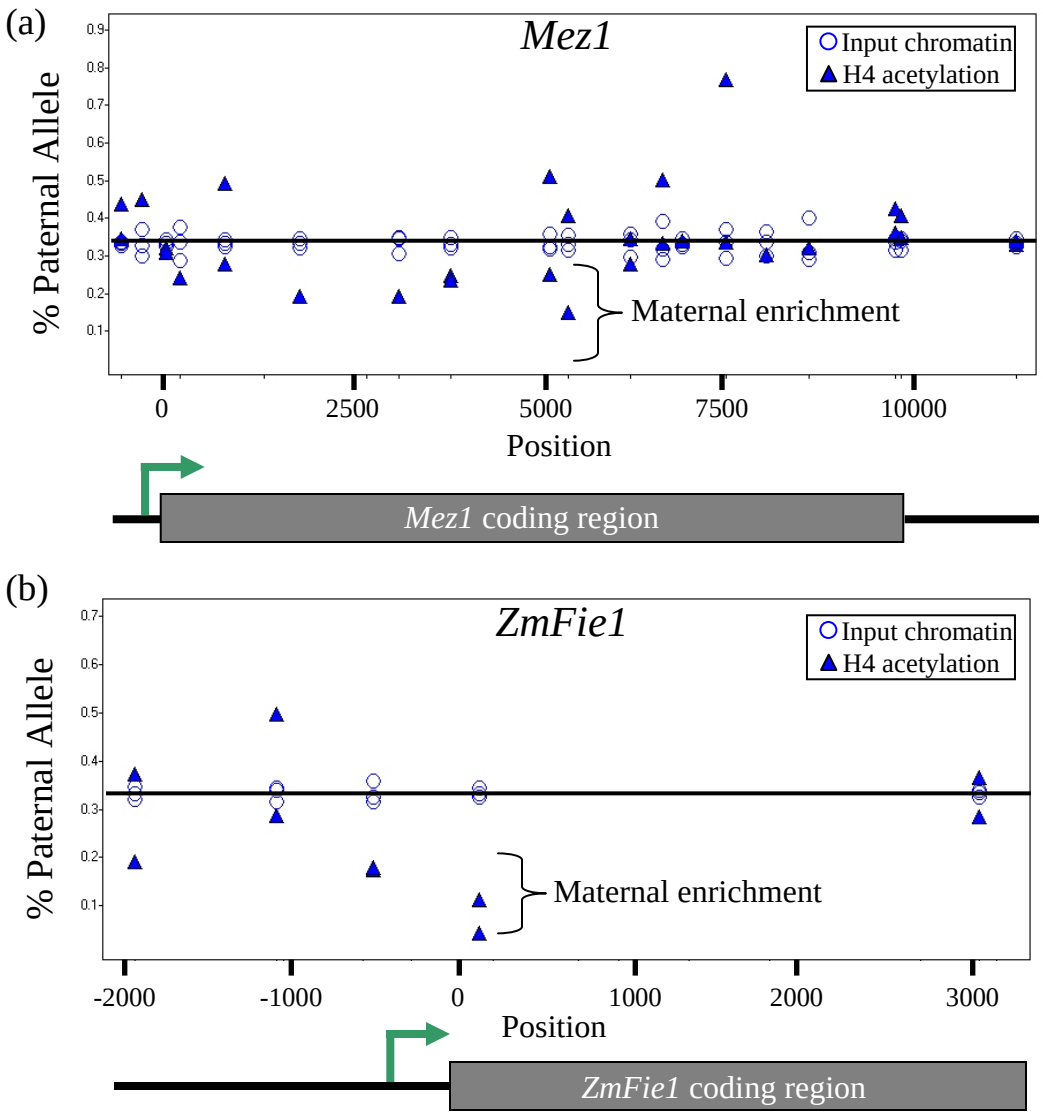

Figure 6. Maternal enrichment for H4 acetylation in the promoter of *Mez1* and *ZmFie1*. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using an antibody that recognizes H3K27me3. The quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with a H4 acetylation antibody (blue triangles). The position of each SNP within the *Mez1* (A) or *ZmFie1* (B) transcript is indicated along the x-axis. The location of the transcription start site is indicated by the green arrow. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 7

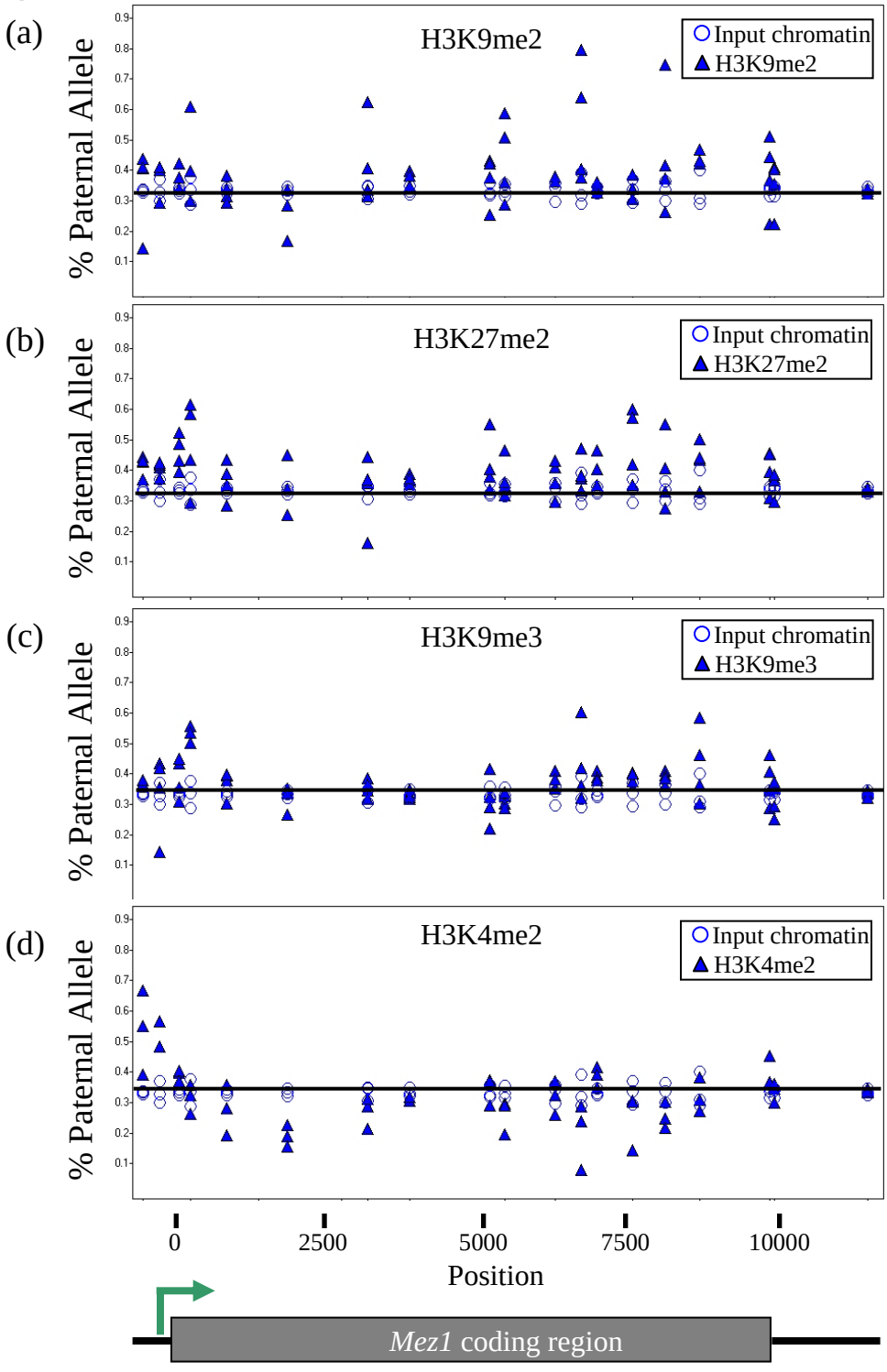
(a)
H3K9me2
Input chromatin
H3K9me2
% Paternal Allele
(b)
H3K27me2
Input chromatin
H3K27me2
% Paternal Allele
(c)
H3K9me3
Input chromatin
H3K9me3
% Paternal Allele
(d)
H3K4me2
Input chromatin
H3K4me2
% Paternal Allele
0
2500
5000
7500
10000
Position
Mez1 coding region

Figure 7. Several histone modifications do not show evidence for allele-specific enrichment at *Mez1*. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using antibodies that recognize H3K9me2 (a), H3K27me2 (b), H3K9me3 (c) or H3K4me2 (d). The quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with specific antibodies (blue triangles). The position of each SNP within the *Mez1* transcript is indicated along the x-axis. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 8

