Imprinting and Seed Development

Mary Gehring, Yeonhee Choi, and Robert L. Fischer[1]

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720-3102

INTRODUCTION

Imprinted genes are expressed predominantly from one allele in a parent-of-origin–specific manner. The endosperm, a seed tissue that mediates the transfer of nutrients from the maternal parent to the embryo, is an important site of imprinting in flowering plants. Imprinted genes have been identified in maize and *Arabidopsis thaliana*, but crosses in a variety of species suggest that the effect of imprinting on seed development is widespread throughout the angiosperms. The mechanism of imprinting in plants, as yet only partially understood, likely involves differences in DNA methylation and chromatin structure between differentially expressed maternal and paternal alleles.

Fertilization

Seeds consist of three genetically distinct components: embryo, endosperm, and seed coat. In plants, mitosis follows meiosis to produce the haploid phase of the plant life cycle, the male and female gametophytes. The angiosperm female gametophyte, the site of fertilization, is completely embedded within the maternal sporophytic tissues of the ovule. The most prevalent type of mature female gametophyte is a seven-celled organism consisting of three antipodal cells, two synergid cells, an egg cell, and a diploid central cell. The function of the antipodal cells is unknown. The micropylar synergid cells help attract the pollen tube to the female gametophyte (Higashiyama et al., 2001). The egg cell lies adjacent to the synergid cells and is the progenitor of the embryo. The large central cell, the progenitor of the endosperm, contains two polar nuclei. These fuse before, or at the time of, fertilization to form a diploid central cell nucleus. The male gametophyte, or pollen, develops in the anther from microspores. A mature male gametophyte consists of two haploid sperm cells encased by a haploid vegetative cell.

Seed development begins upon double fertilization. The pollen tube, formed from the vegetative cell of the male gametophyte, enters the female gametophyte through the micropylar end and releases two sperm into a synergid cell. One sperm fertilizes the egg cell and the other fertilizes the central cell. The resulting embryo and endosperm are genetically identical except for ploidy level: the embryo is diploid and the endosperm is triploid. Fertilization also initiates changes in maternal tissues. The ovary develops into a fruit and the ovule integuments differentiate to form the protective seed coat.

[1] To whom correspondence should be addressed. E-mail: rfischer@uclink.berkeley.edu; fax 510-642-9017.

Endosperm Development

The endosperm, a terminally differentiated tissue that nourishes the embryo during seed development, is the only known site of imprinting in plants. It has been known that the endosperm is a product of double fertilization for >100 years (reviewed by Sargant, 1900), but its evolutionary origin is still a puzzle. The endosperm might be derived from a supernumerary embryo that took on an embryo-nourishing function or from a female gametophyte that was later sexualized (Friedman, 2001).

Arabidopsis endosperm development begins with the division of the triploid primary endosperm nucleus, which precedes the division of the zygote by several hours (Faure et al., 2002). Endosperm nuclei continue to divide without cytokinesis to create a syncytium of nuclei (Brown et al., 1999). Each nucleus is surrounded by dense cytoplasm and organelles; these compose nuclear cytoplasmic domains (Brown et al., 1999). The syncytium, as analyzed by the expression of a histone H2B fusion protein, is organized along the anterior–posterior axis into three distinct mitotic domains: the micropylar endosperm (MCE), the peripheral endosperm (PEN), and the chalazal endosperm (CZE) (Boisnard-Lorig et al., 2001) (Figure 1). The nuclei of each domain divide synchronously with each other and asynchronously with the nuclei of the other domains. The three domains also are distinct morphologically and cytologically. Dense nuclei surround the suspensor and the base of the developing embryo in the micropylar endosperm. The endosperm nuclei of the peripheral endosperm lie in a thin, evenly spaced layer around the large central vacuole. The CZE is organized into individual nodules and, at the most posterior pole, a large multinucleate chalazal cyst. The chalazal cyst has larger nuclei than the rest of the syncytial endosperm, perhaps as a result of DNA replication without nuclear division (endoreduplication) (Boisnard-Lorig et al., 2001). The morphology and position of the chalazal cyst are suggestive of a role in the transfer of resources from mother to seed. The cyst sits atop the maternal nucellar proliferating tissue and underlying vasculature. Projections extend from the basal portion of the chalazal cyst into the maternal tissues (Nguyen et al., 2000). Imprinting may be particularly important in the CZE because of its direct connection with the female parent. The syncytial endosperm begins to cellularize at the heart stage of embryogenesis, in a wave that starts in the micropylar chamber and proceeds through the peripheral endosperm (Brown et al., 1999). The CZE is not affected, remaining syncytial until later during seed maturation (Brown et al., 1999). The embryo begins to absorb the endosperm after cellularization. As the embryo reaches maturity, the cotyledons function as storage organs and the endosperm disappears almost entirely, leaving only one or two cell layers in the mature seed.
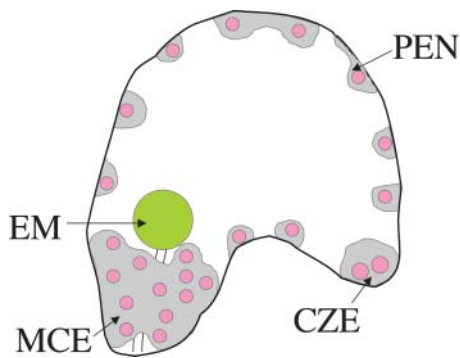
Figure 1. Diagram of the Embryo Sac of a Developing Arabidopsis Seed.

The syncytial endosperm consists of three domains: the micropylar endosperm (MCE), which surrounds the base of the globular-stage embryo (EM), the peripheral endosperm (PEN), and the chalazal endosperm (CZE). Pink circles indicate endosperm nuclei, and gray areas indicate cytoplasm.

Early endosperm development follows a similar pattern in maize. The endosperm, although not organized into distinct mitotic domains, divides synchronously as a syncytium for several days and then cellularizes from the periphery inward. Nuclear divisions continue asynchronously for several more days. Mitosis stops first at the base of the kernel. The cessation then spreads as a wave from the kernel apex downward and then out to the periphery (Kowles and Phillips, 1988). After the mitotic activity of the central region ceases, the cells increase in size and undergo extensive endoreduplication. Endoreduplication is asynchronous, and there is a large amount of heterogeneity in nuclear DNA content (Kowles and Phillips, 1988). The endosperm persists after embryo development is completed and constitutes the major portion of the mature kernel. It stores starch, lipids, and storage proteins and acts as a vital source of nutrients during germination and early seedling development (Lopes and Larkins, 1993).

