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Mutations in the *FIE* and *MEA* Genes That Encode Interacting Polycomb Proteins Cause Parent-of-Origin Effects on Seed Development by Distinct Mechanisms

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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, which replicates to generate the endosperm, a tissue that supports embryo development. The *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) and *MEDEA* (*MEA*) genes encode WD and SET domain polycomb proteins, respectively. In the absence of fertilization, a female gametophyte with a loss-of-function *fie* or *mea* allele initiates endosperm development without fertilization. *fie* and *mea* mutations also cause parent-of-origin effects, in which the wild-type maternal allele is essential and the paternal allele is dispensable for seed viability. Here, we show that FIE and MEA polycomb proteins interact physically, suggesting that the molecular partnership of WD and SET domain polycomb proteins has been conserved during the evolution of flowering plants. The overlapping expression patterns of *FIE* and *MEA* are consistent with their suppression of gene transcription and endosperm development in the central cell as well as their control of seed development after fertilization. Although FIE and MEA interact, differences in maternal versus paternal patterns of expression, as well as the effect of a recessive mutation in the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene on mutant allele transmission, indicate that *fie* and *mea* mutations cause parent-of-origin effects on seed development by distinct mechanisms.

INTRODUCTION

Flowering plant reproduction involves fertilization of two cells (reviewed in van Went and Willemse, 1984). Within the Arabidopsis ovule, the female gametophyte consists 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 is initiated when an entering pollen tube discharges two genetically identical haploid sperm cells. Fertilization of the egg generates the diploid embryo, which passes through morphologically defined stages (globular, heart, torpedo, walking stick, early maturation, and maturation) (Goldberg et al., 1994; Jurgens and Mayer, 1994). During embryo development, two organ systems (axis and cotyledon) and three tissue layers (protoderm, procambium, and ground meristem) are specified (Lindsey and Topping, 1993; Jurgens, 1994; Meinke, 1994).

Fertilization of the central cell generates the triploid endosperm, for which the pattern of development differs dramatically from that of the embryo. Arabidopsis endosperm development is characteristic of nuclear endosperm development in angiosperms (Mansfield and Briarty, 1990a; Webb and Gunning, 1991; Berger, 1999; Brown et al., 1999). The Arabidopsis primary endosperm nucleus replicates without cytokinesis to form a syncytium of nuclear–cytoplasmic domains that migrate to the periphery of the expanding central cell (Brown et al., 1999). When the embryo is at the globular/heart transition stage, the endosperm surrounding the embryo at the micropylar end of the seed begins to cellularize. Cellularization progresses from the micropylar to the chalazal pole of the seed. Finally, periclinal divisions produce successive layers of endosperm that fill most of the central cell (Brown et al., 1999). As the seed matures, the endosperm, except for a thin aleurone layer, is degraded and absorbed by the embryo. The endosperm is

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thought to support embryo development by producing large amounts of storage proteins, starch, and lipids and by sequestering nutrients from maternal tissue (Lopes and Larkins, 1993).

Loss-of-function mutations in the *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) gene allow diploid endosperm development in the absence of fertilization (Ohad et al., 1996, 1999; Chaudhury et al., 1997). This phenotype suggests that the wild-type *FIE* gene functions to suppress endosperm development until fertilization occurs. The FIE polypeptide (Ohad et al., 1999) has seven tryptophan-aspartate (WD) motifs (Ng et al., 1997). FIE is related to polycomb proteins from Drosophila (*EXTRA SEX COMBS* [*ESC*; Gutjahr et al., 1995; Sathe and Harte, 1995]), mammals (*EMBRYONIC ECTODERM DEVELOPMENT* [*EED*; Schumacher et al., 1996; Sewalt et al., 1998]), and Caenorhabditis (*MATERNAL EFFECT STERILE6* [*MES6*; Korf et al., 1998]). Drosophila ESC and mammalian EED promote interactions with other polycomb proteins that repress gene transcription at specific sites within the genome (Wall et al., 1995; Sondek et al., 1996; Ng et al., 1997).

After fertilization, mutations in the *FIE* gene cause parent-of-origin effects on seed development (Ohad et al., 1996; Chaudhury et al., 1997). For example, when a heterozygous *fie*/*FIE* plant is pollinated with wild-type pollen, half of the seed inherit a maternal mutant *fie* allele and subsequently abort their development, even in the presence of the wild-type paternal allele. These aborted seed contain endosperm and a heart-shaped embryo. However, no seed abortion is observed when the reciprocal cross is performed. Thus, the wild-type maternal *FIE* allele is essential, whereas the paternal *FIE* allele plays little or no role during seed development. The mechanism responsible for these parent-of-origin effects is unknown. Perhaps maternal *FIE* gene expression, which is essential for embryo or endosperm development, occurs before fertilization in the female gametophyte. Alternatively, a gene dosage–dependent mechanism might underlie the parent-of-origin effect of *fie* on seed viability; that is, a single copy of the paternal wild-type *FIE* gene may not be able to compensate for the lack of gene activity associated with two maternal mutant *fie* alleles within the triploid endosperm. Finally, *FIE* could be an imprinted gene in which the maternal allele is expressed and the paternal allele is silenced.

Plants with mutations in either the *MEDEA* (*MEA*) or *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*) gene display phenotypes similar to those of *fie* mutant plants (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999). The *MEA* gene encodes a SET (Jenuwein et al., 1998) domain polycomb protein (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999), whereas *FIS2* encodes a zinc finger protein (Luo et al., 1999). The relationships between the functions performed by the *FIE*, *FIS2*, and *MEA* genes have not been elucidated.

To better understand how female gametophyte, endosperm, and embryo development is controlled, we compared the functions of the *FIE* and *MEA* polycomb genes. Here, we show that the FIE WD polycomb protein interacts physically with the MEA SET domain polycomb protein. Their overlapping expression patterns are consistent with their suppression of endosperm development in the central cell as well as their control of seed development after fertilization. Although FIE and MEA probably function together in a polycomb protein complex, differences in maternal versus paternal patterns of expression, as well as the effect of a recessive mutation in the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene on mutant allele transmission, indicate that *fie* and *mea* mutations cause parent-of-origin effects on seed development by distinctly different mechanisms.

RESULTS

FIE and MEA Proteins Interact

Because the phenotypes associated with mutations in the *FIE*, *MEA*, and *FIS2* genes are similar, we considered that the FIE WD polycomb might interact directly with the MEA SET domain polycomb or the FIS2 zinc finger protein. To investigate this possibility, we used the yeast two-hybrid system, in which interacting proteins bring a GAL4 activation domain (GAL4AD) and a DNA binding domain (GAL4BD) together to activate transcription of β-*GALACTOSIDASE* (β-*GAL*) and *HISTIDINE3* (*HIS3*) reporter genes (Chevray and Nathans, 1992). To this end, we expressed all pairwise combinations of FIE, MEA, and FIS2 fused to GAL4AD and GAL4BD. As shown in Figure 1, yeast expressing both GAL4BD-FIE and GAL4AD-MEA activated strong expression of the *HIS3* (Figure 1B) and β-*GAL* (Figure 1C) genes. In contrast, control yeast containing individual constructs did not activate reporter expression. Although MEA was shown to bind weakly to itself (Figure 1E), analogous to the self-association of SET domain polypeptides in Drosophila and human (Rozovskaia et al., 2000), no other strong interactions, including pairwise combinations with FIS2, were detected in the yeast two-hybrid experiments (Figure 1). Therefore, these results suggest that the FIE and MEA proteins interact specifically in a yeast two-hybrid system.