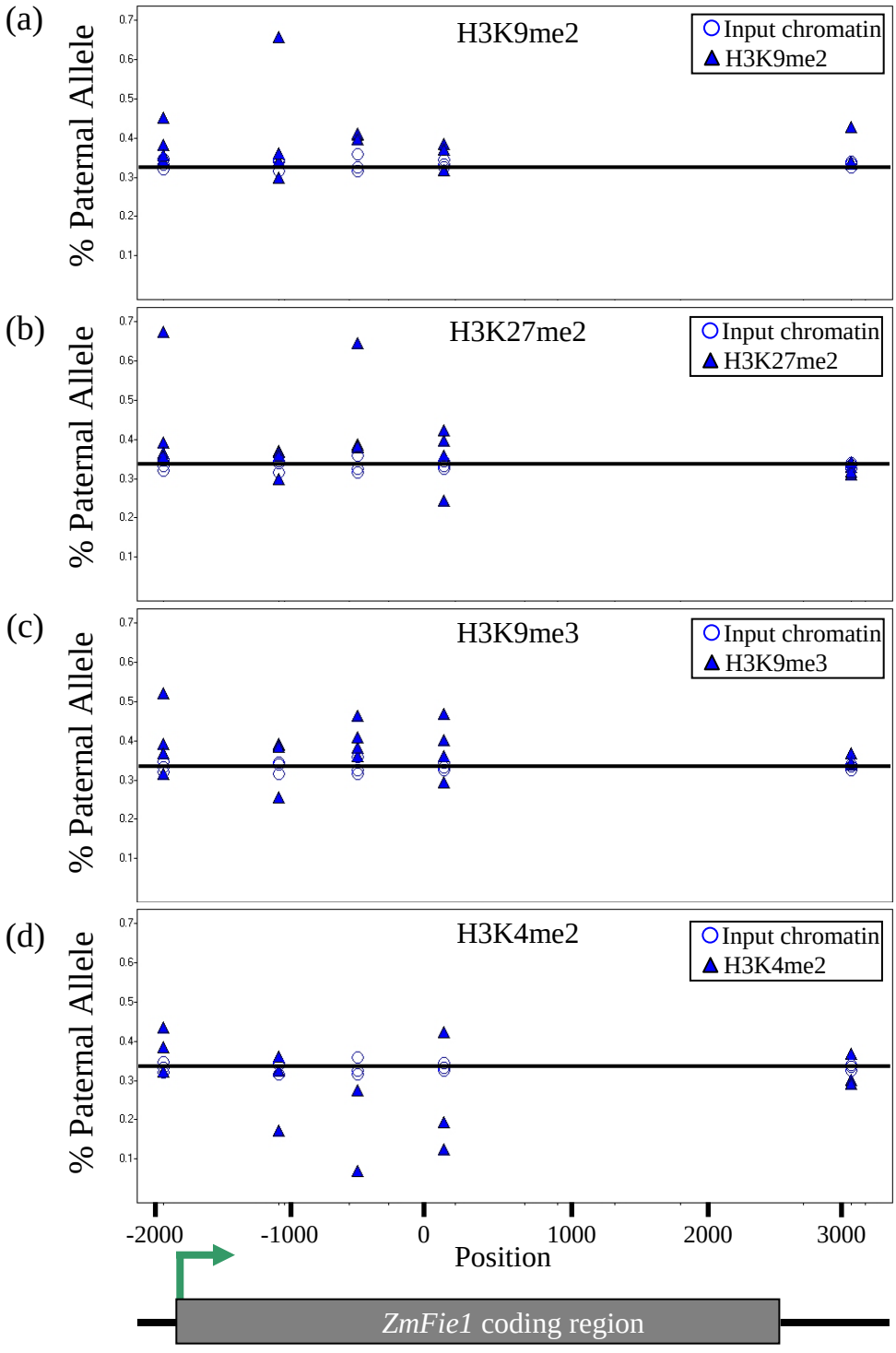
(a)
H3K9me2
Input chromatin
H3K9me2
% Paternal Allele
(b)
H3K27me2
Input chromatin
H3K27me2
% Paternal Allele
(c)
H3K9me3
Input chromatin
H3K9me3
% Paternal Allele
(d)
H3K4me2
Input chromatin
H3K4me2
% Paternal Allele
-2000
-1000
0
1000
2000
3000
Position
ZmFie1 coding region

Figure 8. Several histone modifications do not show evidence for allele-specific enrichment at *ZmFie1*. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using antibodies that recognize H3K9me2 (a), H3K27me2 (b), H3K9me3 (c) or H3K4me2 (d). The quantitative SNP assays were used to determine the percent of the paternal allele (y-axis) detected in input chromatin (open circles) and chromatin precipitated with specific antibodies (blue triangles). The position of each SNP within the *ZmFie1* transcript is indicated along the x-axis. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment. Values from the three different biological replicates are shown for each SNP.

Figure 9

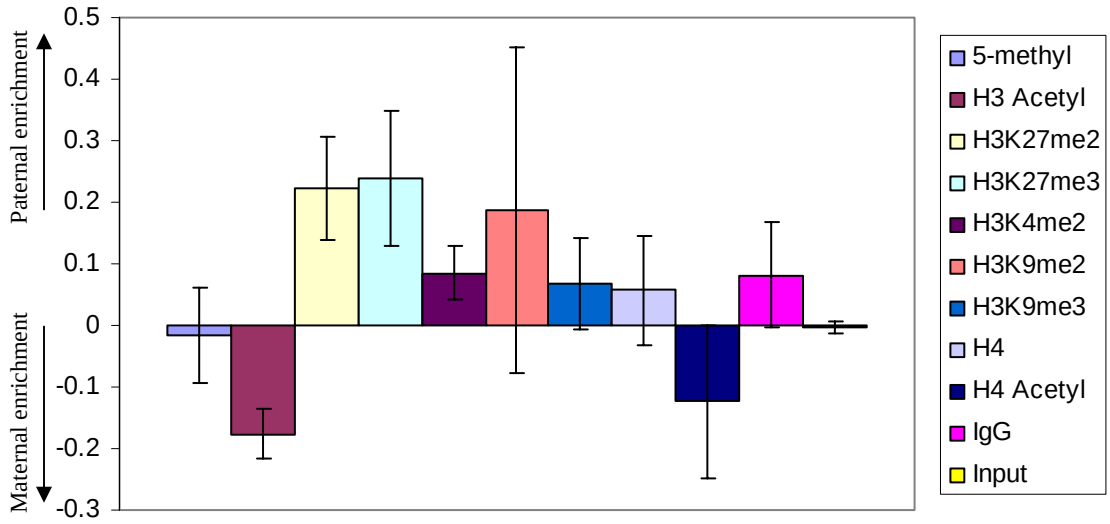

Figure 9. Analysis of allele-specific enrichment for histone modifications in the imprinted *Nrp1* gene. Chromatin immunoprecipitation was performed on chromatin isolated from 14 DAP endosperm tissue using a series of antibodies that recognize histone H4 or specific histone modifications. The allelic enrichment (percent paternal allele detected in immunoprecipitated chromatin minus percent paternal allele detected in input chromatin) was determined for each antibody. The standard deviation (based on three biological replicates) is shown for each antibody. We see evidence for maternal enrichment for H3 and H4 acetylation and paternal enrichment for H3K27me2 and H3K27me3.

Figure 10

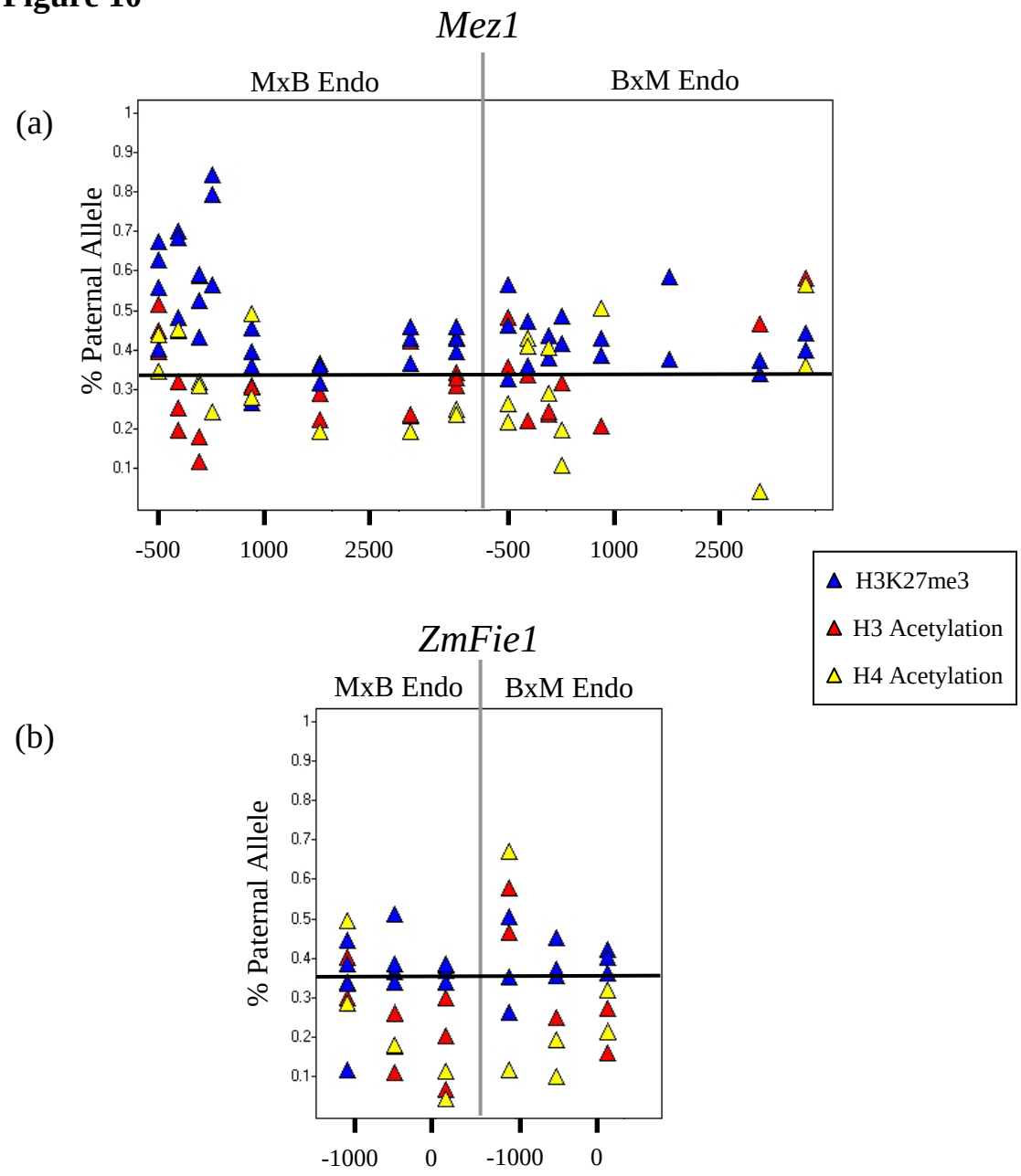

Figure 10. Detailed analysis of H3 acetylation, H4 acetylation and H3K27me3. Data as shown in figures 4, 5 and 6 was analyzed in more detail in the promoter and 5' coding regions of *Mez1* and *ZmFie1*. The percent of the paternal allele (y-axis) for H3 acetylation (red triangles), H4 acetylation (red triangles) and H3K27me3 (blue triangles) is shown. The position of each SNP within the *Mez1* (a) or *ZmFie1* (b) transcript is indicated along the x-axis. The horizontal black line indicates the expected percent of the paternal allele if there is no enrichment.

