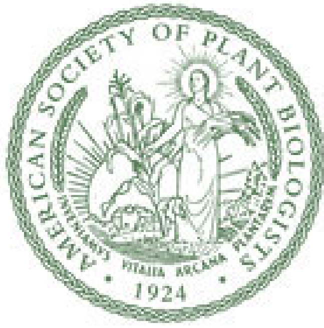

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Imprinting of the *MEDEA* Polycomb Gene in the Arabidopsis Endosperm

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In flowering plants, two cells are fertilized in the haploid female gametophyte. Egg and sperm nuclei fuse to form the embryo. A second sperm nucleus fuses with the central cell nucleus that replicates to generate the endosperm, which is a tissue that supports embryo development. *MEDEA* (*MEA*) encodes an Arabidopsis SET domain Polycomb protein. Inheritance of a maternal loss-of-function *mea* allele results in embryo abortion and prolonged endosperm production, irrespective of the genotype of the paternal allele. Thus, only the maternal wild-type *MEA* allele is required for proper embryo and endosperm development. To understand the molecular mechanism responsible for the parent-of-origin effects of *mea* mutations on seed development, we compared the expression of maternal and paternal *MEA* alleles in the progeny of crosses between two Arabidopsis ecotypes. Only the maternal *MEA* mRNA was detected in the endosperm from seeds at the torpedo stage and later. By contrast, expression of both maternal and paternal *MEA* alleles was observed in the embryo from seeds at the torpedo stage and later, in seedling, leaf, stem, and root. Thus, *MEA* is an imprinted gene that displays parent-of-origin–dependent monoallelic expression specifically in the endosperm. These results suggest that the embryo abortion observed in mutant *mea* seeds is due, at least in part, to a defect in endosperm function. Silencing of the paternal *MEA* allele in the endosperm and the phenotype of mutant *mea* seeds supports the parental conflict theory for the evolution of imprinting in plants and mammals.

INTRODUCTION

Flowering plant reproduction is characterized by the fertilization of two cells (reviewed in van Went and Willemse, 1984). Within the Arabidopsis ovule, a single spore replicates and generates the female gametophyte, which is composed of an egg cell and two synergid cells at the micropylar end, a central cell in the middle, and three antipodal cells at the chalazal end. All are haploid except for the central cell, which contains two polar nuclei that fuse to form a diploid nucleus. Reproduction begins when a pollen tube enters the micropylar opening of the ovule and discharges two haploid sperm cells. Fertilization of the egg generates the diploid embryo, whereas fertilization of the central cell generates the triploid endosperm. Surrounding and protecting the developing embryo and endosperm are maternal cell layers, namely, the seed coat. These cell layers are derived from the ovule integuments.

In Arabidopsis, the fertilized egg undergoes an asymmetric transverse cleavage to produce a small cytoplasmically dense apical cell and a large vacuolated basal cell. The apical cell divides many times and generates cells that comprise most of the embryonic structures found in the mature seed (Bowman and Mansfield, 1994). During embryo development, two primary organ systems (i.e., axis and cotyledon) and three tissue layers (i.e., protoderm, procambium, and ground meristem) are specified (Lindsey and Topping, 1993; Jürgens, 1994; Meinke, 1994). The embryo passes through a series of stages that have been defined morphologically as globular, heart, torpedo, walking stick, early maturation, and maturation (Goldberg et al., 1994). By contrast, the embryonic basal cell divides and forms a single file of cells that comprise the suspensor, which is an ephemeral organ that represents the physical connection between the embryo and maternal tissues during the early stages of embryogenesis (Yeung and Meinke, 1993).

Embryo and endosperm are genetically identical except for their ratio of maternal-to-paternal genomes, which are 1:1 and 2:1, respectively. However, the pattern of endosperm development is quite distinct from that of the embryo. The fertilized central cell nucleus undergoes a series of mitotic divisions to produce a syncytium of nuclei that surround the embryo and fill the expanding central cell (Mansfield and Briarty, 1990a; Webb and Gunning, 1991; Berger, 1999; Brown et al., 1999). At the heart stage, cellularization

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begins, and endosperm cytoplasm and nuclei are sequestered into discrete cells (Mansfield and Briarty, 1990b; Berger, 1999; Brown et al., 1999). Endosperm cells produce high levels of storage proteins, starch, and lipids.