Maternal and Paternal Genomes Are Not Functionally Equivalent in Angiosperms and Mammals

Imprinting exists in placental mammals as well as in angiosperms. Unsuccessful attempts to create mouse embryos from two female pronuclei first revealed parent-of-origin effects on embryogenesis in mammals (McGrath and Solter, 1984; Surani et al., 1984). Mammalian androgenetic and gynogenetic embryos fail to develop because of the failure of embryonic and extraembryonic tissues, respectively. These parent-of-origin effects suggested that imprinted genes might be essential for embryo development.

Plant embryogenesis appears to be more flexible than mammalian embryogenesis. In addition to normal sexual reproduction, plant embryos can arise asexually from somatic tissue (Goldberg et al., 1994). Viable haploid embryos occasionally may arise from an unfertilized egg cell (parthenogenesis) or sperm cell (androgenesis) (Kimber and Riley, 1963; Sarkar and Coe, 1966; Chase, 1969). Additionally, many plant species pro-

duce seeds asexually by apomixis, which exists in several different forms (Nogler, 1984; Koltunow, 1993; Bicknell and Koltunow, 2004). During gametophytic apomixis, the megagametophyte develops from an unreduced megaspore or from a somatic cell inside the ovule. The diploid embryo then develops from an unreduced cell of the megagametophyte. Apomixis can be pseudogamous or autonomous. Both the embryo and the endosperm develop by parthenogenesis in autonomous apomicts. However, pseudogamous apomicts still require fertilization of the central cell for endosperm formation and asexual embryo development. The multiple asexual ways in which a plant embryo can be created suggest that gender-specific epigenetic information from a female and a male parent might not be essential for embryo development.

The endosperm is more sensitive to genomic perturbation than the embryo. After analysis of interspecific crosses in a range of angiosperm families, Brink and Cooper (1947) concluded that endosperm dysfunction is the primary reason for hybrid incompatibility, with embryo death being a subsequent event. Interploidy crosses within species, particularly maize and Arabidopsis, have demonstrated that a balance of maternal and paternal genomes in the endosperm is necessary for seed viability. Lin (1984) used the *indeterminate gametophyte* (*ig*) mutation, which alters the number of polar nuclei in the central cell, to assess the effects of different endosperm ploidy levels on maize kernels. After mating *ig/ig* females to Ig/Ig males, endosperm ploidy ranged from 2x to 9x, with one genome contributed by the male. Mature kernels were either phenotypically normal, with two maternal and one paternal genomes in the endosperm (2m:1p), miniature but plump (3m:1p), or abortive (all other ploidys). Crossing wild-type diploid females to tetraploid males also produces tetraploid endosperm, in this case with two maternal and two paternal genomes (2m:2p). These tetraploid kernels were small, shriveled, mostly inviable, and weighed much less than the tetraploid kernels with a 3m:1p genome ratio, which were normal in morphology and viability but weighed less than control 2m:1p kernels. Thus, the difference between kernels with tetraploid endosperm tissue can be attributed to the parental source of one of the genomes in the endosperm. Furthermore, the only normal mature seeds produced from crosses between *ig/ig* females and tetraploid males were those with hexaploid endosperm (4m:2p). A 2:1 ratio of maternal to paternal genomes in the endosperm (4:2 in hexaploid and 2:1 in triploid), rather than the actual ploidy, is necessary to generate a normal maize kernel, regardless of the ploidy of the embryo or the maternal parent (Lin, 1984).

Lin (1982) also distinguished specific maize chromosome arms that had a parent-specific effect on seed size. Seeds with endosperm that lacked part of a chromosome or contained it in duplicate were created by reciprocal translocations between standard chromosomes and the accessory B chromosomes, which do not disjoin during the second pollen mitosis. Endosperm that was deficient (2m:0p) for the long arm of chromosome 10 (10L) was 50% smaller than normal endosperm (2m:1p for 10L) or endosperm that contained the region in duplicate (2m:2p for 10L). Additionally, endosperm that was 4m:0p for 10L was smaller than endosperm that was 2m:2p for 10L. Thus, it is the parental source of 10L, not the number of copies in the

endosperm, that is the determinant of seed size (Lin, 1982). Maternally inherited 10L cannot compensate for the lack of paternally inherited 10L. The author concluded that these results are evidence of an imprinting effect in which the genes function when inherited paternally.

Scott et al. (1998) also found that the source of the extra parental genome(s) affected the seed phenotype in interploidy crosses of Arabidopsis. Arabidopsis is able to produce viable seeds from a 4x × 2x cross or a 2x × 4x cross. However, these seeds are abnormal and exhibit reciprocal phenotypes. Seeds with maternal genomic excess (4x × 2x) weigh less than seeds produced by diploids. The cell cycle is affected during the development of these seeds: embryo differentiation and endosperm mitosis are slower and the endosperm cellularizes early. Maternal genomic excess has a similar effect in maize, shortening the period of mitotic proliferation and causing early endoreduplication (LeBlanc et al., 2002). In contrast, Arabidopsis seeds with paternal genomic excess (2x × 4x) are heavier than normal seeds. The developing endosperm contains more nuclei, the mitotic phase lasts longer, and cellularization is delayed. Embryos develop at the same rate as in a 2x × 2x cross (Scott et al., 1998).

The failure of interspecific crosses in *Arabidopsis* also demonstrates the need for genomic balance in the endosperm. When *Arabidopsis thaliana*, a diploid, is crossed as a female to *Arabidopsis arenosa*, a tetraploid, no viable seeds are produced. Viable seeds are produced, however, when *A. thaliana* is crossed as a tetraploid female to *A. arenosa* (Comai et al., 2000). The basis for the success or failure of these interspecific crosses lies in the endosperm (Bushell et al., 2003). Endosperm from the diploid *A. thaliana* crossed to tetraploid *A. arenosa* showed phenotypes characteristic of paternal genomic excess, such as a lack of cellularization and prolonged proliferation. Increasing the maternal genomic contribution by using tetraploid *A. thaliana* as the female parent (4x *A. thaliana* × 4x *A. arenosa*) reduced endosperm proliferation and allowed the production of some viable seeds (Bushell et al., 2003).