Figure 11

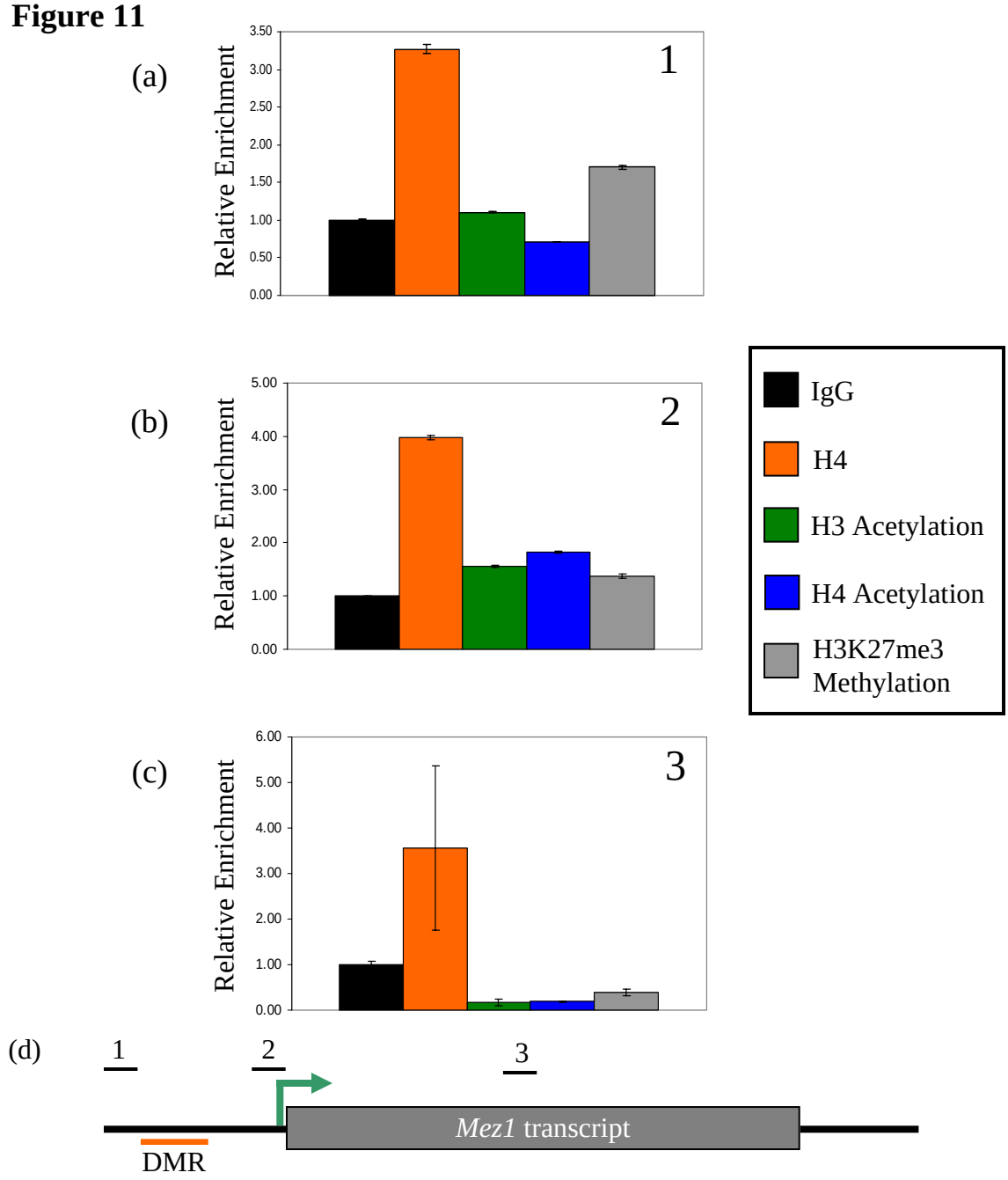
(a)
Relative Enrichment
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
1
(b)
Relative Enrichment
5.00
4.00
3.00
2.00
1.00
0.00
2
IgG
H4
H3 Acetylation
H4 Acetylation
H3K27me3 Methylation
(c)
Relative Enrichment
6.00
5.00
4.00
3.00
2.00
1.00
0.00
3
(d)
1
2
3
Mez1 transcript
DMR

Figure 11. Real-time PCR analyss of histone modification enrichments in three regions of *Mez1*. The relative enrichment of IgG, H4, H3 acetylation, H4 acetylation and H3K27me3 in immunoprecipitated DNA was determined by real-time PCR. The amount of DNA from each immunoprecipitation was calculated from a standard curve and compared to the calculated concentration of input control. Enrichment for each antibody relative to IgG was then calculated. Enrichment for a particular histone modification will result in a relative enrichment greater than 1.00. (a) Relative enrichment of H4 and H3K27me2 in region 5' to the DMR. (b) Relative enrichment of H4, H3 acetylation, H4 acetylation and H3K27me in the region 5' to transcription start. (c) Relative enrichment of only H4 in the fourth exon of the coding region. (d) Genomic map of *Mez1*. 5' and 3' genomic regions are shown as a thick blank like, transcription start site is shown as a green arrow, the three regions analyzed are shown as thin black lines above the figure and the DMR is shown as an orange line below the figure.

Figure 12

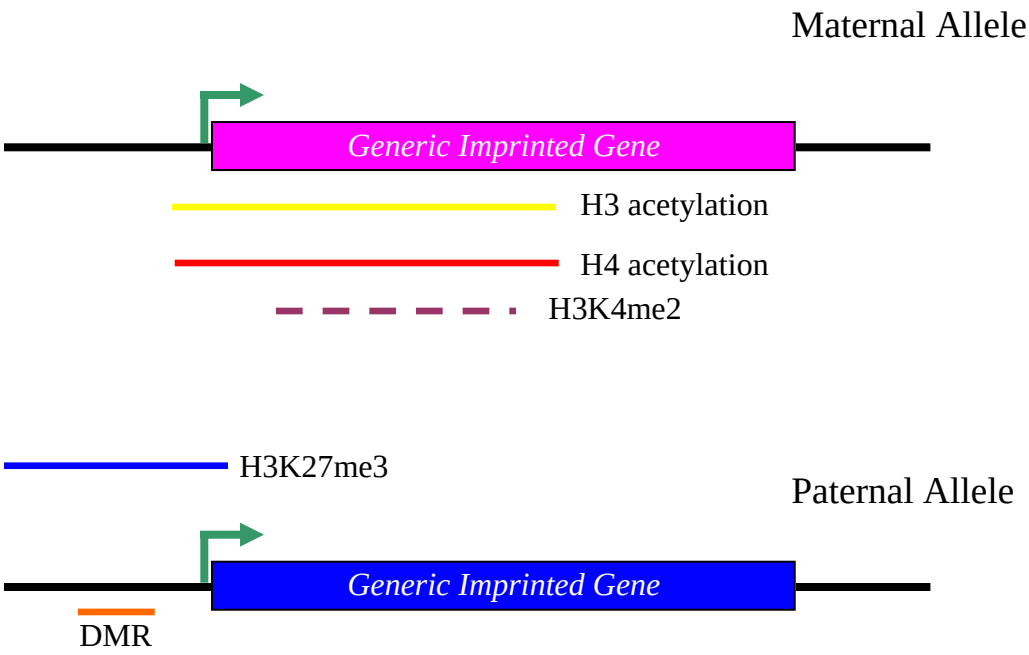

Figure 12. Model for the epigenetic regulation of imprinted loci in plants. A genomic structure for a generic plant imprinted gene is shown, with the coding region represented by the colored rectangle (pink for the maternal allele and blue for the paternal allele). Transcription start is indicated by the green arrow. The location of the regions enriched for either DNA methylation and/or histone modifications are shown as colored lines.

Chapter 4
Loss-of-imprinting is associated with *mu* transposon insertions in the 5'*cis*-proximal region of *Mez1*

INTRODUCTION

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* (Choi *et al*, 2002; Choi *et al*, 2004; Gehring *et al*, 2006). The putative histone methyltransferase encoded by the maternal *MEA* locus is actually important for the silencing of the paternal allele of *MEA*, likely through methylation of H3K27 (Jullien *et al*, 2006). This and other information has been formulated into a model of imprinting mechanisms 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 the normally imprinted loci.

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 (Spillane *et al*, 2004). The DNA regions that are sufficient for imprinting of the *FIS2* and *MEA* loci have been determined using transgenic fusion constructs (Lou *et al*, 2000). 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 (Hermon *et al*, 2007; Gutierrez-Marcos *et al*, 2006). Additionally, Gutierrez-Marcos *et al* (2006) demonstrated imprinted expression and differential DNA methylation of *Fie1*-GUS and *Fie2*-GFP transcriptional fusions containing ~4kb of promoter and downstream sequence elements. Danilevskaya *et al* (2003) proposed a two CpG island rule as a 'mark' for genomic imprinting, however no other plant imprinted genes contain more than one CpG island (Haun *et al*, 2007). A comparison of the primary sequences required for imprinting as defined by transgenic fusions or differential methylation does not identify any conserved sequence motifs (Haun *et al*, 2007).