The endosperm is thought to interact with the embryo during seed development. The chalazal-oriented portion of the endosperm is located next to a pad of maternal proliferative tissue that is adjacent to the vascular tissue. The relative position of these tissues has led to the suggestion that the chalazal-oriented endosperm may be involved in nutrient transfer into the developing seed (Schulz and Jensen, 1971; Berger, 1999; Brown et al., 1999). Also, in many dicot plants, the nutrients produced and stored in the endosperm are ultimately absorbed by the embryo (Lopes and Larkins, 1993). Hence, the endosperm is thought to function to support the growth and development of the embryo.

The Arabidopsis *MEDEA* (*MEA*) gene encodes a SET domain Polycomb protein (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999). The SET acronym is derived from the names of three genes that share a common 130–amino acid motif. These genes are the *Suppressor of position-effect variegation* gene *Su(var)3-9*, the *Enhancer of zeste polycomb group* gene *E(z)*, and the *Trithorax* gene *trx-G* (Jenuwein et al., 1998). In mammals, insects, and fungi, SET domain polycomb proteins have been shown to regulate gene transcription by participating in the formation of complexes at specific sites within the genome (Pirrotta, 1998). In Arabidopsis, *MEA* functions as a suppressor of endosperm development (Kiyosue et al., 1999). Loss-of-function *mea-3* and *mea-4* mutations cause precocious endosperm formation before fertilization and prolonged endosperm nuclear proliferation after fertilization (Kiyosue et al., 1999). Mutations in the *MEA* gene also cause embryo abortion (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). The relationship between the mutant endosperm and embryo phenotypes remains to be elucidated.

Genetic analysis has shown that only the maternal wild-type *MEA* allele, and not the paternal *MEA* allele, is required for proper embryo and endosperm development (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). For example, when a heterozygous plant is pollinated with wild-type pollen, only those seeds inheriting a maternal mutant *mea* allele are defective. When the reciprocal cross is performed, no defective seeds have been observed. One explanation for these parent-of-origin–specific effects is that *MEA* gene expression, which is essential for embryo development, occurs before fertilization in the female gametophyte. Another possibility is that *MEA* could be an imprinted gene whose expression in seeds is dependent on the parent of origin. In this model, the maternal *MEA* allele is expressed and the paternal *MEA* allele is silenced, perhaps due to transcriptional silencing (Martienssen, 1998).

To understand the molecular basis for the parent-of-origin effects of *mea* mutations on reproductive development, we monitored expression of the maternal and paternal wild-type *MEA* alleles in the F_1 progeny of crosses between two Arabidopsis ecotypes. Here, we show that expression of the maternal *MEA* allele is predominant in the endosperm from seeds at the torpedo stage and later. By contrast, both maternal and paternal *MEA* mRNAs were detected in approximately equal concentrations in the embryo from seeds at the torpedo stage, and later, in seedling, leaf, stem, and root. Thus, the paternally derived *MEA* allele is silenced specifically within the endosperm. These results suggest that *mea* embryo abortion is due, at least in part, to a defect in endosperm function. Our results characterizing the phenotype of loss-of-function *mea* mutants (Kiyosue et al., 1999) and describing the silencing of the paternal *mea* allele support the parental conflict theory for the evolution of imprinting in plants and mammals (Haig and Westoby, 1989, 1991).