In the reciprocal two-hybrid experiment, expression of GAL4BD-MEA and GAL4AD-FIE activated HIS3 reporter expression (Figure 1E), but no β-*GAL* reporter expression was detected (Figure 1F). Because this may have been a result of decreased expression or stability of the fusion proteins in this experiment (Fields and Sternglanz, 1994), we used an independent method to verify whether FIE binds to MEA. Glutathione *S*-transferase–tagged FIE fusion protein (GST-FIE) was purified from *Escherichia coli* and tested for binding to in vitro–synthesized radiolabeled MEA protein. As shown in Figure 2A (lane 1), radiolabeled full-length MEA migrated as an ~100-kD polypeptide in our gel electrophoresis system and reacted with an anti-MEA antibody (Figure

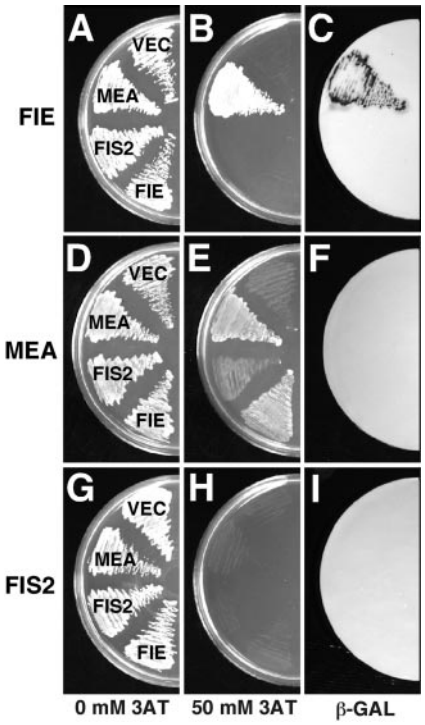

Figure 1. FIE and MEA Interaction in Yeast.

Yeast were transformed with genes encoding GAL4BD fused with full-length FIE (**[A]** to **[C]**), MEA (**[D]** to **[F]**), and FIS2 (**[G]** to **[I]**). Yeast were also transformed with genes encoding GAL4AD fused to MEA, FIS2, and FIE, as shown in **(A)**, **(D)**, and **(G)**, respectively. In **(B)**, **(E)**, and **(H)**, yeast were grown on plates containing synthetic complete medium minus leucine, tryptophan, and histidine. 3-Amino-1,2,4-triazole (3AT) (50 mM) was added to repress the basal activity of the *HIS3* reporter gene, which had resulted in the nonspecific background growth visible in **(A)**, **(D)**, and **(G)** in the absence of exogenous histidine (Kishore and Shah, 1988). The colony colors of the transformants on the plates shown in **(B)**, **(E)**, and **(H)** were determined by the filter lift assay shown in **(C)**, **(F)**, and **(I)**, respectively.

2A, lane 2). Radiolabeled MEA was retained by the immobilized GST-FIE (Figure 2B, lanes 3 to 5) but not by control immobilized GST (Figure 2B, lane 2). Moreover, the quantity of radiolabeled MEA retained correlated with the amount of immobilized GST-FIE used (Figure 2B, lanes 3 to 5). These results indicate that FIE is capable of binding to MEA in vitro.

To investigate the specificity of the interaction between WD and SET domain polycomb proteins, we localized the portion of the MEA polypeptide that binds to FIE. As shown in Figure 3B, we constructed different deletions of GAL4DB-MEA and analyzed their capacity to bind GAL4AD-FIE in the yeast two-hybrid system (Figure 3A). Our results show that the N-terminal 168 amino acids of MEA are sufficient to interact with FIE in the yeast two-hybrid system. Together, these results indicate that the FIE and MEA proteins interact specifically and that this interaction is mediated by the N-terminal region of the MEA protein.

FIE::*GFP* and *FIE*::*GUS* Expression during Ovule, Endosperm, and Embryo Development

If the interaction between FIE and MEA is biologically relevant, then their expression patterns should overlap. Previously, we showed that both *FIE* and *MEA* were expressed in reproductive organ systems, immature floral buds, mature flowers, and young siliques containing developing seed (Kiyosue et al., 1999). Moreover, *MEA* mRNA is detected in the female gametophyte (i.e., central, egg, and synergid cells) (Vielle-Calzada et al., 1999) as well as in the embryo and endosperm (Kiyosue et al., 1999; Vielle-Calzada et al., 1999). To obtain information about the spatial regulation of *FIE* gene expression within the female gametophyte, and after fertilization in the endosperm and embryo, we transformed Arabidopsis plants with three chimeric genes: either 1.3 or 4.9 kb of *FIE* 5′ flanking sequences ligated to a *GREEN FLUORESCENT PROTEIN* (*GFP*) reporter gene (*FIE*::*GFP*) (Heim et al., 1995; Chiu et al., 1996), and 1.3 kb of *FIE* 5′ flanking sequences ligated to a β-*GLUCURONIDASE* (*GUS*) reporter gene (*FIE*::*GUS*) (Jefferson et al., 1987). As shown in Figure 4A, in a line bearing the 1.3-kb *FIE*::*GFP* gene, GFP reporter fluorescence was detected in mature female gametophytes within the central cell. No GFP fluorescence was detected in ovules at earlier stages (data

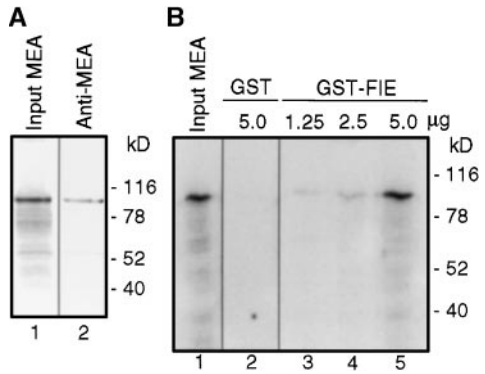

Figure 2. In Vitro Interaction of FIE and MEA Polycomb Proteins.

(A) In vitro–transcribed and –translated ^{35}S-methionine–labeled MEA (lane 1) was subjected to SDS-PAGE, blotted, and incubated with anti-MEA antibody (lane 2).

(B) In vitro–transcribed and –translated ^{35}S-methionine–labeled MEA was incubated with immobilized GST (lane 2) or GST-FIE (lanes 3 to 5), washed, eluted, and subjected to SDS-PAGE and autoradiography. The quantities of GST and GST-FIE proteins immobilized (μg) are indicated. The input MEA (lane 1) represents 10% of the amount of ^{35}S-methionine–labeled MEA incubated with immobilized proteins.