While there has been significant progress towards 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 (Gehring *et al*, 2006; Choi *et al*, 2002; Jullien *et al*, 2006). 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 altered regulation at other genomic loci. A recent study in *Arabidopsis* demonstrated that seed production could occur without 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 increases overall seed size, but also introduces genetic information that may seek to selfishly misallocate 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* (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* (Haun *et al*, 2007). We identified several alleles that contain *Mu* transposons insertions in the 5' proximal sequences of the *Mez1* gene. These transposon insertion alleles were used to probe the causes and effects of imprinted expression at the *Mez1* locus.

RESULTS

Characterization of the *mu*-transposon insertion alleles at the *Mez1* locus. Three *Mutator* transposons insertion alleles for the *Mez1* locus were identified during a screen of Pioneer Hi-Bred's Trait Utility System for Corn (TUSC) mutant collection (provided by Bob Meeley). These three insertion alleles were back-crossed into the B73 and Mo17 genetic backgrounds for at least four generations. 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 in between the *Mez1* transcription start site and the previously identified differentially methylated region (DMR) (Haun *et al*, 2007).The *-m1, -m2* and *-m4* insertions are 38, 151 and 159 base pairs, respectively, upstream of the *Mez1* transcription start site. Based upon the family pedigrees and sequencing of several regions of the *Mez1* locus we were able to infer the likely progenitor allele. 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.

The *mez1-m1* allele is an insertion of a 4.9kb MuDR element into a B73-like *Mez1* allele. The *Mez1* coding sequence linked to the *mez1-m1* insertion contains B73-like SNP polymorphisms and imprinting can be assayed when this allele is crossed to Mo17. The *mez1-m1* allele has been backcrossed to B73 for several generations, so a plant heterozygous for *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 2.2kb *Mu7* and a 2.2 kb *Mu4* element, respectively, into an A632-like *Mez1* allele. 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 several generations, resulting in individuals that are heterozygous for the *mez1-m2* or *mez1-m4* insertions and homozygous for Mo17-like SNPs in the coding region and DMR.

***mez1-mu* insertion lines do not effect *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 (Figure 4d and data not shown). Plants heterozygous and homozygous for the *mez1-mu* insertion alleles showed no abnormal growth phenotype or reduced germination relative to their wild-type siblings (Figure 2a, 3a & 4a). In order to determine if the *mez1-mu* insertions had an affect on overall seed weight, 100 seeds from the ears described above were individually weighed. If the *mez1-mu* insertions resulted in improper seed development, a non-normal distribution of seed weights would have been observed. However, the distribution of individual seed weights for all three *mez1-mu* alleles appeared normal (Figure 2b, 3b & 4b). The 12 heaviest and lightest seeds were germinated and genotyped to verify that certain genotypic classes were not enriched. For each *mez1-mu* allele, the resultant seedlings segregated approximately 1:2:1 for *mez1-mu* in both the heavy and light seeds (data not shown). *Mez1* is imprinted in endosperm tissue, but expression is bi-allelic in all other tissues tested (Springer *et al*, 2002; Haun *et al*, 2007). To better understand the effect of the *mez1-mu* insertions, the expression level of *Mez1* was analyzed in tissue from seedlings segregating for the *mez1-mu* insertion for

all three alleles. Real-Time PCR results did not provide evidence for altered expressed levels of *Mez1* in seedlings homozygous for *mez1-mu* relative to wild-type or heterozygous siblings (Figure 2c, 3c & 4c). The three *Mu* transposons insertion alleles do not appear to have an effect on the expression level of *Mez1* in vegetative tissues.

***Mu* insertion alleles affect imprinting of *Mez1*.** Although the *mu* insertions at the *Mez1* allele did not affect seedling expression levels, we were interested in determining whether the normal imprinted expression pattern was maintained in these alleles. Insertional mutagenesis has been used to generate a loss-of-imprinting phenotype in mouse at the *Dlk1-Gtl2* locus (Steshina *et al*, 2006). The location of the *mu* insertions in the 5'*cis*-proximal region of *Mez1* suggested that imprinted regulation could potentially be disrupted since the location of the DMR is 1.5-4kb further from the transcription start that in the wild-type *Mez1* alleles. We initiated a screen to survey for a loss-of-imprinting at the *Mez1* locus when a *mez1-mu* insertion allele is inherited from the maternal parent or the paternal parent. A total of 67 crosses involving the three *mu* insertion lines and wild-type B73 or Mo17 plants were made. RNA was isolated from a pool of 12-16 sibling endosperms that were isolated 14-16 days after pollination (DAP). The pooled endosperms were segregating 1:1 for kernels that are heterozygous for the *mez1-mu* insertion allele and homozygous for non-insertion alleles. By screening pooled tissue we were able to assess whether imprinting was disrupted in a large number of crosses. Quantitative SNP assays were performed using the MassArray system on *Mez1* transcripts produced by RT-PCR from the pooled endosperm tissue. In several assays of wild-type B73×Mo17 or Mo17×B73 endosperms, expression was detected exclusively

from the maternal allele as expected (data not shown). However, many of the crosses involving *mez1-mu* alleles showed activation of the paternal allele consistent with a segregating loss-of-imprinting (Table 1).

The different *mez1-mu* insertion alleles showed different loss-of-imprinting patterns. We observed a loss-of-imprinting when *mez1-m1* was inherited from the maternal parent but rarely observed imprinting defects when *mez1-m1* was inherited from the paternal parent. The *mez1-m2* allele showed the opposite pattern. We frequently observed a loss-of-imprinting when *mez1-m2* was inherited from the paternal parent but rarely observed effects on imprinting when this allele was inherited from the maternal parent. The *mez1-m4* allele often caused a loss-of-imprinting when inherited from either the maternal or the paternal parent. The results from the imprinting analyses of pooled endosperm tissue suggests the *mu* allele insertions in the 5'*cis*-proximal region of *Mez1* are disrupting the normal mono-allelic expression typically observed in endosperm tissue.

***mez1-m1* causes a loss-of-imprinting when inherited from the maternal parent.** We proceeded to analyze individual endosperms that were segregating for the *mez1-m1* insertion. Two plants that were heterozygous for the *mez1-m1* allele in the B73 genetic background were reciprocally crossed as males and females to wild-type Mo17 plants resulting in four ears. These crosses result in endosperms that are polymorphic for a SNP within the coding region of *Mez1* and are segregating 1:1 for wild-type:heterozygous *mez1-m1* insertions. Both CAPS (Figure 5a) and quantitative SNP (Figure 5b) analyses were used to test for activation of the normally silent paternal allele. The CAPS analysis revealed expression from both the maternal and paternal alleles in endosperms that inherit

the *mez1-m1* allele from the maternal parent (Figure 5a). However, when *mez1-m1* was inherited from the paternal parent only the maternal allele was detected. We confirmed these results using quantitative SNP analyses on at least four heterozygous endosperms and two wild-type endosperms from each of the four ears that were produced. The percent of the *Mez1* transcripts from the paternal allele was determined for each endosperm. We observed significant levels (~20-30%) of transcripts from the paternal allele of *Mez1* when the *mez1-m1* allele was inherited from the maternal parent. The other genotypes showed little or no expression of the paternal allele in these samples. Quantitative Real-Time PCR was used to compare the overall expression of *Mez1* in several individuals of both genotypes (heterozygous *mez1-m1* and wild-type) from all four ears (Figure 5c). We did not notice consistent significant differences in the expression level associated with any of the genotypes. The CAPS and quantitative SNP assays on both individual and bulk endosperm samples suggest that the inheritance of the *mez1-m1* allele from the maternal parent can result in activation of the wild-type paternal allele. These results agree with our survey of imprinting in a number of crosses involving *mez1-m1* (Table 1).

***mez1-m2* causes loss-of-imprinting when inherited from the paternal parent**. The same series of experiments were performed to determine how the *mez1-m2* allele affects imprinting of *Mez1* (Figure 6). The CAPS assays suggested that inheritance of the *mez1-m2* allele through the maternal parent did not have any effect upon imprinted expression. However, when *mez1-m2* was inherited from the paternal parent we observed activation of the paternal allele in both wild-type endosperms (Figure 6a). Interestingly, we also

observed lower levels of paternal *Mez1* transcripts in the wild-type endosperms of the paternal transmission crosses. These results were confirmed by the quantitative SNP data (Figure 6b). The maternal transmission of *mez1-m2* did not affect *Mez1* imprinting; however, when a *mez1-m2* allele was paternally transmitted we observed a significant proportion (20-25%) of paternal *Mez1* transcripts. We saw significantly lower levels of paternal *Mez1* transcripts in the wild-type siblings of the paternal transmission of *mez1-m2* crosses. Quantitative real-time PCR experiments showed slightly lower levels of *Mez1* expression in heterozygous and wild-type endosperms derived from a cross of $B73^{WT}/B73^{WT}$ X $Mo17^{WT}/Mo17^{mez1\text{-}m2}$ than in the reciprocal cross (Figure 6c). However, the *Mez1* expression levels were not different in the endosperms that were heterozygous for the *mez1-m2* allele relative to wild-type siblings.

***mez1-m4* causes loss-of-imprinting when inherited from either parent**. A similar set of experiments were performed on individual endosperms resulting from reciprocal crosses of plants that were heterozygous for the *mez1-m4* allele. In both the CAPS (Figure 7a) and quantitative SNP (Figure 7b) analyses, we observed expression of the paternal *Mez1* allele in heterozygous endosperm samples but not in wild-type siblings. This loss-of-imprinting was observed when the *mez1-m4* allele was inherited from either the maternal parent or from the paternal parent. Interestingly, the level of *Mez1* expression differed in heterozygous endosperms relative to wild-type siblings and the level of expression also varies depending upon which parent transmited the *mez1-m4* allele (Figure 7c). If the maternal parent was heterozygous for the *mez1-m4* allele, then the resulting heterozygous off-spring exhibit lower levels of *Mez1* expression than wild-

type sibling endosperms. However, if the paternal parent was heterozygous for *mez1-m4* then the resulting heterozygous offspring exhibited higher levels of *Mez1* expression than wild-type siblings.