RESULTS

Strategy for Measuring Maternal- and Paternal-Specific *MEA* mRNA Levels

To test the hypothesis that paternal gene silencing is responsible for parent-of-origin–specific effects of *mea* mutations on seed and plant development, we measured maternal and paternal *MEA* mRNA levels in reproductive and vegetative tissues. As shown in Figure 1A, to distinguish maternal and paternal *MEA* RNAs, we identified a DNA sequence polymorphism (i.e., a T versus a G residue) in the *MEA* gene isolated from two Arabidopsis ecotypes, Landsberg *erecta* (L*er*) and RLD (Hardtke et al., 1996). The polymorphism resides in the 17th exon that encodes 3′ untranslated *MEA* mRNA (Figure 1B). A derived cleaved amplified polymorphic sequence primer (Neff et al., 1998), *MEA-R2d* (Figure 1A), was designed to convert this DNA sequence polymorphism to a BamHI restriction endonuclease site polymorphism (Figure 1C). Reverse transcription–polymerase chain reaction (RT-PCR) amplification of L*er* *MEA* sequences followed by BamHI digestion was predicted to produce a 239-bp DNA sequence. For RLD, the same procedure was predicted to produce 207- and 32-bp DNA sequences (Figure 1C). To test this strategy, RNA from L*er* and RLD floral buds was mixed, and *MEA* sequences were amplified and digested as described above. As shown in Figure 1D, the expected size and abundance of restriction fragments were detected, indicating that the assay is semiquantitative and that we can distinguish *MEA* RNAs transcribed from the L*er* and RLD genomes.

Pattern of Maternal and Paternal *MEA* Allele Expression during Plant Development

Previously, it was shown that the *MEA* gene is expressed in siliques with developing seeds (Grossniklaus et al., 1998;

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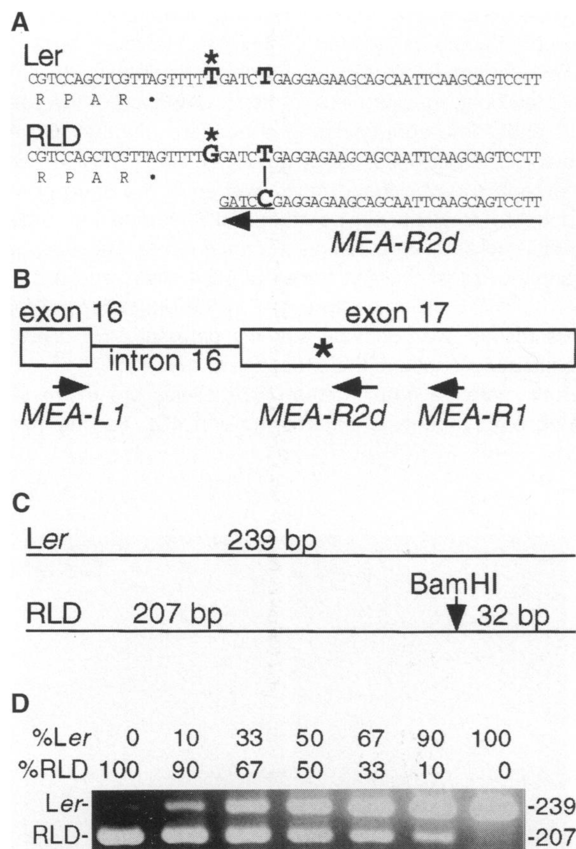

Figure 1. Strategy for Distinguishing Maternal and Paternal *MEA* mRNA.

(A) DNA sequences. Asterisks show the DNA sequence polymorphism between the L*er* and RLD *MEA* genes. The vertical line shows the mismatch position in the derived cleaved amplified polymorphic sequence primer *MEA-R2d* relative to the L*er* and RLD *MEA* genes. Dots represent a translation stop signal. The arrow points to the 3′ end of the *MEA-R2d* primer.

(B) Position of primers. *MEA-L1*, *MEA-R2d*, and *MEA-R1* represent primers used for RT-PCR amplification of *MEA* sequences. The asterisk shows the location of DNA sequence polymorphism. Positions of exons and introns are indicated.

(C) Predicted sizes of restriction fragments after RT-PCR amplification and digestion with BamHI restriction endonuclease.

(D) Amplification of *MEA* sequences from L*er* and RLD floral RNA. RNA was isolated from L*er* and RLD floral buds at stages 0 to 13 (Smyth et al., 1990), mixed in the indicated proportions, and subjected to RT-PCR amplification, BamHI restriction endonuclease digestion, and agarose gel electrophoresis. The lengths of the restriction fragments in base pairs are indicated at right.