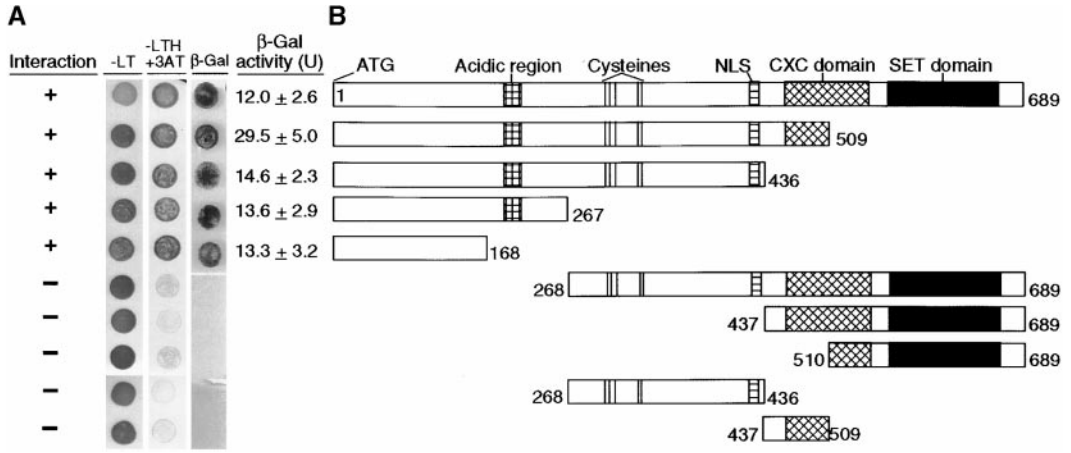

Figure 3. Interaction Domain Analysis of MEA in the Yeast Two-Hybrid System.

(A) Yeast were transformed with GAL4BD-FIE and the GAL4AD plasmid containing the deletion constructs indicated in **(B)**. −LT, synthetic complete medium minus leucine and tryptophan; −LTH +3AT, synthetic complete medium minus leucine, tryptophan, and histidine and supplemented with 50 mM 3-amino-1,2,4-triazole; (+), positive interaction; (−), no detectable interaction; β-Gal activity (U), β-galactosidase activity in liquid cultures measured in units (Kim et al., 1997).

(B) Scheme showing the deletion constructs of MEA. Numbers indicate the positions of the deletions relative to the full-length MEA protein. NLS, nuclear localization sequence.

not shown). Similar results were observed in multiple transgenic lines and in lines bearing a *FIE*::*GFP* gene with 4.9 kb of 5′ flanking sequences. In contrast, histochemical localization of GUS activity in *FIE*::*GUS* plants indicated an earlier stage of female gametophyte development, before the polar nuclei fusion, for the onset of *FIE*::*GUS* activity (Figure 5A). Most likely, GFP and GUS accumulated in the cytoplasm because the FIE-GFP and FIE-GUS fusion proteins did not include the putative FIE nuclear localization sequence (Ohad et al., 1999). Full-length FIE protein fused translationally with GFP localized in the central cell nucleus (T. Kinoshita and R.L. Fischer, unpublished results). This pattern of *FIE*::*GFP* and *FIE*::*GUS* expression is consistent with the hypothesis that FIE protein is present in the central cell, along with MEA (Vielle-Calzada et al., 1999), and functions to prevent the central cell from replicating and forming an endosperm.

After fertilization, *FIE*::*GFP* and *FIE*::*GUS* expression was detected during very early endosperm development, from the primary endosperm nuclear-cytoplasmic domain stage to the eight– to 16–nuclear-cytoplasmic domain stage (Figures 4B to 4G and 5B to 5E). After this stage, GFP fluorescence and GUS activity decreased (Figures 4H to 4J, 5F, 5G, and 6A) until a second phase of *FIE*::*GFP* and *FIE*::*GUS* expression was observed in the endosperm, when the ~100 nuclear-cytoplasmic domains formed a single layer surrounding the endosperm cavity (Figures 6B to 6E and data not shown). GFP fluorescence persisted in the endosperm during the cellularization stage (Figures 6F to 6H) and was also observed in the developing embryo at the torpedo, walking stick, and early maturation stages (Figure 6I and data not shown). Before endosperm cellularization, the only evidence for any transgene activity within the developing seed was a persistent localization of GUS activity in the chalazal cyst (Figures 5E to 5G). The biphasic expression pattern in the endosperm described above was specific to *FIE*::*GFP* and *FIE*::*GUS* and was not detected in endosperm in an enhancer trap line (data not shown), in which *GFP* transcription during endosperm development was controlled by an uncharacterized transcriptional enhancer element (C. Dever and G.N. Drews, unpublished results). The mRNA transcribed from the *FIE*::*GFP* and *FIE*::*GUS* constructs used in this study included essentially all of the *FIE* 5′ untranslated sequences (see Methods). Therefore, these patterns of GFP fluorescence and GUS activity may reflect both transcriptional and translational regulatory processes conferred by *FIE* 5′ flanking sequences. Together, these results indicate a complex pattern of expression for *FIE*::*GFP* and *FIE*::*GUS* before and after fertilization, reflecting the essential role of the FIE protein in seed development.

Parent-of-Origin Effects of *fie* and *mea* Mutations on Seed Development

Mutations in either the *FIE* or *MEA* genes cause parent-of-origin effects on seed development. For *mea* mutations, this probably results, at least in part, from the fact that *MEA* is an imprinted gene in which the paternal *MEA* allele is silenced (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). In particular, previous studies revealed that the paternal *MEA*

allele is silenced specifically in the endosperm of dissected seed from the torpedo to maturation stages (Kinoshita et al., 1999). To determine whether the paternal *FIE* allele is silenced in the same way, we directly measured the amounts of maternal and paternal *FIE* mRNAs in dissected seed. As shown in Figure 7A, to distinguish maternal and paternal *FIE* RNAs, we identified a restriction (MseI) fragment length DNA sequence polymorphism in the *FIE* gene isolated from two Arabidopsis ecotypes, Landsberg *erecta* (L*er*) and RLD (Hardtke et al., 1996). Reverse transcription–polymerase chain reaction (RT-PCR) amplification of L*er FIE* sequences followed by MseI digestion was predicted to produce a 202-bp DNA sequence (Figure 7B). For RLD, the same treatment was predicted to produce 130- and 72-bp DNA sequences (Figure 7B). To test this strategy, RNA from L*er* and RLD floral buds was mixed, and the *FIE* sequences were amplified and digested as described above. As shown in Figure 7C, restriction fragments of the expected size and abundance were detected, indicating that we can distinguish between *FIE* RNAs transcribed from the L*er* and the RLD genomes and that the assay is semiquantitative.

To examine the expression of paternal and maternal *FIE* alleles in seed, reciprocal crosses were performed between L*er* and RLD Arabidopsis plants. RNA was isolated from dissected F_1 seed harvested at 6, 7, and 8 days after pollination—times corresponding to the torpedo, walking stick, and early maturation stages of embryo development (Jurgens and Mayer, 1994). Seed were dissected, and RNA was isolated from the embryo and endosperm-plus-seed-coat components. Using the procedures described above, we measured the amounts of maternal and paternal *FIE* mRNA. Both maternal and paternal *FIE* allele expression was detected in embryos at all stages tested (Figure 7D) and in endosperm plus seed coat at the torpedo stage of embryo development (Figure 7E). In contrast, using the same RNAs, we had previously detected biallelic *MEA* expression in the embryo but only the maternal *MEA* mRNA in the endosperm of seeds containing torpedo, walking stick, and early-maturation embryos (Kinoshita et al., 1999). Thus, the paternal *MEA* allele, and not the paternal *FIE* allele, is silenced at this stage of endosperm development. These results support the hypothesis that different mechanisms are responsible for *fie* and *mea* parent-of-origin effects on seed development.