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 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 alleles of the imprinted *Mez1* gene to further probe the mechanisms of imprinting. Interestingly, these alleles can result in a loss-of-imprinting phenotype. These findings allow us to discuss the biological role of imprinting and to add to the plant imprinting mechanism.

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 a handful of 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 transposons 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. The *mez1-mu* alleles had no affect on germination or seedling growth (Figures 2a, 3a & 4a), nor was the weight of heterozygous or homozygous *mez1-mu* seeds different from that of wild-type seeds (Figures 2b, 3b & 4b). The insertions also did not alter the bi-allelic expression levels of *Mez1* in non-imprinted tissues (Figures 2c, 3c & 4c). 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 (Figure 4d).

These results may suggest the silencing of paternal *Mez1* is not necessary for normal endosperm and seed development. The kinship theory of genomic imprinting (Haig and Westoby, 1989; Haig, 2004) suggests imprinting evolved as a parental 'tug-of-war' to control the expression of certain developmental genes. Nutrient-acquiring genes would be down-regulated by the mother to ensure equal distribution of nutrients to all her offspring. Under this theory, one of the consequences a loss-of-imprinting should result in the over-acquisition of nutrients and a growth advantage. Our results demonstrate that activation of the silent allele of one imprinted gene has no detectable phenotypic consequence. Several possibilities exist to explain our results and the lack of a phenotype associated with paternal *Mez1* expression. The *Mez1* gene was originally identified as a

maize homolog to the *Drosophila* PcG gene *Enhancer-of-Zeste [E(z)]* (Springer *et al*, 2002). Based on genetic and biochemical evidence in *Arabidopsis* and *Drosophila* the *E(z)*-like genes often function in a protein complex containing an *esc*-like, *Su(z)12*-like and *p55*-like gene. Evidence from *Arabidopsis* has shown the imprinted genes *MEA* and *FIS2* are members of a plant PcG protein complex (Spillane *et al*, 2000; Köhler *et al*, 2003; Wang *et al*, 2006). A similar complex in maize may contain the imprinted genes *Mez1* and *Fie1/Fie2*. For the plant PcG protein complexes, redundant imprinting of multiple members of the complex could explain the lack of a phenotype when one of the members is bi-allelically expressed. Paternal activation of *Mez1* may not be sufficient to increase the level of the functional PcG complex and therefore does not alter endosperm phenotype.

***mez1-mu* alleles provide new insight 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 (Haun *et al*, 2007; Vielle-Calzada *et al*, 1999; Xiao *et al*, 2003), histone methylation (Gehring *et al*, 2006; Jullien *et al*, 2006) and self-imprinting autoregulation (Baroux *et al*, 2006). The expected and unexpected results presented here open the door for a variety of theories to expand upon the existing model for the regulation of imprinted expression in plants.

Paternal inheritance of mez1-mu results in activation of paternal Mez1. The activation of paternal *Mez1* allele when *mez1-mu* was inherited paternally was not unexpected and

seems to fit with the current model for the imprinting mechanism in plants. 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) suggests that the *Mez1* imprinting mechanism may involve targeted paternal-allele silencing. Paternal activation is an obvious consequence of an insertion that displaces the silencing epigenetic modifications from the coding region. Displacement of the DMR may have prevented a hypothetical methyl-binding silencing factor from localizing to the paternal *Mez1* promoter. Without this silencing factor, the expression of paternal *Mez1* could have been driven from the normal promoter or from a cryptic promoter within the *mu* transposon.

Maternal inheritance of mez1-mu results in activation of the paternal allele. It was unexpected to observe a loss-of-imprinting when *mez1-mu* was inherited maternally, as was the case for *mez1-m1* and *mez1-m4* (Figures 5 & 7). This scenario suggests some sort of communication is occurring between the maternal and paternal alleles of *Mez1*. This communication between the maternal and paternal alleles could occur through the production of protein, small RNA or a DNA pairing mechanism. Recent studies have suggested the MEA protein produced by the maternal allele is involved in establishing and maintaining the silencing of the paternal allele (Gehring *et al*, 2006; Jullien *et al*, 2006; Baroux *et al*, 2006). 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. The *mez1-mu* insertions might be reducing the amount of maternal *Mez1* or altering temporal/spatial expression patterns. If a certain level of maternal *Mez1* transcript at a precise developmental time point (ie high

expression immediately after fertilization) were required to sufficiently silence paternal *Mez1*, it is possible to suspect the *mez1-mu* alleles could disrupt that chain of events.

mez1-m1, mez1-m2 and mez1-m4 result in a different pattern of paternal Mez1 activation. We were surprised by the fact that each of the three *mez1-mu* alleles analyzed in this study affected *Mez1* imprinting in slightly different ways. The *mez1-m1* allele only affected imprinting when maternally transmitted, the *mez1-m2* allele only affected imprinting when paternally transmitted and the *mez1-m4* allele affected imprinting when maternally or paternally transmitted. There are several potential explanations for the differences between the three *Mu* insertion alleles. The different sizes of the three *Mu* elements may contribute to the variation. The different effects could have been a consequence of insertion site, however, *mez1-m2* and *mez-m4* were inserted in a nearly identical location and displayed different effects. Since each *mez1-mu* allele was the result of a different class of *Mu* transposon, the sequences of the individual *Mu* transposons could give rise to the inconsistent phenotypes. Similarly, the individual *Mu* transposon insertions could have resulted in a different type of chromatin environment at each allele. The extent to which the epigenetic environment was altered could partially explain the variation observed.

The exact mechanism by which the *Mu* transposons insertions affect *Mez1* imprinting is not clear. The simple displacement of *cis*-acting elements may affect normal imprinted expression. The *Mu* transposons insertions could disrupt the normal expression pattern of the MEZ1 protein. Alternatively, the *Mu* transposons may generate a novel type of chromatin within the *Mez1* promoter that blocks or enhances the

formation of different chromatin structures during the process of imprinting. It is clear that these alleles offer a unique system for probing the mechanisms and consequences of imprinting in plants.

EXPERIMENTAL PROCEDURES

Plant Materials and tissue collection. Maize inbred and *mez1-mu* lines were grown using standard field conditions in the summer of 2006 and 2007 in St. Paul, MN. All three *mez1-mu* insertion alleles were independently backcrossed to B73 and Mo17 for at least four generations prior to use in loss-of-imprinting assays. Reciprocal crosses between B73 or Mo17 and the heterozygous *mez1-mu* lines were performed to generate endosperm tissue in which all kernels are polymorphic for coding region SNPs (see below). A total of 67 crosses were made for *mez1-m1* to/by Mo17, *mez1-m2* to/by B73 and *mez1-m4* to/by B73. Ears were harvested at 14-16 days after pollination (DAP) 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 12 individual endosperms. All isolated endosperm tissue was flash frozen in liquid nitrogen and stored at -80°C 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 was extracted from greenhouse grown 14 day-old seedlings. Seedlings were genotyped for *mez1-mu* insertion alleles. All above-ground tissue was harvested, flash frozen in liquid nitrogen and stored at -80°C. Tissue was ground to a fine powder and RNA extracted with Trizol (Invitrogen) according to manufacturer's protocol. For the imprinting assays from pooled or individual endosperms, the tissue was

ground in liquid nitrogen and the tissue divided between the DNA and RNA extractions. DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Valencia, CA, USA) 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, USA).

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, USA). 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, USA) and heated to 70°C for 5 minutes followed by 1 minute on ice. First-strand cDNA synthesis was performed in a total volume of 30 µl by adding 6 µl 5X reaction buffer, 1.5 µl RNaseOut (Invitrogen), 0.8 µl 25mM dNTPs, and 1 µl M-MLV reverse transcriptase (Invitrogen). This reaction was incubated at 42°C for 50 minutes followed by 70°C for 15 minutes. The resulting cDNA was purified by phenol:chloroform extraction followed by ethanol precipitation and resuspended in 30 µl ddH_2O.

Characterization and genotyping of mez1-mu insertion alleles. In order 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. A primer specific to the *mu* TIR 9242 (see table 2 for all primer

sequences) and a *Mez1*-specific primer Mez1R31 downstream of the *mu* insertions were used to assay the presence of *mu*. In order to distinguish between heterozygous *mez1-mu* and homozygous *mez1-mu*, a separate PCR reaction was performed on each sample using a *Mez1*-specific primer Mez1F32 upstream of the *mu* insertion sites with the Mez1R31 primer. A band in both the 9242/Mez1R31 and the Mez1F32/Mez1R31 reactions indicates a heterozygous *mez1-mu* insertion. A band only in 9242/Mez1R31 or only in Mez1F32/Mez1R31 indicates homozygous *mez1-mu* or homozygous wild-type, respectively. PCR reactions were performed in a 15 µl total volume, containing approximately 25 ng of DNA, 2.5 pmol of each primer, 0.33 units of HotStarTaq Polymerase (Qiagen), 1.5 µl 10X reaction buffer and 0.1 µl of 25mM dNTPS. PCR conditions were as follows: 94°C for 15 min, 35 cycles of 94°C for 1 min, 65°C for 30 sec and 72°C for 1 min, followed by 72°C 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 leaf tissue of plants homozygous for each of the *mez1-mu* insertion alleles. 1µl DNA 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. Based on the sequence, the location of the *Mu* insertions were identified by examining where *Mez1* sequence terminated and *Mu* TIR sequence began. These same sequence reads were used to obtain approximately 45 base pairs of the *Mu* TIR sequence and used for identification of the *Mu* element responsible for each insertion allele.

In order 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 and Mez1R33 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 and Mez1R52 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, USA). 2 µl of cDNA was amplified using an Applied Biosystems 7900HT Real-Time PCR System in a 20 µl reaction volume. A 2-fold 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* and to the maize *GAPC* gene (Genbank #X07156). 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 based on

the differences between *Mez1* and *GAPC* 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 formula $2^{-\Delta\Delta Ct}$.