Kiyosue et al., 1999). To examine the expression of paternal and maternal *MEA* alleles in seeds, we performed reciprocal crosses between L*er* and RLD Arabidopsis plants. RNA was isolated from F_1 seeds harvested at 4, 6, 7, and 8 days after pollination, and *MEA* sequences were amplified by using RT-PCR. These time points correspond to the heart, torpedo, walking stick, and early maturation stages of embryo development (Goldberg et al., 1994). To distinguish expression from maternal and paternal *MEA* alleles, we digested RT-PCR products with BamHI and subjected them to agarose gel electrophoresis. As shown in Figure 2, both maternal and paternal *MEA* RNAs were detected at all stages of seed development tested, although the level of maternal *MEA* mRNA appeared to be higher than the level of paternal *MEA* mRNA. These results show that both paternal and maternal *MEA* mRNAs accumulate during seed development.

During double fertilization, the embryo inherits one paternal *MEA* allele and the endosperm inherits another paternal *MEA* allele. To determine whether one or both paternal *MEA* alleles are expressed during seed development, we harvested F_1 seeds from reciprocal crosses between L*er* and RLD plants at 6, 7, and 8 days after pollination. Seeds (Figure 3A) were dissected, and RNA was isolated from the embryo (Figure 3C) and endosperm plus seed coat (Figure 3B) components. Using the procedures described above, we measured the level of maternal and paternal *MEA* mRNA.

As shown in Figure 3D, both maternal and paternal *MEA* gene expression was detected in embryos at all stages tested. Thus, the paternal *MEA* allele from the sperm that fused with the egg is expressed during seed development. However, a very different pattern of expression was observed for the paternal *MEA* allele inherited by the endosperm. As shown in Figure 3E, when the female parent was of the L*er* ecotype, only the 239-bp product associated with the maternal L*er MEA* allele was detected at all stages tested. Moreover, when the female parent was of the RLD ecotype, the 207-bp product associated with the maternal RLD *MEA* allele was detected at 6 and 7 days after pollination. Only at 8 days after pollination was a low level of paternal L*er MEA* mRNA observed. Finally, in a control experiment, both maternal L*er* and paternal RLD *αVACUOLAR PROCESSING ENZYME* gene (Kinoshita et al., 1999) expression were detected in endosperm plus seed coat

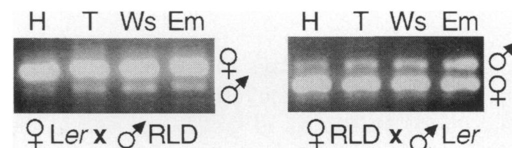

Figure 2. Pattern of Paternal and Maternal *MEA* mRNA Accumulation in Seeds.

Reciprocal crosses between L*er* and RLD plants were performed, and F_1 seeds were harvested at 4, 6, 7, and 8 days after pollination, corresponding to the heart (H), torpedo (T), walking stick (Ws), and early maturation (Em) embryo stages, respectively. RNA was isolated from seeds and subjected to RT-PCR amplification with *MEA* primers, BamHI restriction endonuclease digestion, and agarose gel electrophoresis.

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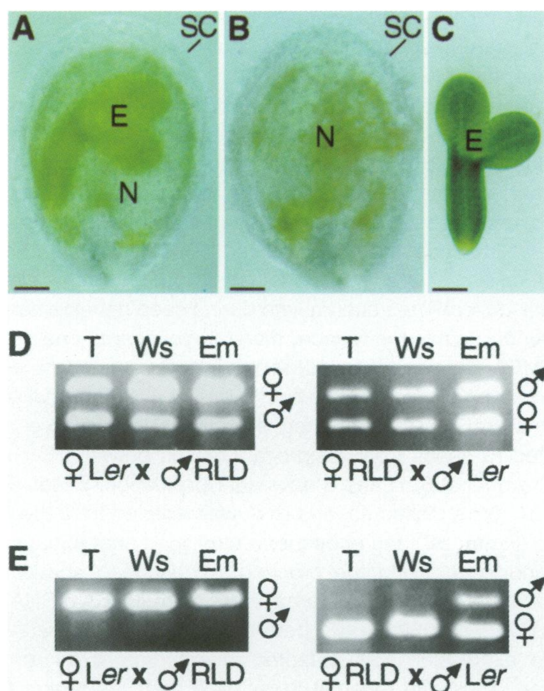

Figure 3. Pattern of Paternal and Maternal *MEA* mRNA Accumulation in Dissected Seeds.