The endosperm of intraspecific, interploidy crosses also must be in a 2m:1p balance to achieve seed viability in potato (Johnston et al., 1980, and references therein). However, the situation is slightly different in interspecific crosses. Some tetraploids do not form hybrids with other tetraploids but produce normal seeds when crossed with diploids. To explain this apparent violation of the 2m:1p rule, Johnston et al. (1980) proposed the endosperm balance number (EBN) hypothesis. The genome of each species is assigned a specific value in the endosperm based on its ability to cross successfully with other species. This effective ploidy can differ from the numerical ploidy. It is the EBN that must be in a 2:1 ratio in the endosperm, not the actual genomes. By altering the numerical ploidy to change the EBN of one of the parents, crosses between two species that were previously incompatible became compatible and could produce viable progeny (Johnston and Hanneman, 1982).

Parent-of-Origin Effects and Imprinting Theories

The parental conflict theory has been proposed to explain imprinting in plants and mammals. According to the hypothesis, the reciprocal seed phenotypes observed in intraploidy and interploidy crosses are the result of a conflict among maternal, paternal, and offspring interests over resource allocation from mother to seed (Haig and Westoby, 1989, 1991). The mother, which may have progeny by multiple fathers, will attempt to provision resources equally among her offspring, to which she is equally related. The father will try to direct the maximal amount of maternal resources to his offspring, ensuring his genetic continuance. Thus, genes that promote maternal resource allocation should be expressed paternally. As a counterbalance, genes that restrict resource distribution from mother to seed will be expressed maternally but not paternally. Therefore, the endosperm, as the resource-acquiring tissue, is a probable site of parent-of-origin–specific gene expression, or imprinting. This theory can explain why the effects of interploidy and intraploidy crosses are observed in the endosperm and agrees with observations that maternal genomic excess is associated with slow growth of the endosperm and small seeds and paternal genomic excess leads to large seeds (Lin, 1982; Scott et al., 1998; Bushell et al., 2003).

The parental conflict theory does not explain the ability of functional endosperm to be formed in autonomous apomicts, which do not require fertilization by a male parent. It also is not clear, according to this theory, why imprinting would exist in a selfing species such as *A. thaliana*, in which the maternal and paternal parents usually are the same individual and should share the same interest. Arabidopsis does outcross occasionally (Pigliucci, 2002, and references therein), and this may be sufficient to maintain a system of imprinting. Or, Arabidopsis might have once been an outcrossing species. Imprinting can serve as a barrier to interspecies hybridization, and this also might promote its maintenance.

The parental conflict theory can explain many, but not all, of the seed phenotypes observed in reciprocal crosses. Maize endosperm (2m:2p) resulting from the cross between a diploid female and its autotetraploid male causes kernel abortion 10 to 12 days after pollination (DAP) (Lin, 1984). Phenotypic examination of the tetraploid endosperm before seed abortion indicates that transfer cell layer development is virtually absent (Charlton et al., 1995). These cells facilitate the transport of nutrients from the maternal parent to the endosperm and thus are likely to be a site of parental conflict and subject to parent-of-origin effects. This phenotype, however, contradicts the hypothesis that paternal genomic excess will be associated with an increased transfer of nutrients from mother to seed. However, the authors also reported that tetraploid (2m:2p) endosperm accumulates starch at an earlier stage than their triploid (2m:1p) counterparts, so the classification of the phenotype as one of growth inhibition might not be entirely straightforward.

Other theories have been proposed to explain imprinting. These include imprinting as a defense against chromosome loss or gain or as a means to accurately control gene expression (Hurst, 1997). Or, imprinting could be a by-product of maintaining chromatin structural differences between homologous chromosomal regions, which could be important for some cellular processes, such as identifying each chromosome during replication (Paldi, 2003).

IMPRINTING OF SPECIFIC GENES

Work with entire genomes or with parts of chromosomes indicates that the parental source of genetic information is important in determining its function. Imprinted genes whose expression varies based on the parental mode of inheritance have been identified in maize and Arabidopsis. All are imprinted in the endosperm, and some have effects on endosperm and seed size, as predicted by the parental conflict theory. Two types of imprinting have been described, allelic imprinting, in which only alleles from a certain background are subject to parent-of-origin–specific gene expression, and locus imprinting, in which all known alleles from different backgrounds are under parent-of-origin control.

Allelic Imprinting

For many years, the only example of an imprinted angiosperm gene was in alleles of the maize *R* gene. The *R* gene conditions anthocyanin accumulation in the aleurone (the outer cell layer of the endosperm) of maize kernels. When an *RR* female (red) is mated to a *rr* male (colorless), all of the kernels have a fully colored aleurone. However, the reciprocal cross gives rise to kernels with mottled aleurone pigmentation, indicative of irregular anthocyanin distribution (Kermicle, 1970). This phenomenon is specific to the endosperm, and no reciprocal differences are observed in embryos or seedlings (Brink et al., 1970). Kermicle (1970) demonstrated that the *R*-mottled phenotype is not a dosage effect (i.e., *RR/r* endosperm versus *rr/R* endosperm) but is attributable to the mode of inheritance of the *R* allele. Kernels are mottled regardless of the number of *R* alleles inherited paternally and are always solidly colored if an *R* allele is inherited maternally. However, this phenomenon is observed only with certain *R* alleles; others (i.e., Rst) respond in a dosage-dependent, sex-independent manner.