Allele-Specific Expression analyses. PCR-based assays for monitoring allele-specific expression based on SNPs were designed using the MassArray platform from Sequenom (San Diego, CA, USA) (see table 2 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, three technical replicates were performed for individual endosperm cDNA. PCR and extension PCR reactions on cDNA templates were performed as described Haun *et al* (2007). 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 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°C overnight. The enzyme was heat-inactivated at 65°C for 20 minutes and separated by electrophoresis in a

TBE gel containing 2.5% Metaphor (FMC Bioproducts, Rockland, ME, USA) and observed by ethidium bromide staining.

CAPS Analysis of Allele-Specific DNA methylation. DNA from individual heterozygous *mez1-mu* and wild-type sibling endopserms was digested with 10 units of the methyl-sensitive restriction enzyme *Bst*UI overnight at 60°C. 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°C 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.

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Table 1. Loss of imprinting is associated with *Mez1-mu* insertion alleles				
Allele	*Mu* element	Location[a]	Maternal loss-of-imprinting[b]	Paternal loss-of-imprinting[b]
mez1-m1	MuDR	-38	**5 (9)**	2 (9)
mez1-m2	Mu7	-151	2 (11)	**8 (9)**
mez1-m4	Mu4	-158	**12 (13)**	**12 (16)**

[a]The location of the Mu insertion relative to the transcription start site is indicated.

[b]In each cell the number of ears with a loss-of-imprinting is shown and the total number of ears tested is shown in parentheses.

Table 1. Loss-of-imprinting is associated with *mez1-mu* insertion alleles.

Primer	Sequence (5' 3')
mez1F33	CACAAAGAAGCAGATAAGCGTGGAAAG
mez1R33	TTGGGGCCTATGAATGACAGGTTC
muTIR9242	AGAGAAGCCAACGCCAWCGCCTCYATTTCGTC
mez1F55	GGGGGCTAAAACTTGATAATAAACA
Mez1R52	CGGTCTCTGTGCATTTGTCCTT
Mez1F32	GACGAGCACACTGGTTTTTCCTACC
Mez1R31	CATCACCCGTGGAAACCCTAGC
MEZ1-MZ11F*	CCGGTCTCGCCCATCTAG
MEZ1-MZ11R*	AGCTCGCACAGCCGAATTA
MEZ1-MZ11M1*	CCGCCCAGGTCACCAG
GAPC-GAP4F*	CCTCACCGTCAGAATCGAGAAG
GAPC-GAP4R*	CACATAACCCATGATACCCTTGAGT
GAPC-GAP4M2*	CTCGGAAGCAGCCTTAAT
Mez_1a_F**	ACGTTGGATGATGCACCCTGCATAGTCTTG
Mez_1a_R**	ACGTTGGATGGAGAAAGGACTTTCGTCTTG
Mez_1a_UEP**	CCGGTTGACAAAGATGGTGG
Mez_1b_F**	ACGTTGGATGTCGTGAGAACTCATCGTTCC
Mez_1b_R**	ACGTTGGATGTCAGCTTGTCACCCATTCTG
Mez_1b_UEP**	TCCTTTTCAACCTGAACAA
Mez_1c_F**	ACGTTGGATGTCTACGATTCCCGCTATGAG
Mez_1c_R**	ACGTTGGATGTTGCCCATCATCCTTTGCTC
Mez_1c_UEP**	GCTATGAGCCTGACAGAGC

Table 2. Primers used for CAPS imprinting assays, genotyping *mez1-mu* insertion alleles, DMR analyses, *Mez1* real-time PCR Taqman(*) assays and Sequenom(**) assays.

Figure 1

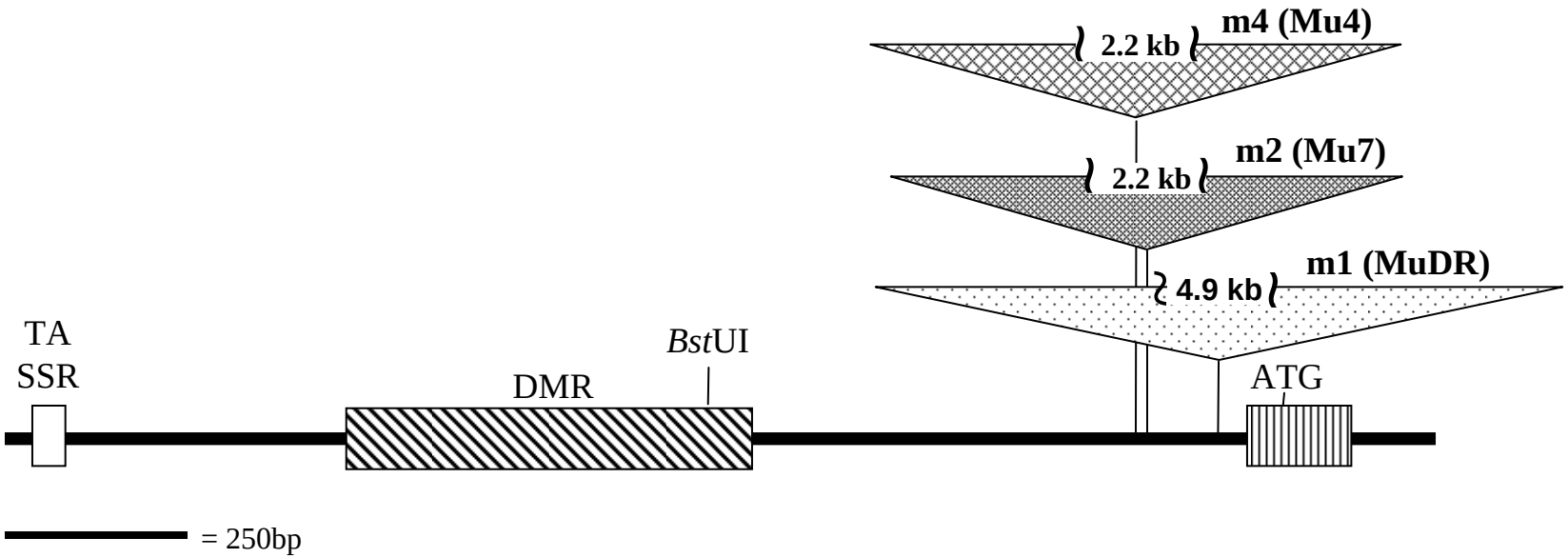


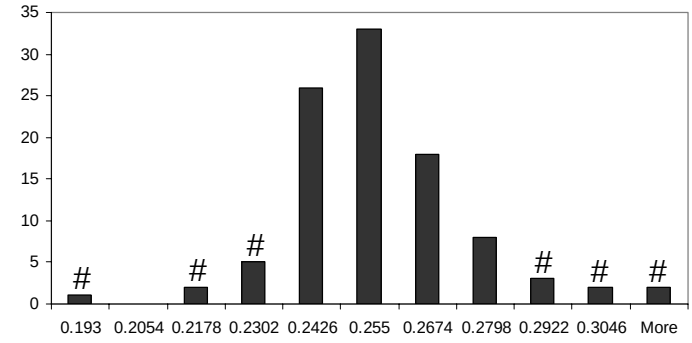
Figure 1. *Mu* transposon insertions at *Mez1*. Approximately 2,000 bp of the *Mez1* 5'-*cis* proximal region is indicated by the bold line. The first exon, differentially methylated region (DMR) and TA single sequence repeat (SSR) is shown as shaded rectangles. The location of each *mu* transposon is shown by triangles, indicating the size of each insertion. The location of the methyl-sensitive *Bst*UI restriction site used for the methylation assay is also indicated.

Figure 2

(a)

Wild-type | Heterozygous *mez1-m1* | Homozygous *mez1-m1*

(b)



(c)

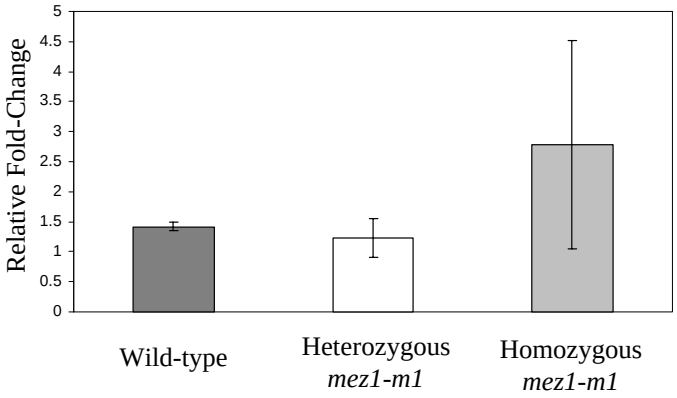
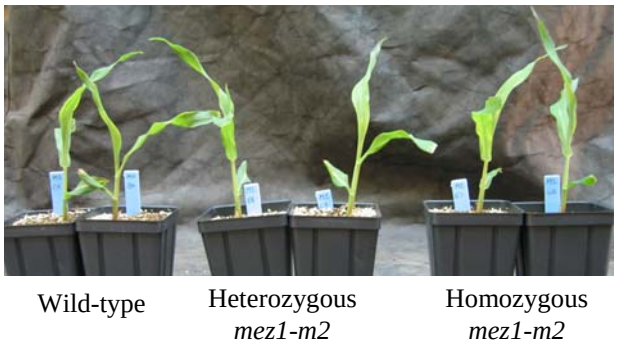


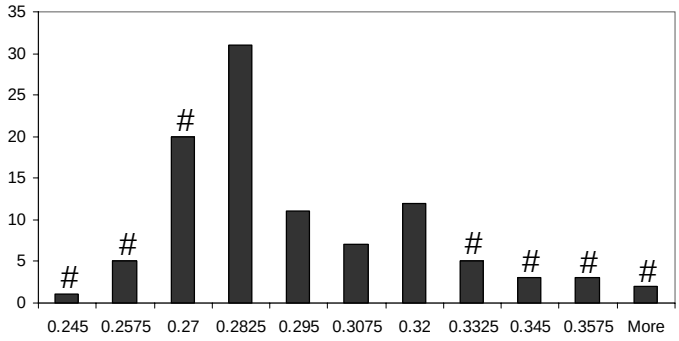
Figure 2. *mez1-m1* insertions do not negatively impact plant health, seed weight or alter *Mez1* expression in non-imprinted tissue. (a) Seedlings from a segregating 1:2:1 ear shown at 12 days after germination. *mez1-m1* heterozygotes and homozygotes are phenotypically indistinguishable from wild-type siblings. (b) The weights of 100 seeds from a segregating 1:2:1 ear show a normal distribution, indicating loss-of-imprinting does not affect seed development. "#" indicates classes of seeds that were grown and genotyped (see text). (c) Real-time PCR results showing no effect on *Mez1* expression in seedling of *mez-m1* heterozygotes or homozygotes relative to wild-type siblings.