Reciprocal crosses between L*er* and RLD plants were performed, and F_1 seeds were harvested at 6, 7, and 8 days after pollination, corresponding to the torpedo (T), walking stick (Ws), and early maturation (Em) embryo stages, respectively. Seeds were dissected into embryo and endosperm plus seed coat components. RNA was isolated and subjected to RT-PCR amplification, BamHI restriction endonuclease digestion, and agarose gel electrophoresis.

(A) Intact RLD seed 7 days after pollination.

(B) Endosperm plus seed coat from an RLD seed harvested 7 days after pollination.

(C) Embryo from an RLD seed harvested 7 days after pollination.

(D) Accumulation of allele-specific *MEA* mRNA in embryos.

(E) Accumulation of allele-specific *MEA* mRNA in the endosperm plus seed coat.

E, embryo; N, endosperm; SC, seed coat. Bars in **(A)** to **(C)** = 0.2 mm.

dissected from seeds 7 days after pollination (data not shown). Taken together, these results show that expression of the paternal *MEA* allele is greatly reduced in the endosperm.

To conclude that the paternal *MEA* allele is specifically silenced in the endosperm, we had to show that maternal *MEA* mRNA accumulates in the endosperm and not merely in the seed coat, which is maternal tissue. To address this issue, we crossed female RLD plants to male L*er* plants and harvested F_1 seeds 7 days after pollination. Embryos were dissected from the F_1 seeds. Because the Arabidopsis seed coat has considerable tensile strength, it was possible to separate the seed coat from the endosperm. In this way, endosperm tissue that was visibly free from contaminating seed coat could be isolated (Figure 4A). However, because the endosperm is cellularized, a portion tended to adhere to the seed coat, resulting in a fraction comprised of the seed coat and some contaminating endosperm (Figure 4B). As shown in Figure 4C, only maternal *MEA* mRNA was detected in the endosperm as well as in the seed coat–endosperm fractions. By contrast, both paternal and maternal *MEA* mRNAs were detected in the embryo. These results strongly suggest that the maternal *MEA* allele, and not the paternal *MEA* allele, is expressed in the endosperm. From these results, we conclude that the paternal *MEA* allele is specifically silenced in the endosperm.

Both maternal and paternal *MEA* alleles are expressed during embryogenesis (Figures 3D and 4C). To determine

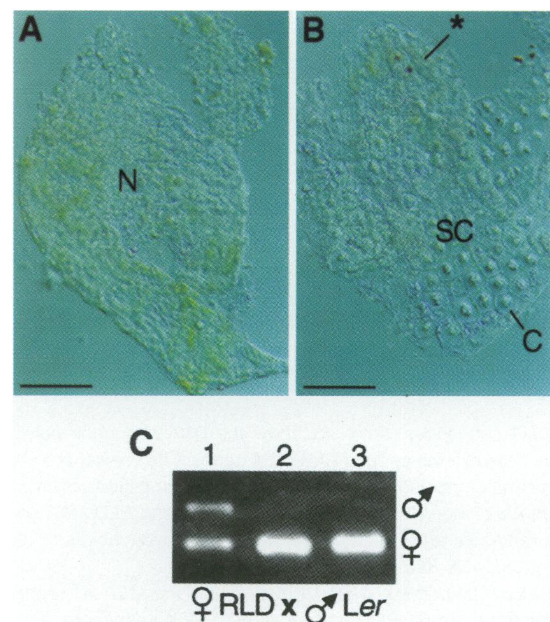

Figure 4. Pattern of Paternal and Maternal *MEA* mRNA Accumulation in Dissected Endosperm and Seed Coat.

(A) Endosperm (N) from an RLD seed harvested 7 days after pollination. Bar = 0.15 mm.

(B) Seed coat (SC) plus endosperm isolated from an RLD seed harvested 7 days after pollination. C, polygonal cell with central elevation, namely, the columella, that is associated with the Arabidopsis seed coat (Leon-Kloosterziel et al., 1994); asterisk, endosperm that adhered to the seed coat. Bar = 0.15 mm.