Alleles of other maize genes, *dzr1* and α-zein, also are imprinted in the endosperm. The *dzr1* locus post-transcriptionally regulates the accumulation of 10-kD α-zeins in the endosperm (Chaudhuri and Messing, 1994). Zeins are the major storage proteins of cereal endosperm and are not expressed in the embryo (Lopes and Larkins, 1993). *dzr1* conditions different levels of zein accumulation in different inbred backgrounds, high in BSSS53 and low in MO17 (Chaudhuri and Messing, 1994). If a BSSS53 female is crossed to a MO17 male, zein RNA and protein accumulation are high, as in BSSS53. RNA and protein accumulation are low in the reciprocal cross, as in MO17. A simple dosage explanation for this effect was ruled out by using B translocations to introduce two copies of MO17 *dzr1* or BSSS53 *dzr1* through the male. Regardless of the number of paternal copies of BSSS53 *dzr1* present, endosperm that receives maternal MO17 *dzr1* has low zein accumulation. The reverse is true of endosperm that maternally inherits BSSS53 *dzr1*, regardless of the number of paternal copies of MO17 *dzr1*. However, in crosses to a different background, BSSS53 *dzr1*, unlike MO17 *dzr1*, behaved in a dosage-dependent manner (Chaudhuri and Messing, 1994). The authors concluded that the MO17 allele of *dzr1* is imprinted such that it has an effect when inherited maternally but not when inherited paternally. Although the high and low accumulation of the 10-kD zein is linked to *dzr1*, it is not known if the MO17 *dzr1* locus itself is actually expressed differentially depending on its parent of origin.

Imprinting of specific α-zein alleles also has been found in the maize endosperm. Using RNase protection assays, it was demonstrated that members of the SF2 subfamily (α-zeins are divided into four subfamilies based on their sequence homology) are expressed maternally but not paternally. This is specific to the W64A inbred background (Lund et al., 1995).

Locus Imprinting

The imprinting of the Arabidopsis *MEDEA* (*MEA*) gene has been the subject of intense study. Mutations in *MEA* were isolated in screens based on two different phenotypes: silique elongation (reproductive development) without fertilization (Chaudhury et al., 1997; Kiyosue et al., 1999) and seed abortion (Grossniklaus et al., 1998). In the absence of fertilization, the diploid central cell nucleus of the *mea* female gametophyte divides to form a multinucleate central cell, reminiscent of syncytial endosperm, that develops to the point of cellularization (Chaudhury et al., 1997; Kiyosue et al., 1999). There is no evidence that the proliferating central cell constitutes an endosperm tissue capable of nourishing an embryo (Friedman, 2001). Sporophytic fertilization programs also are activated in the absence of fertilization: the maternal seed coat develops and the silique elongates. The seed-like structures eventually atrophy. This phenotype is only partially penetrant (Kiyosue et al., 1999). Thus, one function of MEA is to prevent replication of the central cell nucleus in the absence of fertilization.

Additional MEA functions also can be deduced based on the post-fertilization phenotype. Seeds from fertilized *mea* female gametophytes undergo endosperm overproliferation, embryo arrest, and eventual abortion. *mea* endosperm nuclei continue to proliferate after the wild-type endosperm has ceased to replicate, resulting in large, balloon-like developing seeds (Kiyosue et al., 1999) in which endosperm cellularization is delayed (Grossniklaus et al., 1998). Compared with that in the wild type, the *mea* CZE is specifically enlarged and expanded to more anterior regions (Sørensen et al., 2001). Thus, another function of MEA is to restrict endosperm proliferation after fertilization. Also, each morphogenetic stage is lengthened in the embryo, and it arrests at the heart stage (Grossniklaus et al., 1998; Kiyosue et al., 1999). Eventually, the endosperm collapses around the embryo and the seed aborts. It is unknown whether the embryo and endosperm phenotypes are both direct consequences of the *mea* mutation or whether one is a primary defect and the other a downstream event.

MEA encodes a SET-domain Polycomb group protein that is homologous with *Drosophila Enhancer of Zeste* [*E(z)*] (Grossniklaus et al., 1998; Luo et al., 1999; Kiyosue et al., 1999). Polycomb group proteins form complexes that can modify histones and maintain repressed states of gene expression (Orlando, 2003). The mutant phenotypes suggest that MEA maintains the repression of genes involved in cell proliferation. The type I MADS box gene *PHERES1* was identified recently as a direct target of MEA (Köhler et al., 2003).

The *mea* mutation exhibits parent-of-origin effects on seed development. Phenotypic consequences arise only when *mea* is inherited through the female. If a *MEA/mea* female is crossed to a wild-type male, ∼50% of the seeds abort. If a wild-type female is crossed to a *MEA/mea* male, all of the seeds are normal and viable. Thus, seed viability depends only on the genotype of the maternal *MEA* allele; the paternal allele is dispensable and cannot zygotically rescue a seed that has inherited a mutant maternal *mea* allele. Occasionally, *mea* can be transmitted to the next generation through the female, allowing the generation of *mea/mea* plants with between 95 and 100% seed abortion.

MEA is imprinted in the endosperm. The maternal allele is expressed and the paternal allele is silenced. Kinoshita et al. (1999) used ecotype polymorphisms in the *MEA* coding sequence to distinguish maternal and paternal allele expression by reverse transcription (RT) PCR. Seeds were dissected into embryo and endosperm plus maternal seed coat at 6, 7, and 8 DAP, corresponding to the torpedo, walking stick, and early maturation stages of embryo development. Expression from both maternal and paternal alleles was found at all stages of embryo development. Only the maternal allele was expressed in the endosperm. *MEA* is expressed from both alleles in vegetative tissues, including the seedling, rosette leaf, stem, and root (Kinoshita et al., 1999).

Other experiments have suggested that *MEA* also is silenced paternally in the embryo (Vielle-Calzada et al., 1999). Using a RT-PCR approach designed to amplify only RNA expressed from the mutant *mea-1* allele and not from the wild-type *MEA* allele, *mea-1* RNA was detected in whole siliques at 52 to 56 h after pollination (HAP) when a *mea-1* female was crossed to a wild-type male. No *mea-1* expression was detected from siliques of the reciprocal cross. Because siliques contain whole seeds, the authors concluded that the paternal *MEA* allele is silenced in both the embryo and the endosperm. However, Vielle-Calzada et al. (2000) later suggested that the entire paternal genome is silenced until 80 HAP, although examples of paternal genes that are expressed soon after fertilization exist (Weijers et al., 2001). Thus, it is possible that a number of genes are not expressed paternally at 52 HAP and that the inability to detect paternal *MEA* expression in whole siliques at this time reflects a genome-wide phenomenon not specific to the *MEA* locus itself.