Figure 3

(a)



(b)



(c)

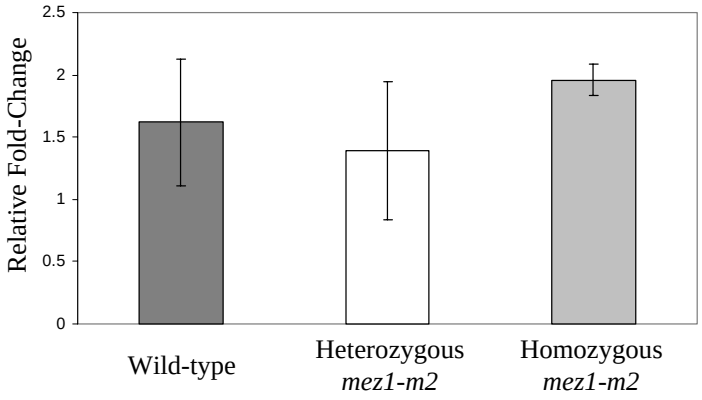


Figure 3. *mez1-m2* insertions do not negatively impact plant health, seed weight or alter *Mez1* expression in non-imprinted tissue. (a) Seedlings from a segregating 1:2:1 ear shown at 12 days after germination. *mez1-m2* heterozygotes and homozygotes are phenotypically indistinguishable from wild-type siblings. (b) The weights of 100 seeds from a segregating 1:2:1 ear show a normal distribution, indicating loss-of-imprinting does not affect seed development. "#" indicates classes of seeds that were grown and genotyped (see text). (c) Real-time PCR results showing no effect on *Mez1* expression in seedling of *mez-m2* heterozygotes or homozygotes relative to wild-type siblings.

Figure 4

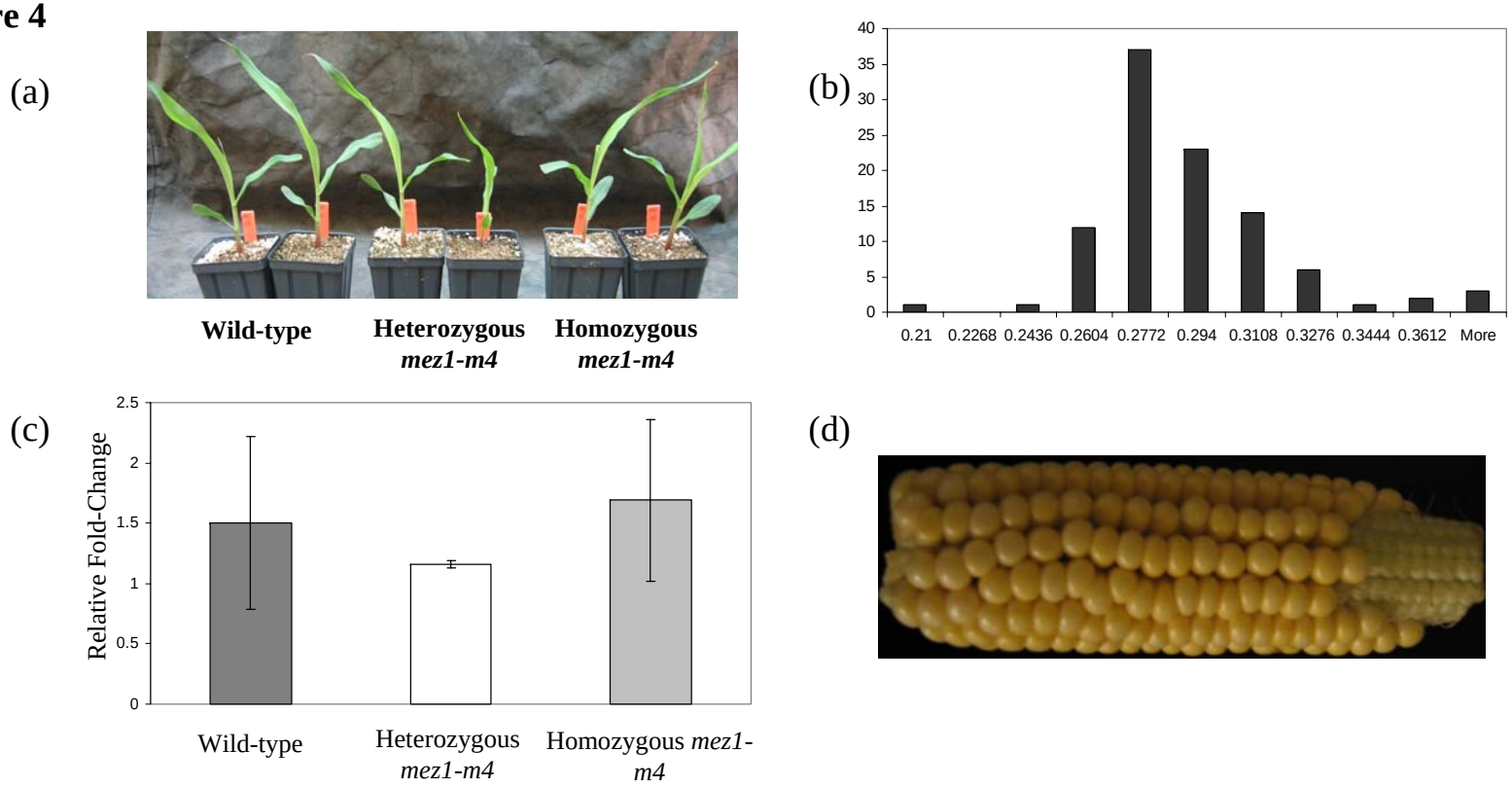


Figure 4. *mez1-m4* insertions do not negatively impact plant health, seed weight or alter *Mez1* expression in non-imprinted tissue. (a) Seedlings from a segregating 1:2:1 ear shown at 12 days after germination. *mez1-m4* heterozygotes and homozygotes are phenotypically indistinguishable from wild-type siblings. (b) The weights of 100 seeds from a segregating 1:2:1 ear show a normal distribution, indicating loss-of-imprinting does not affect seed development. "*" indicates classes of seeds that were grown and genotyped (see text). (c) Real-time PCR results showing no effect on *Mez1* expression in seedling of *mez-m4* heterozygotes or homozygotes relative to wild-type siblings. (d) Ear resulting from a selfed *mez1-m1* heterozygous plant. No segregating kernel abnormalities were observed.