To obtain clues about earlier patterns of allele-specific *FIE* gene expression, we performed reciprocal crosses between wild-type and transgenic plants bearing a *FIE*::*GFP* transgene and then monitored the GFP fluorescence in F_1 progeny seed. As described below, similar results were observed with transgenes having either 1.3 or 4.9 kb of *FIE* 5′ flanking sequences. As shown in Figure 8A (before fertilization), we detected GFP fluorescence in the central cell of a *FIE*::*GFP* ovule before pollination. After pollination of the *FIE*::*GFP* line with wild-type pollen, maternal *FIE*::*GFP* expression was detected up to the 16–nuclear cytoplasmic domain stage (Figures 8B to 8D) in seed with a four- to 16-cell embryo (data not shown). After an interval during which no GFP was detected (Figures 8E and 8F), maternal *FIE*::*GFP* expression resumed in the 100–nuclear cytoplasmic domain stage endosperm (Figures 8G and 8H). Thus, the maternal *FIE*::*GFP* allele was expressed in a biphasic pattern (Figure 8Q), as had been observed in self-pollinated *FIE*::*GFP* plants (Figures 4 and 6).

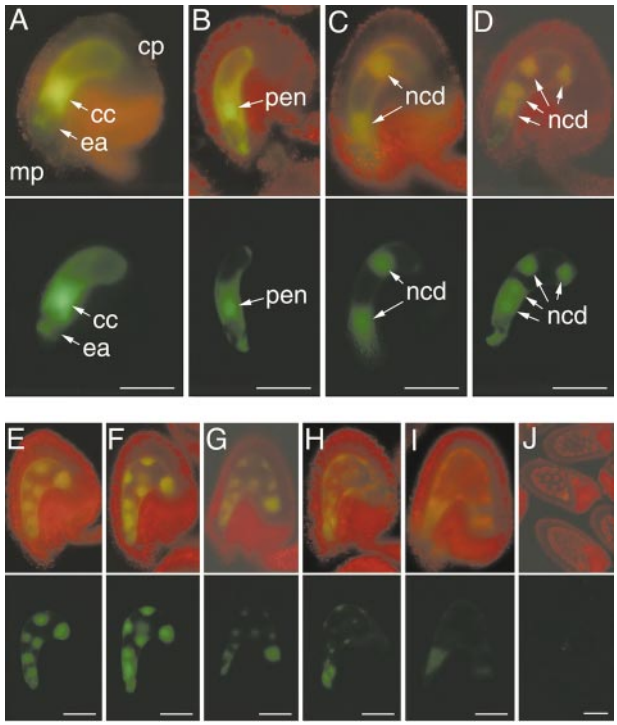

Figure 4. Pattern of *FIE*::*GFP* Expression in an Unfertilized Ovule and Early Endosperm Development.

Unfertilized ovules and developing seeds from transformed plants bearing a transgene with 1.3 kb of *FIE* 5′ flanking sequences fused to *GFP* were analyzed by using fluorescence microscopy, as described in Methods.

(A) Fluorescence micrograph of an intact unfertilized ovule with a mature female gametophyte, indicating *FIE*::*GFP* expression in the cytoplasm of the central cell.

(B) to **(J)** Fluorescence micrographs of intact developing seed corresponding to early stages of endosperm development, including the formation of the primary endosperm nucleus after fertilization **(B)** and its subsequent division (**[C]** to **[J]**) to form a syncytium of nuclear cytoplasmic domains (Mansfield and Briarty, 1990a; Brown et al., 1999). Images were obtained by using fluorescein isothiocyanate (FITC) (chloroplast plus GFP fluorescence; top rows) and GFP (GFP fluorescence; bottom rows) filter sets on a compound microscope (see Methods). The images of the ovules and the developing seed in this figure and in Figures 5, 6, and 8 are oriented in a uniform manner, with the micropylar pole oriented toward the left side and the chalazal pole oriented toward the right side of each figure. cc, central cell; cp, chalazal pole; ea, egg apparatus; mp, micropylar pole; ncd, nuclear cytoplasmic domain; pen, primary endosperm nucleus. Bars in **(A)** to **(I)** = 50 μm; bar in **(J)** = 100 μm.

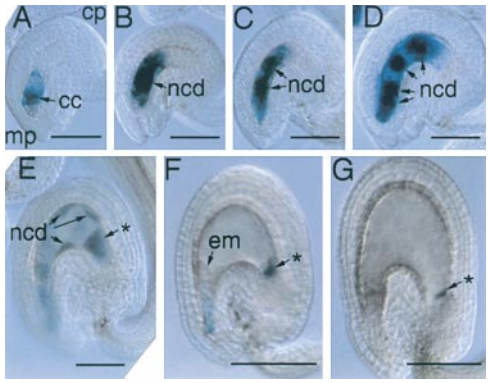

Figure 5. Pattern of *FIE*::*GUS* Expression in an Unfertilized Ovule and Early Endosperm Development.

Unfertilized ovules and developing seeds from transformed plants bearing a transgene with 1.3 kb of *FIE* 5′ flanking sequences fused to *GUS* were analyzed by using light microscopy, as described in Methods.

(A) Light micrograph of an intact unfertilized ovule with an eight-nucleate stage of female gametophyte development (Christensen et al., 1997), indicating *FIE*::*GUS* expression in the cytoplasm of the central cell.

(B) to **(G)** Light micrographs of intact developing seed corresponding to early stages of endosperm development, including the formation of the primary endosperm nucleus after fertilization **(B)** and its subsequent division (**[C]** to **[G]**) to form a syncytium of nuclear cytoplasmic domains (Mansfield and Briarty, 1990a; Brown et al., 1999).

Images were obtained by using differential interference contrast microscopy of unfixed, intact ovules and early seeds after a histochemical reaction for the detection of GUS activity (see Methods). cc, central cell; cp, chalazal pole; em, embryo; mp, micropylar pole; ncd, nuclear cytoplasmic domain; (*), chalazal cyst. Bars in **(A)** to **(E)** = 50 μm; bars in **(F)** and **(G)** = 100 μm.

For the reciprocal cross, as expected, no GFP fluorescence was observed in a wild-type nontransgenic ovule before pollination (Figure 8I). After pollination with *FIE*::*GFP* pollen, no GFP fluorescence was detected until the 100–nuclear cytoplasmic domain stage (Figure 8O), corresponding to the latter phase of *FIE*::*GFP* expression observed in self-pollinated *FIE*::*GFP* plants (Figure 6). In particular, the early phase of maternal *FIE*::*GFP* expression, from fertilization in the central cell until the 16–nuclear cytoplasmic stage of endosperm development, was not detected for the paternal *FIE*::*GFP* allele (cf. Figures 8B to 8D with 8J to 8L). These results show that the expression of the maternal and paternal *FIE*::*GFP* alleles is not equivalent.