Imprinting of *MEA* in the endosperm agrees with the parental conflict theory of parent-of-origin–specific gene expression (Haig and Westoby, 1989). The *mea* phenotype resembles paternal genomic excess: endosperm mitotic activity is prolonged, cellularization is delayed, and the chalazal endosperm is enlarged. The wild-type function is to restrict the growth of the embryo-nourishing endosperm. Growth restriction is in the maternal interest, not in the paternal interest, so *MEA* is expressed maternally but not paternally. Additionally, MEA, a putative chromatin-modifying enzyme that maintains gene repression, might establish imprinting of genes that promote endosperm proliferation (Schubert and Goodrich, 2003). In accordance with the parental conflict theory, these genes would be silenced maternally through the action of MEA and expressed paternally. Polycomb group proteins are known to function in imprinting in mammals (Wang et al., 2001; Mager et al., 2003).

Mutations in two other genes, *FERTILIZATION-INDEPEN-DENT ENDOSPERM* (*FIE*) and *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*), have very similar phenotypes to *mea*: central cell nuclei proliferation if fertilization is prevented and endosperm overproliferation and embryo arrest after fertilization (Ohad et al., 1996; Chaudhury et al., 1997). *FIE* encodes a WD-40 repeat protein homologous with the *Drosophila* Polycomb group protein Extra sex combs (Esc) (Ohad et al., 1999). FIS2 is a zinc-finger transcription factor (Luo et al., 1999) homologous with Suppressor of Zeste12 [Su(z)12] (Birve et al., 2001). Esc, E(z), and Su(z)12 function together in a complex in *Drosophila* to repress gene transcription. The complex has histone deacetylase activity (Tie et al., 2003), and the SET domain of E(z) is responsible for methylating Lys residues 9 and 27 of histone H3 (Czermin et al., 2002; Müller et al., 2002). These histone modifications are indicative of repressive chromatin structure (Jenuwein and Allis, 2001; Turner, 2002). Like Esc and E(z), FIE and MEA have been shown to interact in vitro (Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000).

Mutations in *FIE* and *FIS2* also show parent-of-origin effects on seed development. A wild-type maternal allele is required for normal seed development, whereas the genotype of the paternal allele is irrelevant. Although paternal expression of a *FIE promoter*:*GREEN FLUORESCENT PROTEIN* (*GFP*) transgene is delayed compared with maternal expression, the *FIE* locus is not imprinted, as is *MEA* (Yadegari et al., 2000). By RT-PCR, *FIE* is expressed biallelically in the endosperm at the torpedo stage and biallelically in the embryo at the torpedo, walking stick, and early maturation stages. *FIE* is not expressed from either allele in walking stick– or early maturation–stage endosperm (Yadegari et al., 2000). Unlike *mea*, a mutant maternal *fie* allele is never transmitted through the female (Ohad et al., 1999). This likely indicates an absolute requirement for FIE in the female gametophyte before or soon after fertilization, which probably is the source of the parent-of-origin effects.

Luo et al. (2000) analyzed maternal and paternal *FIS2* expression by visualizing the expression of a maternally or paternally inherited *FIS2 promoter*:*β-GLUCURONIDASE* (*GUS*) transgene. Maternally, *GUS* was expressed in the unfused polar nuclei and central cell nucleus before fertilization and in the dividing endosperm nuclei after fertilization. Expression ceased before endosperm cellularization except in the chalazal cyst. Paternally, GUS was not detected in ovules at 24 HAP. It is not known if the endogenous *FIS2* gene is imprinted like the *FIS2 promoter:GUS* transgene.

The *FWA* gene also is expressed maternally and silenced paternally in the endosperm of Arabidopsis, although FWA has not been ascribed a function in the endosperm (Kinoshita et al., 2003). *FWA* encodes a homeodomain-containing transcription factor that is normally silent during almost all stages of plant development. Ectopic expression of *FWA* causes late flowering (Soppe et al., 2000).

A homolog of Arabidopsis *FIE* is the first maize gene found to be subject to locus-specific imprinting (Danilevskaya et al., 2003). Maize has two *FIE* homologs (*fie1* and *fie2*) that are 78% similar to each other in exonic sequences but are not homologous in their introns or promoter sequences. *fie2* is biallelically expressed in embryo, endosperm, and vegetative

tissues (Danilevskaya et al., 2003). Maize *fie1* is expressed specifically in the endosperm starting at 6 DAP. Polymorphisms between *fie1* alleles in four different inbred lines of maize were used to distinguish maternal and paternal allele expression in the endosperm between 2 and 15 DAP. *fie1* is expressed only from the maternal allele in all inbred lines (Danilevskaya et al., 2003).

A mutation in the maize *fie1* gene is not available, but it is tempting to speculate that FIE1 also might function to repress endosperm development. The fact that the Polycomb group genes *MEA* (in Arabidopsis) and *fie1* (in maize) both are imprinted in the endosperm suggests that it is the imprinting of the function of the repressive Polycomb group complex that is important, not the imprinting of a particular component.

IMPRINTING MECHANISMS

What is the mechanism of genetic imprinting? How are two alleles that reside in the same nucleus and share the same DNA sequence distinguishable from one another? Although imprinting in angiosperms and imprinting in mammals likely evolved independently, it is informative to briefly consider what is known about epigenetic imprinting mechanisms in mammals.

Mammalian Imprinting Mechanisms

More than 60 imprinted autosomal genes have been identified in the mouse (http://www.mgu.har.mrc.ac.uk/research/imprinted/imprin.html), many of which control fetal growth and neuro-behavioral traits (Tycko and Morison, 2002). Imprinted genes usually are found in chromosomal clusters and often are associated with differential DNA methylation at *cis*-acting imprinting control regions (Reik and Walter, 2001). The imprinting of many genes is disrupted in embryos null for *DNA methyltransferase 1* (*Dnmt1*), the major DNA maintenance methyltransferase (Ferguson-Smith and Surani, 2001). Symmetric DNA methylation is attractive as a distinguishing epigenetic mark because it is heritable through rounds of DNA replication. This methylation often is a means of gene silencing (Reik and Walter, 2001). In addition to blocking the access of transcription factors to DNA, DNA methylation can recruit methyl-CpG binding proteins, which form complexes with histone deacetylases, histone methyltransferases, or chromatin-remodeling proteins to generate repressive chromatin structure (Li, 2002). Expressed alleles of some imprinted genes are hyperacetylated on histones H3 and H4, whereas silent alleles are hypoacetylated (Li, 2002).