(C) Accumulation of allele-specific *MEA* mRNA in dissected tissues. RLD females were crossed with L*er* males. Seeds were harvested 7 days after pollination, corresponding to the walking stick embryo stage (Goldberg et al., 1994). Seeds were dissected into embryo (lane 1), endosperm (lane 2), and seed coat plus endosperm (lane 3) components. RNA was isolated and subjected to RT-PCR amplification, BamHI restriction endonuclease digestion, and agarose gel electrophoresis.

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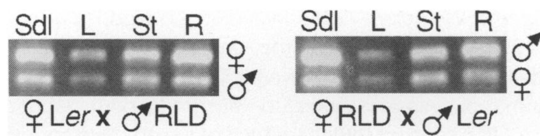

Figure 5. Pattern of Paternal and Maternal *MEA* mRNA Accumulation in Vegetative Tissues.

Reciprocal crosses between L*er* and RLD plants were performed, and tissue was harvested from F_1 progeny. RNA was isolated and subjected to RT-PCR amplification with *MEA* primers, BamHI restriction endonuclease digestion, and agarose gel electrophoresis. Sdl, 4-day seedling; L, rosette leaf; St, stem; R, root.

whether both maternal and paternal *MEA* alleles are expressed after germination in vegetative tissues, we performed reciprocal crosses between L*er* and RLD plants, and RNA was isolated from F_1 seedling, rosette leaf, stem, and root tissue. As shown in Figure 5, we detected maternal and paternal *MEA* mRNAs in all vegetative tissues tested. Thus, silencing of the paternal *MEA* allele was only observed in the endosperm and not in the embryo or in postembryonic vegetative tissues.

DISCUSSION

The Role of the Endosperm in Parent-of-Origin Effects on Seed Development

Loss-of-function mutations in the *MEA* gene display parent-of-origin effects on seed development (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). A seed that inherits a wild-type maternal *MEA* allele develops normally, regardless of the genotype of the paternal *MEA* allele. Conversely, a seed that inherits a mutant maternal *mea* allele, regardless of the genotype of the paternal *MEA* allele, develops abnormally. To understand the molecular basis for these parent-of-origin effects, we compared the level of expression of maternal and paternal *MEA* alleles during seed and plant development. We found that both maternal and paternal *MEA* alleles are expressed in the embryo, seedling, leaf, stem, and root. By contrast, maternal allele expression predominated in the endosperm. These results suggest that the *mea* parent-of-origin effects on seed development are the result, at least in part, of silencing of the paternal allele specifically in the endosperm.

In certain situations, it appears that the silenced paternal *MEA* allele in the endosperm can be activated and expressed. For example, genetic background may have an effect on silencing. Eight days after RLD plants were pollinated with L*er* pollen, paternal *MEA* allele expression was detected in the endosperm of F_1 seeds (Figure 3E). No paternal *MEA* expression was detected in the F_1 progeny of the reciprocal cross. In this regard, it is interesting that we have observed effects on the transmission of the mutant maternal *mea* allele in the progeny of crosses between different ecotypes (data not shown). We are currently determining whether there is a correlation between specific ecotypes, transmission of the maternal mutant *mea* allele, and the level and timing of paternal *MEA* allele expression in the endosperm.

Although our experiments suggest that paternal silencing in the endosperm is important, other mechanisms may also contribute to the parent-of-origin effects of *mea* mutations on seed development. First, it is possible that essential maternal *MEA* expression takes place before fertilization within the female gametophyte. Second, the paternal *MEA* allele may be silenced at an important period within the embryo before the torpedo stage, which is the earliest stage we were able to investigate (Figure 3). Further experiments are required to determine the status of these latter two mechanisms.