Differential Effects of *ddm1* on *fie*- and *mea*-Mediated Seed Abortion

Gene silencing is known to be associated with hypermethylation, and a recessive mutation in the *DECREASE IN DNA METHYLATION1* (*DDM1*) gene, *ddm1-2*, encoding a chromatin-remodeling SWI2/SNF2-like protein (Jeddeloh et al., 1999), has been shown to rescue mutant *mea* seed in self-pollinated plants (Vielle-Calzada et al., 1999). To determine whether a similar relationship exists between genome hypomethylation and *fie* phenotype, we analyzed the ability of the *ddm1-2* mutation to rescue *fie* mutant seed. As shown in Table 1, when control *mea*/*MEA* heterozygous plants were pollinated with wild-type pollen, most of the F_1 seed inheriting a maternal mutant *mea* allele aborted, and only 6% of the F_1 progeny were *mea*/*MEA* heterozygotes. In contrast, pollination of *mea*/*MEA* heterozygous plants with homozygous *ddm1*/*ddm1* pollen decreased the proportion of seed abortion, and 33% of the F_1 progeny inherited the maternal mutant *mea* allele (Table 1). This fivefold increase is consistent with *DDM1* playing a role in silencing the paternal wild-type *MEA* allele during pollen development. In contrast to *MEA*, when *fie*/*FIE* heterozygous plants were pollinated with either wild-type or homozygous *ddm1* mutant pollen, all of the F_1 seed inheriting a maternal *fie* allele aborted, and none of the F_1 progeny were *fie*/*FIE* heterozygotes (Table 1). Thus, *ddm1* pollen suppressed *mea*-mediated, but not *fie*-mediated, seed abortion. Therefore, the *ddm1-2* mutation distin-

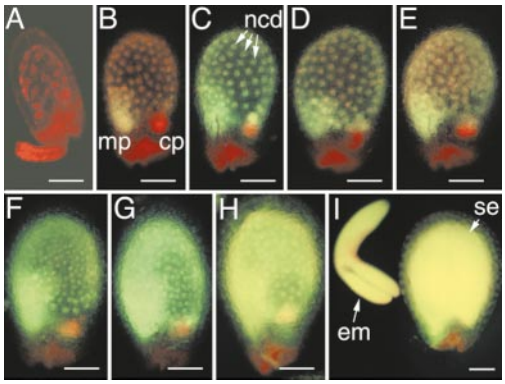

Figure 6. Pattern of *FIE*::*GFP* Expression during Mid to Late Endosperm and Embryo Development.

Developing seed from transformed plants bearing a transgene with 1.3 kb of *FIE* 5′ flanking sequences fused to *GFP* were analyzed by fluorescence microscopy, as described in Methods. Fluorescence micrographs are shown for intact developing seed at various stages of development (Mansfield and Briarty, 1990c; Brown et al., 1999).

(A) Preglobular stage embryo/free nuclear endosperm.

(B) Late globular or transition stage embryo.

(C) to **(F)** Heart stage embryos/cellularizing endosperm.

(G) and **(H)** Late heart or early torpedo stage embryos.

(I) Maturation stage embryo/degenerating cellular endosperm.

Images were obtained using an FITC filter set (chloroplast and GFP fluorescence) on a fluorescence stereomicroscope (see Methods). cp, chalazal pole; em, embryo; mp, micropylar pole; ncd, nuclear cytoplasmic domain; se, intact seed containing an embryo and endosperm. Bars = 100 μm.

guishes the distinct mechanisms responsible for *fie* and *mea* parent-of-origin effects on seed development.

DISCUSSION

Molecular Function of the FIE Protein

The Arabidopsis FIE (Ohad et al., 1999) polycomb protein belongs to a family of WD repeat polypeptides conserved during the evolution of diverse organisms (Ng et al., 1997). The WD repeats in this family of proteins form a circular structure known as a β-propeller, which forms surface loops used as a scaffold for the generation of protein complexes (Komachi et al., 1994; Wall et al., 1995; Sondek et al., 1996). The closest related members to FIE in model genetic organisms are the polycomb proteins ESC in Drosophila (Sathe and Harte, 1995), EED in mouse (Schumacher et al., 1996), and MES6 in Caenorhabditis (Korf et al., 1998). Drosophila ESC and mouse EED have been shown to interact with the SET domain (Jenuwein et al., 1998) polycomb proteins ENHANCER OF ZESTE (E[Z]) (Jones et al., 1998) and ENX1/ENX2 (van Lohuizen et al., 1998), respectively. In the case of Drosophila, yeast two-hybrid experiments, in vitro binding studies, demonstration of coimmunoprecipitation of proteins from embryos, colocalization of proteins on polytene chromosomes, and identification of mutations that disrupt both binding and function all indicate that ESC and E(Z) interact directly and that this interaction is an essential aspect of their function (Jones et al., 1998; Tie et al., 1998). An analogous set of studies demonstrates a direct physical interaction between the mouse WD and SET domain homologs (Denisenko et al., 1998; Jones et al., 1998; Sewalt et al., 1998; van Lohuizen et al., 1998).

Here, we show that the Arabidopsis homologs FIE WD polycomb and MEA SET domain polycomb interact in the yeast two-hybrid system (Figure 1) and in vitro (Figure 2). We also show that an interacting domain is present at the N terminus of MEA (Figure 3), similar to that found in Drosophila and mouse SET domain homologs (Tie et al., 1998; van Lohuizen et al., 1998). In addition, these results imply that the MEA interaction domain is distinct from other conserved regions of the protein, such as the cysteine-rich domain, the nuclear localization domain, the CXC domain, and the SET domain (Grossniklaus et al., 1998). Moreover, the pattern of *FIE*::*GFP* and *FIE*::*GUS* expression (Figures 4 to 6) and FIE mRNA accumulation (Figure 7) overlaps with the pattern of MEA RNA accumulation in the central cell of the female gametophyte, endosperm, and embryo (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). Thus, FIE and MEA polypeptides probably are present in the same cells, which allows for interaction. Together, these results suggest that FIE and MEA interact and that a fundamental biochemical mechanism, the molecular partnership of WD and SET domain polycomb proteins, has been conserved during the evolution of flowering plants.

In Drosophila and mouse, long-term repression of homeotic genes requires the activity of polycomb group proteins (Pirrotta, 1998), and a distinct temporal sequence of events involved in the assembly of the complex has been defined. Initially recruited, although not in a direct interaction (Kehle et al., 1998; Zhang et al., 1998), by a zinc finger transcription factor (HUNCHBACK), ESC functions transiently (Struhl and Brower, 1992; Gutjahr et al., 1995; Sathe and Harte, 1995) to recruit other polycomb group proteins, including the SET domain protein E(Z), which form large, stable complexes at specific sites within the genome (Sewalt et al., 1998; van der Vlag and Otte, 1999; Ng et al., 2000). The complexes, perhaps by the action of a histone deacetylase component (van der Vlag and Otte, 1999), are thought to remodel chromatin into a condensed form that prevents gene transcription (Pirrotta, 1998). By analogy, a similar process may occur in plants, with FIS2 taking the role of the zinc finger protein to indirectly recruit FIE. FIE, in turn, may function transiently to establish a stable complex that includes the SET domain polycomb MEA and ultimately results in the stable repression of gene transcription.

Function of FIE in the Female Gametophyte

Although the biochemical mode of action of interacting pairs of WD and SET domain polycomb proteins is highly conserved, the processes that they regulate are not conserved during evolution. The Drosophila and mouse WD and SET domain polycomb proteins have a somatic role and control pattern formation during embryogenesis by suppressing the transcription of homeotic genes (Gutjahr et al., 1995; Schumacher et al., 1996). In contrast, the Caenorhabditis WD and SET domain homologs MES6 and MES2, respectively, act as transcriptional repressors (Kelly and Fire, 1998) and are essential for germ line development (Holdemann et al., 1998; Korf et al., 1998). Thus, it is not surprising that the plant FIE and MEA interacting pair has been used to repress distinct developmental pathways, as described below.