There are many reports demonstrating a mechanistic link between DNA hypermethylation, histone deacetylation, and the induction of transcription repression. DNA methylation, however, is not always associated with silencing. For example, the expressed *insulin-like growth factor type 2* (*Igf2*) paternal allele is methylated at a downstream imprinting control region and an upstream differentially methylated region. The silent maternal allele is not methylated in these regions. Methylation of the imprinting control region blocks the binding of the enhancer-blocking zinc-finger protein CCCTC binding factor (CTCF) (Bell and Felsenfeld, 2000; Hark et al., 2000). The unmethylated imprinting control region on the maternal allele acts as a chromatin boundary. The maternal *Igf2* allele is insulated from downstream enhancers and is not expressed when CTCF is bound (Bell and Felsenfeld, 2000; Hark et al., 2000). DNA methylation in the differentially methylated region upstream of *Igf2* also allows paternal allele expression by blocking the binding of a repressor protein to a silencer element (Eden et al., 2001).

Noncoding imprinted antisense RNAs are associated with some imprinted genes. The full-length antisense RNA *Air*, transcribed off the paternal allele from an intron in the imprinted gene *insulin-like growth factor type 2 receptor* (*Igf2r*), is required for silencing of the paternal *Igf2r* allele and two adjacent genes not overlapped by *Air* (Sleutels et al., 2002). On the maternal chromosome, the imprinting control region adjacent to the *Air* promoter is methylated, *Air* cannot be transcribed, and *Igf2r* is expressed maternally. Imprinted micro-RNAs also can direct imprinting. mir-127 and mir-136, which are located near two CpG islands in a mouse retrotransposon-like gene, *Rt1*, are expressed in an antisense orientation exclusively from the maternal allele. In turn, *Rt1* is expressed only from the paternal allele. This suggests that the RNA interference machinery could regulate imprinting (Seitz et al., 2003).

Polycomb group complexes also are involved in imprinting. Mutations in the Polycomb group gene *Embryonic ectoderm development* (*Eed*; the mouse homolog of Arabidopsis *FIE*) led to reactivation of the inactive X chromosome in extraembryonic lineages (Wang et al., 2001) and a loss of imprinting of a subset of autosomal paternally silenced genes (Mager et al., 2003). Parent-of-origin methylation still is present in *eed*–/– embryos, but the pattern of methylation is changed. This finding suggests that, for some genes, it is not the overall presence of methylation itself that is important for imprinting but the specific arrangement of the methylation (Mager et al., 2003).

The life cycle of mammals requires that established imprints also are capable of being erased in the germ cells, so that germline imprints properly reflect the organism's gender. Demethylation of primordial germ cells erases methylation imprints. Remethylation and establishment of the imprints takes place during sperm and oocyte development. After fertilization, both the paternal and maternal genomes are demethylated extensively and then remethylated at the blastocyst stage. Methylated and unmethylated alleles of imprinted genes are protected, by unknown mechanisms, from these global changes that occur after fertilization (Reik et al., 2001).

Angiosperm Imprinting Mechanisms

Differences in the life cycle between plants and mammals, in particular the presence of a multicellular haploid gametophyte generation, have implications for the establishment of imprinting. Imprints could be established during meiosis and remain unaltered in haploid mitotic descendants, or imprints could be established in only some cells (i.e., the central cell) after mitosis. This could happen in either the male or the female gametophyte. An imprinting cycle in plants would be different from that in mammals if imprinting occurs only in the endosperm. The endosperm is a terminally differentiated tissue that does not pass on its genetic and epigenetic information to the next generation, rendering imprint erasure unnecessary. If imprinting does occur in other parts of the plant, imprints would have to be erased and

reset when the lineages that give rise to the male and female gametes diverge.

Methylation and Imprinting

There is substantial evidence that DNA methylation plays a role in angiosperm gene imprinting. Plants contain multiple de novo and maintenance methyltransferase genes. Three classes of methyltransferases are present in Arabidopsis: the *MET1* (methyltransferase) family, the *CMT* (chromomethylase) family, and the *DRM* (domains rearranged methyltransferases) family (Cao et al., 2000; Finnegan and Kovac, 2000).

MET1 (*METHYLTRANSFERASE1*), the homolog of mammalian *Dnmt1*, is the predominant maintenance methyltransferase in Arabidopsis (Kankel et al., 2003). *met1* mutants have reduced CpG and CpNpG methylation at centromeric repeats, rDNA gene repeats, and single-copy gene sequences (Kankel et al., 2003). Although *met1* mutations cause an overall decrease in cytosine methylation, some genes can become hypermethylated in the mutant background (Jacobsen et al., 2000). *met1* is capable of creating epialleles that are stably inherited even when introduced into a wild-type *MET1* genetic background (Kankel et al., 2003). Plants that carry an antisense transgene of *MET1* (*MET1* a/s) display developmental abnormalities, including reduced height and apical dominance, shorter roots, and homeotic transformation of floral organs (Finnegan et al., 1996). *MET1* also is necessary for the maintenance of methylation during gametogenesis. If a megaspore inherits a *met1* mutation, the DNA becomes passively demethylated by DNA replication in the mitotic descendants during megagametogenesis. This produces gametes with different epigenetic states as a result of differences in DNA methylation (Saze et al., 2003).

Chromomethylases are unique to plants (Henikoff and Comai, 1998). Global cytosine methylation is reduced by 13% in maize *Zea methyltransferase2* chromomethylase mutants. Bisulfite sequencing of a 180-bp knob repeat showed that the mutation specifically affects CpNpG methylation, not CpG or asymmetric methylation (Papa et al., 2001). In Arabidopsis, *cmt3* mutants display a loss of methylation in CpNpG and asymmetric sequence contexts but change little at CpG dinucleotides (Bartee et al., 2001; Lindroth et al., 2001). The *DRM* genes are responsible for de novo CpG, CpNpG, and asymmetric DNA methylation of direct and inverted repeats of transgenes and maintain CpNpG and asymmetric methylation at some loci (Cao and Jacobsen, 2002a, 2002b).