Support of Embryo Development by the Endosperm

Within the Arabidopsis seed, the endosperm and maternal seed coat serve to support the growth and development of the embryo. Early in embryogenesis, the suspensor is thought to play a critical role, perhaps by acting as a conduit for nutrients moving from maternal tissues to the embryo (Yeung and Meinke, 1993). Later, it is thought that chalazal-oriented endosperm might facilitate transfer of nutrients from maternal vascular tissue to the embryo (Schulz and Jensen, 1971; Berger, 1999; Brown et al., 1999). Finally, as in most other dicot seeds, the Arabidopsis endosperm is ephemeral, and most of its stored nutrients are absorbed by the embryo as the seed matures (Berger, 1999; Brown et al., 1999). Hence, it is thought that embryo development is dependent on support provided by endosperm and maternal tissues.

Verification of the idea that embryo development requires a functional endosperm has come from the analysis of the phenotype of mutant *mea* seeds and parent-of-origin *MEA* gene expression. Previously, it was shown that when a seed inherits a maternal mutant *mea* allele, the embryo aborts even when the paternal *MEA* allele is wild type (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). Here, we show that within the seed, the paternal *MEA* allele is expressed in the embryo (Figures 3D and 4C) and is silenced in the endosperm (Figures 3E and 4C). Thus, the compartment in the defective seed that most likely lacks MEA activity is the endosperm, and as a result, development of the embryo aborts. Hence, the parent-of-origin effects of *mea* mutations on embryo development are likely to be indirect and result from defects in endosperm function. These results demonstrate that proper endosperm development is a prerequisite for embryogenesis in dicot seeds.

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Endosperm Imprinting and the Parental Conflict Theory

The parental conflict theory attempts to explain the existence of genomic imprinting in flowering plants and mammals (Haig and Westoby, 1989, 1991; Moore and Haig, 1991). During reproduction in these organisms, a mother sometimes has offspring by more than one father. Moreover, in both flowering plants and mammals, the embryo acquires a significant amount of resources from maternal tissues. As a result, fathers strive to extract the maximal amount of resources for their own offspring, whereas the mother endeavors to allocate resources equally among all offspring. According to the parental conflict theory, imprinting arose from this conflict between the maternal and paternal genomes in relation to the transfer of nutrients from the mother to the embryo via specialized acquisitive tissues—the endosperm in plants and the placenta in mammals. According to this theory in plants, a gene that tends to suppress endosperm development (e.g., *MEA*; Kiyosue et al., 1999) and restricts nutrient flow to the embryo would be preferentially expressed by the maternal allele, whereas the paternal allele would be silenced (Figure 6). By contrast, a gene that tends to promote endosperm development and therefore increases nutrient flow to the embryo would be preferentially expressed by the paternal allele, and the maternal allele would be silenced (Figure 6).

Results from both genetic and molecular analyses of the *MEA* gene support the parental conflict theory. Loss-of-function mutations in the maternal *mea* allele result in precocious endosperm development before fertilization and endosperm overproduction after fertilization, suggesting that the wild-type maternal *MEA* gene suppresses endosperm development (Kiyosue et al., 1999). As predicted by the parental conflict model for genes that suppress endosperm development, the maternal *MEA* allele is expressed, whereas the paternal *MEA* allele is silenced in the endosperm (Figures 3E and 4C).

In support of the parental conflict model in plants, analysis of progeny from interploidy crosses in many plant species (Haig and Westoby, 1991), including Arabidopsis (Scott et al., 1998), reveals that paternal genomic excess is associated with overproduction of endosperm, whereas maternal genomic excess is associated with endosperm reduction. Our results suggest that the effects on seed development of changing the ratio of maternal to paternal genomes may be due to changes in the ratio of maternal to paternal alleles of specific genes, such as *MEA*. Ultimately, successful reproduction in many plant species (Haig and Westoby, 1991), including Arabidopsis (Scott et al., 1998), may require the proper balance of expression of maternal and paternal alleles of multiple genes within the endosperm.

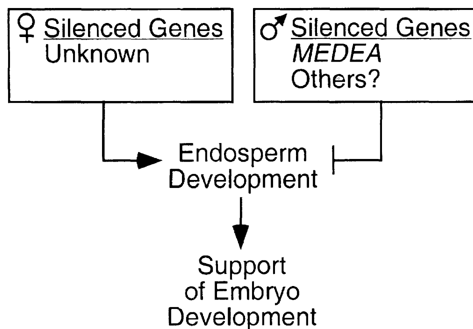

Figure 6. Model for Imprinting during Seed Development.