Loss-of-function mutations in the *FIE* (Ohad et al., 1999) and *MEA* (Kiyosue et al., 1999; Luo et al., 1999) genes allow for the central cell in the female gametophyte to replicate and begin endosperm development. Moreover, FIE and MEA interact (Figures 1 to 3) and are expressed in the central cell (Figures 4 and 5) (Vielle-Calzada et al., 1999). These results suggest that before fertilization, FIE and MEA participate in the formation of a polycomb complex that represses the transcription of genes required for replication of the central cell nucleus and subsequent endosperm development. The fact that *FIE*::*GFP* expression was detected in the central cell and not in the egg cell (Figure 4) suggests that *FIE* may not be expressed in the egg cell, which is consistent with the observation that concomitant autonomous embryo development was not observed in *fie* mutant ovules (Ohad

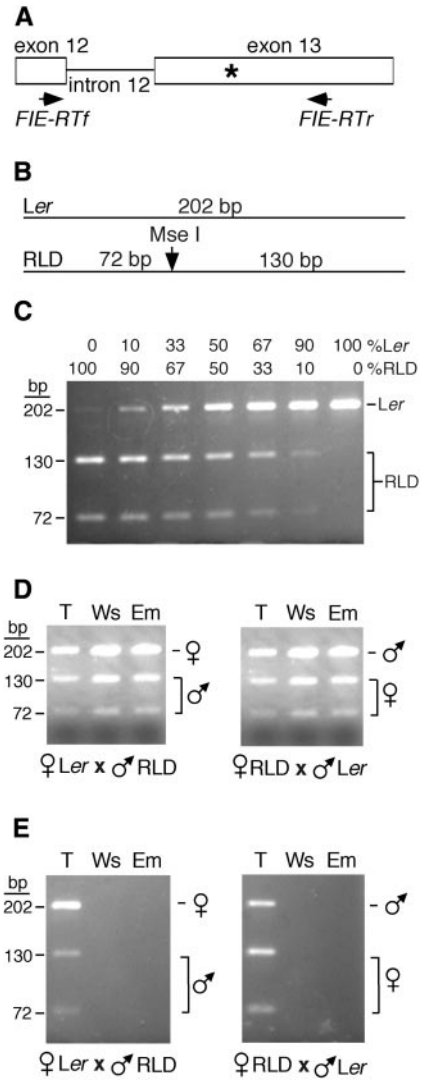

Figure 7. Pattern of Paternal and Maternal *FIE* mRNA Accumulation in Dissected Seed.

(A) Strategy for distinguishing maternal and paternal *MEA* mRNA. *FIE-RTf* and *FIE-RTr* represent primers used for RT-PCR amplification of *FIE* sequences. The asterisk indicates the DNA sequence polymorphism between the L*er* and RLD *MEA* genes. Positions of exons and introns are indicated.

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

(C) Amplification of *FIE* 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, MseI restriction endonuclease digestion, and agarose gel electrophoresis. L*er*- and RLD-specific bands are indicated at right, and the lengths of the restriction fragments (in base pairs) are indicated at left.

(D) Pattern of paternal and maternal *FIE* mRNA accumulation in embryos. Reciprocal crosses between L*er* and RLD plants were made, and F_1 seed 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. Embryos were dissected from seed. RNA was isolated and subjected to RT-PCR amplification, restriction endonuclease digestion, and agarose gel electrophoresis. Restriction fragments associated with maternal and paternal alleles are indicated at left.

(E) Pattern of paternal and maternal *FIE* mRNA accumulation in endosperm plus seed coat. Reciprocal crosses were performed as described in **(D)**. Embryos at the indicated stages were removed from seed. RNA was isolated from the remaining endosperm-plus-seed-coat portion and analyzed as described in **(D)**. Restriction fragments associated with maternal and paternal alleles are indicated at left.

et al., 1996). Together, these results suggest that different proteins and mechanisms may be responsible for the activation of endosperm and embryo development.

Function of FIE after Fertilization

The *FIE*::*GFP* and *FIE*::*GUS* genes display a complex pattern of expression during seed development. They display a biphasic pattern during endosperm development (Figures 4 to 6 and 8), with the latter phase of activity also present in the embryo (Figure 6 and data not shown). This complex pattern suggests that FIE might function, and have multiple roles, during endosperm and embryo development. Because *MEA* is also expressed in the developing embryo and endosperm (Kinoshita et al., 1999; Vielle-Calzada et al., 1999), FIE and MEA may interact after fertilization. We had shown previously that the MEA polycomb protein functions to suppress endosperm growth after fertilization (Kiyosue et al., 1999). Possibly FIE recruits MEA into a polycomb complex in the endosperm for that purpose.

Many studies have focused on the regulation of early embryogenesis by the Drosophila ESC and mouse EED WD proteins. However, Drosophila ESC is also detected in mid and late embryogenesis as well as in larval and pupal stages, during which it is thought to play a role in imaginal disc formation (Struhl and Brower, 1992; Ng et al., 2000). The mouse *EED* gene also is expressed in many tissues during mouse development (Schumacher et al., 1996), and EED protein has been associated with the chromatin-remodeling enzyme histone deacetylase (van der Vlag and Otte, 1999). These studies suggest that ESC and EED may be globally involved in chromatin regulation at many points during the development of Drosophila and mouse, respectively (Ng et al., 2000).

The broad pattern of *FIE* RNA accumulation in both reproductive and vegetative tissues (Kiyosue et al., 1999; Ohad et al., 1999; R. Yadegari and R.L. Fischer, unpublished results) suggests that the FIE polycomb protein may also participate in a general repression mechanism, perhaps by interacting with various SET domain polypeptides or histone deacetylase genes at different times during plant development (Goodrich