At the global level, crosses between wild-type and hypomethylated genomes phenocopy maternal or paternal genomic excess in Arabidopsis, depending on the direction of the cross. The methylation level of plants that carry an antisense *MET1* transgene is reduced to 13% of normal. The seeds from a wild-type female crossed to a *MET1* a/s male are lighter weight than the seeds of a *MET1* a/s female crossed to a *MET1* a/s male. A *MET1* a/s female crossed to a wild-type male produces heavier seeds than a *MET1* a/s female crossed to a *MET1* a/s male (Adams et al., 2000). These results resemble the results from interploidy crosses with maternal genomic excess and paternal genomic excess, respectively (Scott et al., 1998). Furthermore, when the hypomethylated parent is male, the endosperm produces less peripheral endosperm nuclei and cellularizes earlier (Adams et al., 2000). When the hypomethylated parent is female, more peripheral endosperm nuclei are produced, cellularization is delayed, and the chalazal endosperm is enlarged (Adams et al., 2000). Hypomethylation has an analogous effect on interspecies crosses. Crossing a *MET1* a/s 4x *A. thaliana* female to a 4x *A. arenosa* male produces shriveled seeds that rarely germinate (Bushell et al., 2003). Hypomethylation effectively offsets the extra *A. thaliana* maternal contribution, causing an imbalance that resembles the 2x *A. thaliana* $\times$ 4x *A. arenosa* cross. All of these results accord with the theory that endosperm-inhibiting genes are expressed maternally and silenced paternally and that endosperm-promoting genes are expressed paternally and silenced maternally (Haig and Westoby, 1989, 1991). The epigenetic state of each genome is specific to its gender, so changes in methylation alter the male and female in unique ways. If methylation is responsible for gene silencing, then a hypomethylated male genome would express usually silent endosperm-inhibiting genes, mimicking maternal excess, and a hypomethylated female genome would express usually silent endosperm-promoting genes, mimicking paternal excess.

There also is a link between methylation status and the expression of alleles of imprinted maize genes. Using methylation-sensitive restriction enzymes followed by a DNA gel blot, Lund et al. (1995) found that some maternal zein alleles are hypomethylated in the endosperm compared with paternal alleles in the endosperm, pollen alleles, or maternal and paternal embryo alleles. However, it is not known if the hypomethylation is correlated with increased expression.

Chromatin Structure and Imprinting

Differences in chromatin structure between maternal and paternal alleles are increasingly the focus of mammalian imprinting research and likely play a role in plant imprinting as well. DNA methylation and chromatin structure are intimately connected. Histone methylation at histone H3 Lys 9 is associated with silent chromatin. Methylation of histone H3 Lys 4 is associated with transcriptionally competent regions of the genome. In *Neurospora*, DNA cytosine methylation is abolished in a histone H3 methyltransferase mutant (Tamaru and Selker, 2001). In Arabidopsis, *KRYPTONITE* (*KYP*), a histone H3 methyltransferase specific for Lys 9 (K9), is required for CpNpG methylation by *CMT3* (Jackson et al., 2002). *DECREASE IN DNA METHYLATION1* (*DDM1*) and *KYP* are required for H3 K9 methylation at centromeric repeats, retrotransposons (Johnson et al., 2002), and a heterochromatin knob (Gendrel et al., 2002). *DDM1* encodes an ATP-dependent chromatin-remodeling protein of the SWI2/SNF2-like family (Jeddeloh et al., 1999). Recombinant DDM1 can bind to free DNA and nucleosomes in vitro, stimulating ATPase activity, and also can induce the movement of a histone octamer along DNA in an ATP-dependent manner (Brzeski and Jerzmanowski, 2003). *ddm1* was identified originally as a mutation that reduced 5-methylcytosine levels by 70% (Vongs et al., 1993), again emphasizing the effect a chromatin-remodeling enzyme can have on DNA methylation.

The precise relationship between histone and DNA methylation remains unclear. Histone methylation has been proposed to

precede DNA methylation (Gendrel et al., 2002; Johnson et al., 2002) and vice versa (Soppe et al., 2002), and a recent report, using a *MET1* null mutant, suggests that CpG methylation is required for H3 K9 methylation in heterochromatin (Tariq et al., 2003). Thus, the connection between DNA methylation and H3 K9 methylation in Arabidopsis is proving to be complex and might be specific to the type of sequence examined. These processes might be important for *MEA* paternal allele silencing and the silencing of other alleles. A paternally inherited *ddm1* allele can partially suppress seed abortion caused by the maternal inheritance of *mea* (Vielle-Calzada, et al., 1999; Yadegari et al., 2000). However, whether the paternal *MEA* allele is expressed in a *ddm1* endosperm is not known. *DDM1* could allow seed rescue by having a direct effect on *MEA* paternal allele silencing or an effect on *MEA* downstream target genes.

Control of *MEA* and *FWA* Imprinting

The discovery of the *DEMETER* (*DME*) gene (Choi et al., 2002) has shed light on the mechanism of imprinting of two Arabidopsis genes. Expression of the maternal *MEA* (Choi et al., 2002) and *FWA* (Kinoshita et al., 2004) alleles in the endosperm is under the control of DME.

dme was identified as a mutation that causes parent-of-origin effects on seed viability. Like *mea*, *fis2*, and *fie*, inheritance of a *dme* mutant allele from the female causes endosperm overproliferation, embryo arrest at approximately the heart stage, and seed abortion. During reproduction, *DME*, as assayed by RT-PCR and *DME promoter*:*GFP/GUS* fusions, is expressed primarily in the central cell of the female gametophyte. Interestingly, *DME* RNA decreases quickly after fertilization, and no further *DME promoter*:*GFP* or *DME promoter*:*GUS* expression is detected in the developing seed. The effect of *dme* on seed development is at least partially attributable to a loss of *MEA* expression. DME is required for *MEA* expression in developing flower buds, as well as for *MEA promoter*:*GFP* expression in the central cell before fertilization and in the endosperm of the developing seed after fertilization, even when *DME* is no longer expressed (Choi et al., 2002). This finding suggests that one of the primary functions of DME is to activate *MEA* in the central cell. This activation, presumably through an unknown epigenetic function exerted by DME before fertilization, is sufficient for subsequent maternal *MEA* allele expression during endosperm development. DME also is required for *FWA* expression in ovules, as assayed by RT-PCR, and for the expression of a *FWA promoter*:*FWA-GFP* transgene in the central cell before fertilization. DME might exert a similar epigenetic effect on *FWA* as on *MEA* (Kinoshita et al., 2004).