Figure 5

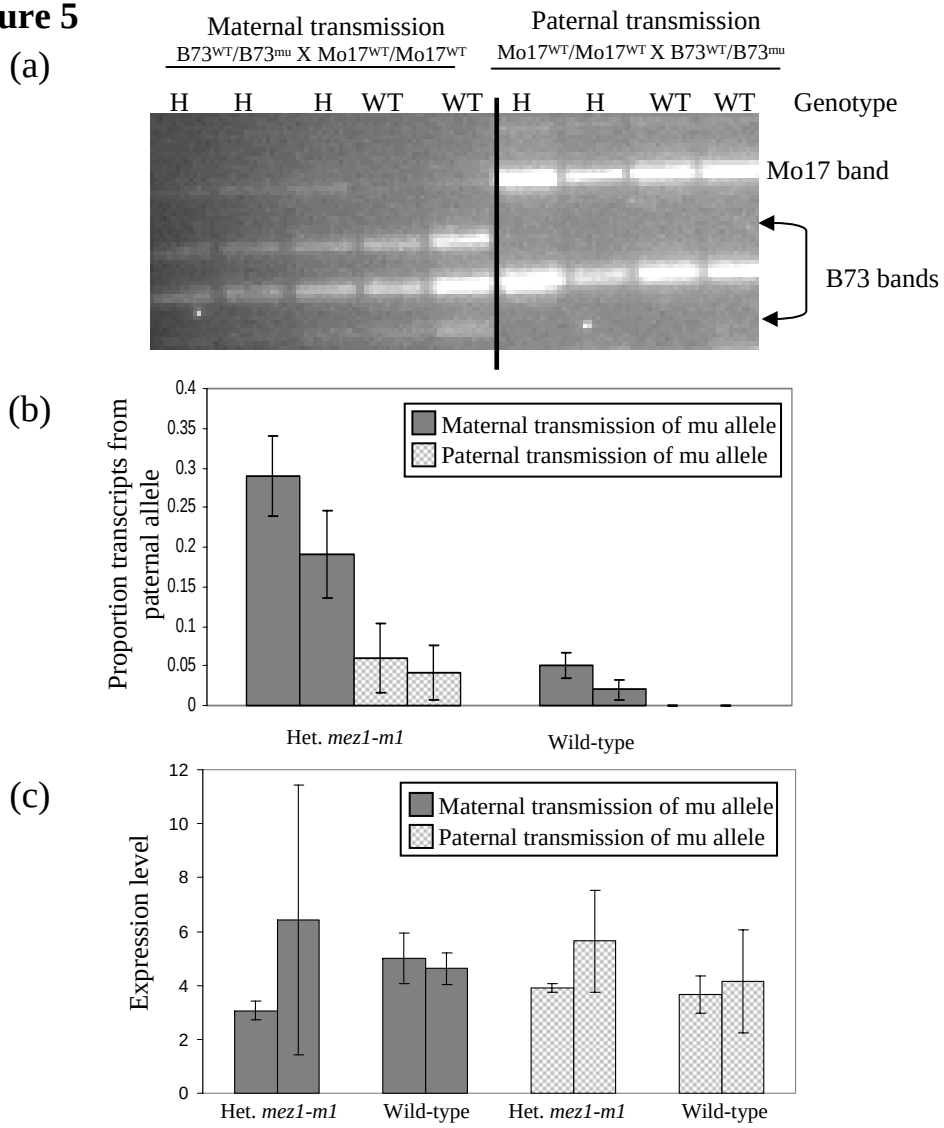


Figure 5. Maternal transmission of *mez1-m1* results in loss-of-imprinting. (a) Allele-specific expression was monitored in *mez1-m1* heterozygote and wild-type endosperm tissue. The expression in the wild-type endosperms is exclusively from the maternal parent. In heterozygous endosperms transmitted maternally, a faint paternal band is observed, indicating a loss-of-imprinting. (b) Quantification of paternal allele activation using Sequenom. RNA from the same endosperms as shown in (a) was analyzed with Sequenom. The proportion of transcripts from the paternal allele in wild-type tissue is virtually zero. The proportion of transcripts from the paternal allele in maternally-inherited *mez1-m1* heterozygous endosperms is 17-38%. (c) Quantification of total *Mez1* expression in heterozygous *mez1-m1* and wild-type endosperm tissue. Real-time PCR results show no difference in overall *Mez1* expression in loss-of-imprinting endosperms compared to wild-type. Expression level is relative to *Mez1* expression in wild-type seedling tissue.

Figure 6

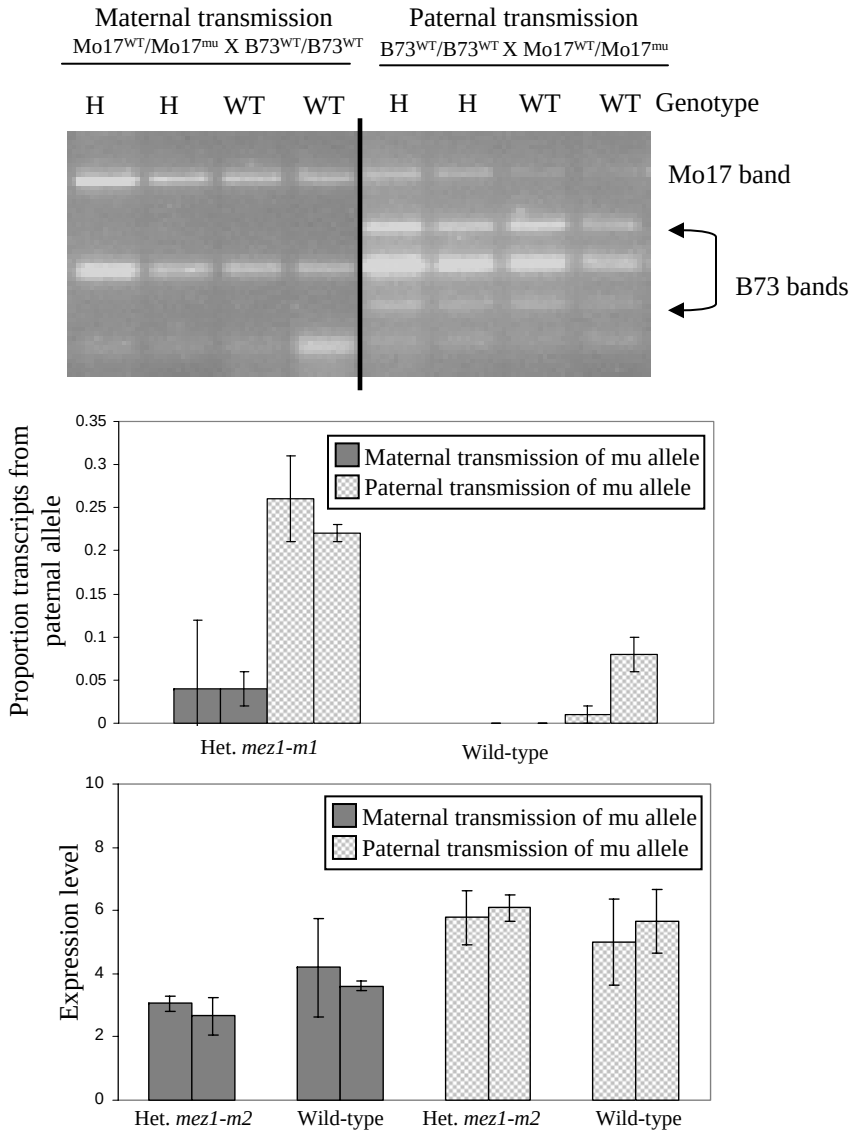


Figure 6. Paternal transmission of *mez1-m2* results in loss-of-imprinting in heterozygous and wild-type endosperms. (a) Allele-specific expression was monitored in *mez1-m2* heterozygote and wild-type endosperm tissue. In heterozygous endosperms transmitted paternally, a faint paternal band is observed, indicating a loss-of-imprinting. Wild-type sibling endosperms from the same cross also show a faint paternal band. (b) Quantification of paternal allele activation using Sequenom. RNA from the same endosperms as shown in (a) was analyzed with Sequenom. The proportion of transcripts from the paternal allele in maternally-inherited *mez1-m1* heterozygous endosperms is 21-33%. The proportion of transcripts from the paternal allele in wild-type tissue is 0-10%. (c) Quantification of total *Mez1* expression in heterozygous *mez1-m1* and wild-type endosperm tissue. Real-time PCR results show no difference in overall *Mez1* expression in loss-of-imprinting endosperms compared to wild-type. Expression level is relative to *Mez1* expression in wild-type seedling tissue.

Figure 7

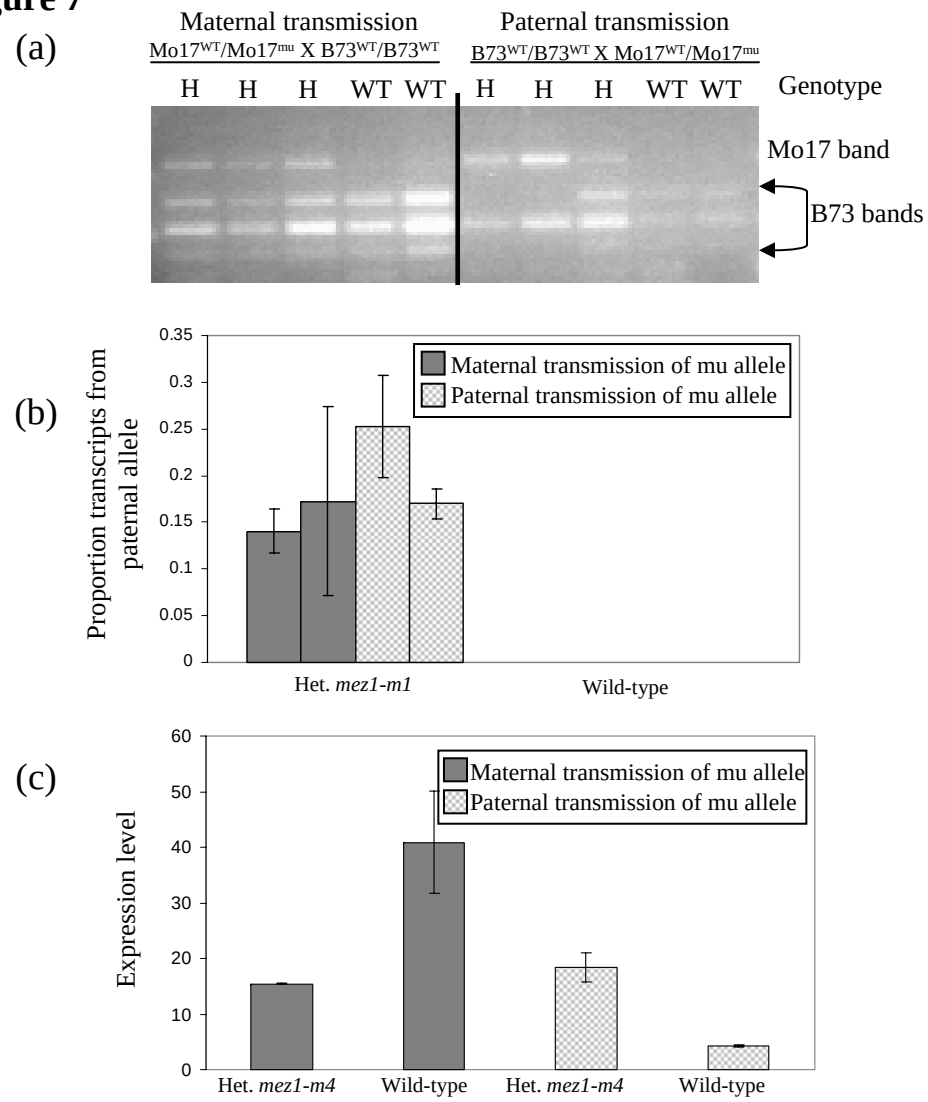


Figure 7. Maternal and paternal transmission of *mez1-m4* results in loss-of-imprinting. (a) Allele-specific expression was monitored in *mez1-m4* heterozygote and wild-type endosperm tissue. The expression in the wild-type endosperms is exclusively from the maternal parent (no paternal transcript was detected). In heterozygous endosperms transmitted maternally, a faint paternal band is observed, indicating a loss-of-imprinting. (b) Quantification of paternal allele activation using Sequenom. RNA from the same endosperms as shown in (a) was analyzed with Sequenom. The proportion of transcripts from the paternal allele in wild-type tissue is zero. The proportion of transcripts from the paternal allele in maternally-inherited *mez1-m4* heterozygous endosperms is 14-16% and 16-24% when inherited paternally. (c) Quantification of total *Mez1* expression in heterozygous *mez1-m4* and wild-type endosperm tissue. Real-time PCR results show maternally inherited *mez1-m*4 endosperms

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