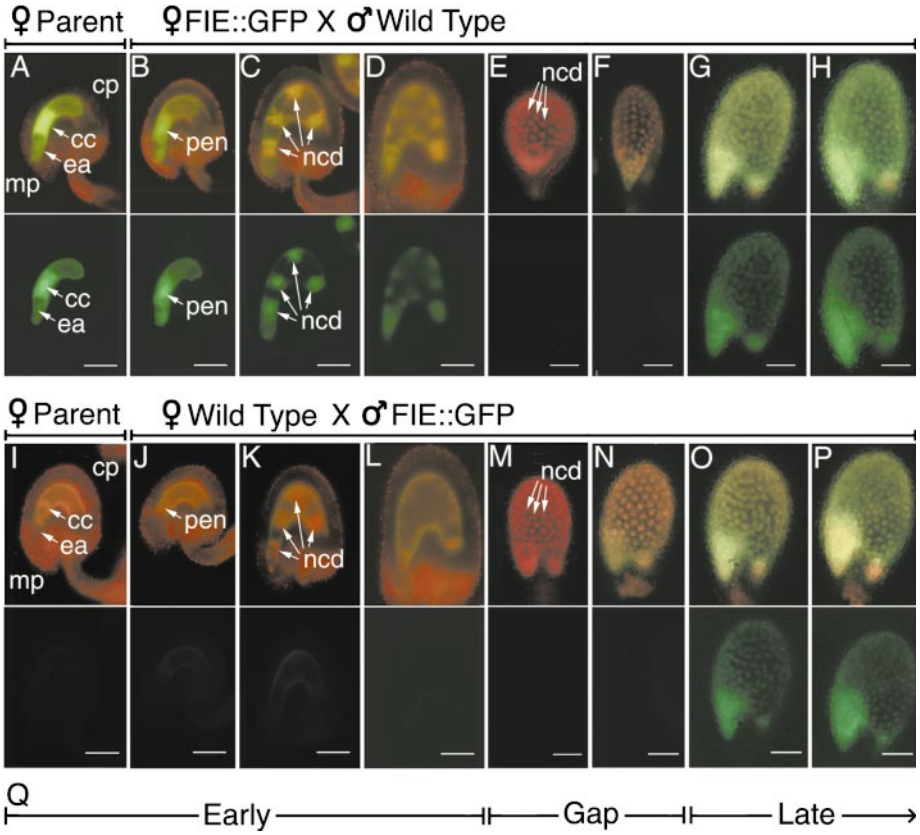

Figure 8. Pattern of Paternal and Maternal *FIE*::*GFP* Expression during Endosperm and Embryo Development.

Developing F_1 progeny seed resulting from reciprocal crosses between wild-type and transgenic plants bearing a transgene with 4.9 kb of the *FIE* 5′ flanking sequences fused to *GFP* were analyzed by fluorescence microscopy, as described in Methods.

(A) and **(I)** Intact unfertilized ovules with mature female gametophytes are shown from the maternal parents in each cross, one bearing a *FIE*::*GFP* transgene **(A)** and the other a wild-type/nontransgenic plant **(I)**.

(B) to **(H)** Intact developing seed resulting from fertilizing a transgenic plant **(A)** with wild-type pollen.

(J) to **(P)** Intact developing seed resulting from fertilizing a wild-type plant **(I)** with pollen from a transgenic plant **(A)**.

(Q) Scheme of early and late periods of maternal *FIE*::*GFP* allele expression separated by a gap.

Images were obtained by using FITC (chloroplast plus GFP fluorescence; top rows) and GFP (GFP fluorescence; bottom rows) filter sets on a compound microscope (**[A]** to **[D]**) and (**[I]** to **[L]**) and a stereomicroscope (**[E]** to **[H]**) and (**[M]** to **[P]**) as described in Methods. cc, central cell; cp, chalazal pole; ea, egg apparatus; mp, micropylar pole; ncd, nuclear cytoplasmic domain; pen, primary endosperm nucleus. Bars in **(A)** to **(D)** and **(I)** to **(L)** = 50 μm; bars in **(E)** to **(H)** and **(M)** to **(P)** = 100 μm.

et al., 1997; Wu et al., 2000). However, because loss-of-function mutations in the *FIE* gene are not transmitted to the next generation (Table 1), it has not been possible to address this point by generating homozygous mutant *fie* plants to study the function of *FIE* during vegetative plant development.

Mechanism for Parent-of-Origin Effects of *fie* Mutations on Seed Development

Mutations in both the *FIE* and *MEA* polycomb genes result in parent-of-origin effects on seed development. Specifically, a seed inheriting a mutant *mea* or *fie* allele will abort, even if the paternal allele is wild type. For *MEA*, this is probably a result, at least in part, of the silencing of the paternal *MEA* allele (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). However, several lines of evidence suggest that *fie*- and *mea*-mediated parent-of-origin effects are caused by distinct mechanisms. First, the parent-of-origin effects associated with *fie* mutations are more stringent than those associated with *mea* mutations. That is, the maternal mutant *mea* allele is occasionally transmitted (Chaudhury et al., 1997; Kiyosue et al., 1999; Vielle-Calzada et al., 1999), whereas the maternal mutant *fie* allele is not (Table 1) (Ohad

Table 1. Effect of a Paternal *ddm1-2* Mutant Allele on Transmission of a Maternal *fie-1* or *mea-3* Mutant Allele

Maternal Parent	Paternal Parent	Progeny with Maternal Mutant Allele	Progeny with Maternal Wild-Type Allele	Percent[a]
mea/MEA	*MEA/MEA* (wild type)	9 *mea/MEA*	150 *MEA/MEA*	6
mea/MEA	*MEA/MEA*, *ddm1/ddm1*	44 *mea/MEA*, *ddm1/DDM1*	89 *MEA/MEA*, *ddm1/DDM1*	33
fie/FIE	*FIE/FIE* (wild type)	0 *fie/FIE*	52 *FIE/FIE*	0
fie/FIE	*FIE/FIE*, *ddm1/ddm1*	0 *fie/FIE*, *ddm1/DDM1*	46 *FIE/FIE*, *ddm1/DDM1*	0

[a] Percentage of transmission is 100 × (number of *fie* or *mea* heterozygotes/total progeny of cross).

et al., 1996; Chaudhury et al., 1997). Second, despite a report that homozygosity of *ddm1* in the developing seed is required to suppress the *mea* mutant phenotype (Vielle-Calzada et al., 1999), we find that pollen from a homozygous *ddm1* paternal parent is sufficient for suppression (Table 1). In contrast, pollen from a *ddm1* parent does not suppress the *fie* mutant phenotype (Table 1), indicating that *FIE*, unlike *MEA*, is not influenced by *DDM1* during pollen donor development. Third, the wild-type paternal *MEA* is silenced specifically in the endosperm during the mid to late stages of endosperm development (Kinoshita et al., 1999). In contrast, the paternal *FIE* allele is expressed during these stages (Figure 7). The different patterns of paternal *FIE* and *MEA* allele expression within the endosperm (Figures 7 and 8) indicate differences in the regulation of their expression. For example, paternal *FIE* and *MEA* alleles might be initially silenced at the same time before fertilization, but the underlying molecular mechanisms responsible for silencing might be sufficiently different to include a longer period of silencing for the paternal *MEA* allele. Together, these results suggest that the mechanisms for *fie* and *mea* parent-of-origin effects on seed development differ.

Why is the maternal *FIE* allele essential and the paternal *FIE* allele dispensable? Only the maternal *FIE*::*GFP* allele is expressed in the unfertilized central cell of the female gametophyte and during very early endosperm development until approximately the 16–nuclear cytoplasmic domain stage (Figure 8). During this interval, the paternal *FIE*::*GFP* allele is either not present (i.e., in the unfertilized ovule) or not active (Figure 8). Perhaps the parent-of-origin phenotype of *fie* mutants results from a similar difference in the expression of maternal and paternal *FIE* alleles during this interval. In support of this hypothesis, a transgene consisting of 1.6 kb of 5′ flanking sequences ligated to a full-length *FIE* cDNA fused to GFP showed a pattern of early GFP expression similar to that of the *FIE*::*GFP* transgenes and rescued a mutant *fie* seed only when inherited maternally (T. Kinoshita and R.L. Fischer, unpublished results). Therefore, we propose that maternal *FIE* allele gene expression—transcription, translation, protein modification (Ng et al., 2000)—during this interval results in the generation of maternal FIE protein, which is necessary for seed development. As a result, mutations in *fie* cause parent-of-origin effects on seed development. As described below, three models can be considered.