The restricted expression pattern of *DME* suggests a model for the regulation of *MEA* and *FWA* imprinting in the endosperm. DME activates the maternal alleles in the central cell before fertilization, and the alleles are expressed. *DME* is not expressed in the stamen, the male gametophyte–producing organ; therefore, the paternal alleles cannot be activated and expressed. After fertilization, the epigenetically "marked" maternal alleles can be expressed throughout endosperm development, whereas the paternal alleles still cannot be expressed because of the lack of DME in the endosperm. Choi et al. (2002) tested this

model for *MEA* by using the *35S* promoter of *Cauliflower mosaic virus* to express *DME* in the endosperm. Weak expression of the normally silenced paternal *MEA* allele was detected in the endosperm at 7 DAP. In addition, *MEA* is also expressed ectopically in the cauline leaves of *35S promoter*:*DME* plants (Choi et al., 2002). Therefore, in those tissues, ectopic *DME* expression is sufficient for *MEA* expression. Lack of expression of the paternal *MEA* allele in the stamen and endosperm presumably is attributable, at least in part, to the absence of DME. The discovery of *DME* supports the idea that both maternal and paternal *MEA* and *FWA* alleles are silenced by default after gametogenesis. Then, only the maternal *MEA* and *FWA* alleles are exposed to activation by DME in the central cell of the female gametophyte (Choi et al., 2002; Dickinson and Scott, 2002; Kinoshita et al., 2004).

Expression of *MEA* in the embryo must not be subject to the same *DME*-mediated activation. *MEA* is expressed from both maternal and paternal alleles in the embryo, but *DME promoter*:*GFP* expression is not detected in the egg cell before fertilization or in the developing embryo (Choi et al., 2002). This suggests that the egg cell and the central cell are epigenetically distinct.

DME encodes a large protein containing a DNA glycosylase domain. Most DNA glycosylases function in DNA repair and excise modified, damaged, or mispaired bases, creating an abasic site. Apurinic/apyrimidinic (AP) endonucleases create a nick 5′ of the abasic site, and then DNA polymerases and DNA ligases complete the repair process by removing the abasic site and the nick. Ectopic *DME* expression results in nicks in the *MEA* promoter, indicative of a base excision repair process that occurs at these sites (Choi et al., 2002). DME-induced nicking has not been reported for *FWA*. *REPRESSOR OF SILENCING 1* (*ROS1*), a *DME*-like gene, excises 5-methylcytosine in vitro (Gong et al., 2002), raising the possibility that DME activates *MEA* by excising 5-methylcytosine. However, it is unknown how the DME DNA glycosylase marks the maternal *MEA* or *FWA* alleles for activation.

Two recent articles suggest that DNA methylation is an important component of the *MEA* and *FWA* imprinting mechanisms, although in somewhat different ways for each gene (Xiao et al., 2003; Kinoshita et al., 2004). Silencing of *FWA* is associated with hypermethylation of *FWA* promoter repeats. These repeats are hypomethylated in mutants that ectopically express *FWA* (Soppe et al., 2000). Kinoshita et al. (2004) found that *FWA* is specifically maternally expressed in the endosperm of developing wild-type seeds. The promoter repeats are hypomethylated in this tissue but are methylated in other seed tissues in which *FWA* is not expressed (i.e., embryo and seed coat). When a wild-type female is crossed to a *met1* male, expression from the paternal *FWA* allele is detected in the endosperm and the embryo. Thus, paternal *MET1* is required for *FWA* paternal allele silencing in the embryo and endosperm. Paternally inherited mutations in *cmt3* or *drm1* and *drm2* do not alter *FWA* paternal allele expression.

Xiao et al. (2003) identified *met1* mutations in a screen for suppressors of the *dme* seed-abortion phenotype. Suppression of *dme* by *met1* is specific to the female gametophyte and requires a wild-type maternal *MEA* allele. This finding indicates

that maternal *met1* suppression of *dme* acts upstream of or at the *MEA* locus. Expression of *MEA* in flowers and of *MEA promoter:GFP* in seeds, which is absent in *dme* mutants, is restored in a *dme met1* double mutant. These data suggest that *MEA* imprinting is controlled in the female gametophyte by antagonism between the two DNA-modifying enzymes: MET1 methyltransferase and DME DNA glycosylase. Three regions of the *MEA* promoter are subject to DNA methylation, and this methylation is reduced in *met1* mutant seeds. However, it is unknown whether or not *MEA* promoter methylation regulates *MEA* expression directly and how DME overcomes the MET1-mediated repression of *MEA*.

Epigenetic modification of maternal alleles in the central cell need not be reset each generation, because chromosomes inherited by the endosperm are not transmitted to progeny. In this regard, the imprinting mechanism in plants is fundamentally different from that in mammals, in which the epigenetic modification of imprinted genes is reset at every generation (Xiao et al., 2003; Kinoshita et al., 2004).

CONCLUSIONS

Reciprocal interspecies and interploidy crosses, as well as reciprocal crosses between plants differing in methylation status, clearly demonstrate the nonequivalence of maternal and paternal genomes and the importance of proper endosperm formation for the development of viable seeds. Imprinted genes, including Polycomb group genes, have been identified in maize and Arabidopsis. The discovery of *DME*, a regulator of maternal allele expression of the imprinted *MEA* and *FWA* genes, is a step toward understanding the mechanism of imprinting in Arabidopsis. Further insights into the influence of imprinting on seed development will be gained with the identification of more imprinted genes in Arabidopsis, maize, and a wider range of angiosperm species as well as with the identification of new mutations that disrupt or alter imprinting.

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