The T bar and arrow symbols represent the inhibition and activation of endosperm development, respectively.

Differential Imprinting in the Embryo and Endosperm

Because of double fertilization, reproduction in flowering plants involves inheritance of two paternal gametes, one by the embryo and one by the endosperm. The question remains as to why the embryo-inherited paternal *MEA* allele is expressed, whereas the endosperm-inherited paternal *MEA* allele is silenced. One possibility is that analogous to mammalian gene imprinting in the germ line (Pagel, 1999; Tilghman, 1999), silencing of paternal *MEA* alleles occurs during pollen development, perhaps by methylation of specific *MEA* sequences. However, after fertilization of the egg, the silenced state of the paternal *MEA* allele may not be sustained during embryogenesis. This could be the result of failing to maintain the methylated state of the paternal *MEA* allele as embryonic cells replicate their DNA and divide. By contrast, the silenced state of the paternal *MEA* allele may be sustained in the endosperm. This could be accomplished by maintaining the methylation pattern of the paternal *MEA* allele as endosperm cells proliferate.

It is interesting that plant embryos tend not to be susceptible to the effects of parental imprinting (Martienssen, 1998). By contrast, among the parentally imprinted genes studied to date in plants, such as *MEA*, the paternal allele tends to be silenced in the endosperm (Kermickle and Alleman, 1990; Chaudhuri and Messing, 1994). Thus, a fundamental difference between the egg and central cells may be their capacity to maintain the silenced state of parentally imprinted genes after fertilization. Alternatively, it is possible that silencing of the *MEA* gene is only established in the endosperm after fertilization. Experiments designed to elucidate when paternal imprinting occurs will make it possible to distinguish between these models.

METHODS

Plant Material

The two *Arabidopsis thaliana* ecotypes used in these experiments are Landsberg *erecta* (Ler) and RLD (Hardtke et al., 1996). The amino

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acid sequences predicted from the L*er* and RLD *MEA* genes are 99.9% identical (data not shown), and parent-of-origin effects on seed development of the *mea-3* mutation were observed in both the L*er* (Kiyosue et al., 1999) and RLD (data not shown) genetic backgrounds.

Plants were grown in greenhouses under 16-hr-light and 8-hr-dark photoperiods generated by supplemental lighting. Plants were selected for reciprocal crosses 1 week after bolting. Flowers were pollinated 2 days after removal of anthers. F_1 seeds were harvested from siliques at 4, 6, 7, or 8 days after pollination, as described in the text. Seeds were dissected into embryo, endosperm, and seed coat components by using a stereomicroscope. To confirm seed stage, we cleared seeds and visualized them with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) with Nomarski optics (Ohad et al., 1999).

RNA Preparation and Reverse Transcription–Polymerase Chain Reaction Amplification

Tissue samples were ground in Trizol (Life Technologies, Inc., Gaithersburg, MD) reagent with a microglass homogenizer, and total RNA was isolated as described by the manufacturer. Total RNA was further purified by LiCl precipitation and resuspended in 25 μL of RNase-free water (Sambrook et al., 1989). All reverse transcription–polymerase chain reactions (RT-PCRs) were performed with 5 μL of RNA as starting material, using a RETROscript kit (Ambion, Inc., Austin, TX). Primers to amplify *MEA* sequences were *MEA-R1* (5′-GGTTTAGTAACACAAAATAGCATTAC-3′) and *MEA-L1* (5′-GACCTAACTGCTACGCCAAG-3′), which span the 16th intron of the *MEA* gene. All PCR reactions were performed as follows: 1 min at 94°C; 40 cycles (94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec) followed by 72°C for 7 min. To determine the ratio of RLD to L*er MEA* sequences, we performed a second PCR amplification by using the conditions described above with the *MEA-L1* primer and the *MEA-R2d* (5′-AAGGACTGCTTGAATTGCTGCTTCTCCTCGGATC-3′) derived cleaved amplified polymorphic sequence primer (Neff et al., 1998). PCR products were digested with BamHI restriction endonuclease and subjected to electrophoresis on a 3% Metaphor (FMC BioProducts, Rockland, ME) gel.

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