One model suggests that essential expression of the *FIE* maternal allele occurs in the central cell of the female gametophyte before the introduction of the paternal *FIE* allele by fertilization. Indeed, the early pattern of GFP fluorescence (Figures 8A to 8D and 8Q) might reflect the translation of maternal *FIE*::*GFP* mRNA transcribed before fertilization. If so, FIE would be similar to PROLIFERA (PRL), an Mcm7 protein essential for DNA replication; accumulation of PRL in the female gametophyte is thought to be responsible for parent-of-origin effects of *prl* mutations (Springer et al., 2000).

An alternative model suggests that a gene dosage-dependent mechanism regulates the expression of *FIE* (and possibly other regulatory genes). That is, a single copy of the paternal wild-type *FIE* gene might be incapable of compensating for the lack of gene activity associated with two mutant *fie* maternal alleles within the triploid endosperm of a *fie*/*FIE* heterozygous plant. However, *fie*/*FIE* maternal plants, when fertilized with pollen having a wild-type *FIE* allele plus extra *FIE* transgenes capable of complementing the *fie* mutation when maternally inherited (Ohad, et al., 1999), did not display any decrease in the *fie*-mediated seed abortion phenotype (M. Hannon and R.L. Fischer, unpublished data). Moreover, in reciprocal crosses between wild-type and *FIE*::*GFP* transgenic plants (Figure 8), pollen donors with single or multiple copies of the transgene exhibited the same pattern of early paternal silencing during early endosperm development, followed by reactivation of *FIE*::*GFP* activity before endosperm cellularization (R. Yadegari and R.L. Fischer, unpublished data). We think it unlikely, therefore, that a *FIE* gene dosage model would account for the parent-of-origin effect observed for *fie* during seed development.

A final model to account for the *fie* mutation's parent-of-origin effects on seed development suggests that essential expression of the *FIE* maternal allele takes place in the endosperm soon after fertilization, although not later than the 16–nuclear cytoplasmic domain stage (Figure 7). Vielle-Calzada et al. (2000) proposed that during this interval, paternal allele expression of many Arabidopsis genes may be delayed. However, the scarcity of "embryo-lethal" mutations

that cause parent-of-origin effects on seed viability (Jurgens et al., 1991) implies that late expression of the paternal wild-type allele is sufficient for almost all genes required for seed viability. Therefore, if this model of *fie*-mediated parent-of-origin effects is correct, then the essential early requirement for *FIE* expression must be very stringent to explain why later expression of the paternal *FIE* allele does not result in viable seed. Experiments designed to elucidate more precisely when *FIE* is transcribed and translated and when active FIE protein accumulates will make it possible to distinguish between these models of *fie*-mediated parent-of-origin effects on seed development.

In summary, although the mechanisms responsible for parent-of-origin effects caused by *fie* and *mea* mutations are probably different, we propose that FIE and MEA interact directly in wild-type plants to control female gametophyte, endosperm, and embryo development.

METHODS

Growth Conditions

Arabidopsis thaliana plants were grown in greenhouses under a 16-hr light/8-hr dark photoperiod generated by supplemental lighting.

Yeast Two-Hybrid Clones and Assays

Full-length *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*), *MEDEA* (*MEA*), and *FERTILIZATION-INDEPENDENT SEED2* (*FIS2*) cDNA clones were subcloned into the pBI880 vector (pGAL4BD) containing the GAL4 DNA binding domain and the pBI771 vector (pGAL4AD) containing the GAL4 activation domain (Kohalmi et al., 1997). Interactions between FIE, MEA, and FIS2 proteins were tested by introducing the appropriate pairs of constructs into Y190 yeast cells. Transformants were plated on synthetic complete medium that lacked leucine, tryptophan, and histidine amino acids and was supplemented with 3-amino-1,2,4-triazole as noted. The surviving yeast colonies were then transferred to filters containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and the amount of β-galactosidase activity was determined by scoring the intensity of blue pigment produced. Deletions of MEA were constructed by using restriction endonuclease sites and were ligated in frame in pBI771. β-Galactosidase assays were performed as described previously (Kohalmi et al., 1997).

In Vitro Binding of FIE and MEA

A cDNA encoding a full-length FIE protein (Ohad et al., 1999) was ligated into the pGEX-4T-1 vector (Amersham Pharmacia Biotechnology, Piscataway, NJ), giving rise to glutathione *S*-transferase (GST)–FIE, which was introduced into *Escherichia coli* BL21 cells. GST-FIE fusion protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (Bio Lab Ltd., Falmouth, MA) at 30°C for 12 to 18 hr. Similarly, control GST protein was produced from Escherichia BL21 cells containing the pGEX-4T-1 vector. Cells were harvested and frozen at −20°C. Cells were resuspended in one-tenth volume of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.3) containing protease inhibitor mix (Complete; Roche Molecular Biochemicals, Indianapolis, IN) and were lysed by using a French press. After centrifugation at 11,000*g*, the soluble fraction was incubated with glutathione agarose beads (Sigma, St. Louis, MO) for 30 min at 4°C while being rotated. Agarose beads were washed two times with PBS plus 1% (w/v) Triton X-100 and three times in PBS. GST-FIE protein was shown to migrate as a single band after PAGE and staining with Gel-Code (Pierce, Rockford, IL).

Full-length MEA cDNA sequences (Kiyosue et al., 1999) were amplified by polymerase chain reaction (PCR) and ligated into pGEM-T Easy vector (Promega, Madison, WI) at the BamHI-XhoI site in the polylinker. The pGEM-MEA plasmid was digested with SalI and used as a template for in vitro transcription and translation in a wheat germ extract system (Promega) by procedures provided by the manufacturer. Redivue ^{35}S-methionine (Amersham Pharmacia Biotechnology) was used to label the MEA protein.

Purified GST-FIE (1.25, 2.5, or 5.0 μg) or control GST (5.0 μg) proteins were immobilized on glutathione agarose beads, washed with binding buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), resuspended in 250 μL of binding buffer, and incubated for 2 hr at 4°C with equal amounts of ^{35}S-methionine–labeled MEA protein. Beads were washed four times with the binding buffer and resuspended in SDS–polyacrylamide gel sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromphenol blue, and 100 mM DTE). Samples were boiled for 2 min at 98°C, separated by electrophoresis on a 10% SDS–polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) Immobilion-P membrane (Millipore, Bedford, MA). Labeled MEA protein was detected with a PhosphorImager (model BAS-1000; Fuji, Tokyo, Japan) using trans-screen-LE intensifying screens (Kodak, Rochester, NY).

For protein blot analysis, PVDF membranes were blocked for 1 hr in TBS (25 mM Tris-HCl, pH 7.5, 136 mM NaCl, and 2.68 mM KCl) containing 5% powdered milk and incubated with anti-MEA antibody for 1 hr at room temperature. Membranes were washed three times for 10 min in TBS. A second anti–goat antibody conjugate (Sigma) was added to the membrane after the third wash and incubated for 30 min at room temperature. Blots were then washed three times with TBS. Antibodies were detected by alkaline phosphatase reaction.

To generate MEA antibodies, a 1.3-kb *MEA* cDNA clone encoding amino acids 1 through 436 was inserted into the pET28a vector (Novagen, Madison, WI). The resulting histidine tag (HIS)–MEA fusion protein was purified by SDS-PAGE. A single protein band was eluted from the gel and used as an immunogen in rabbits. MEA antibodies were affinity-purified by reacting sera with GST-MEA fusion protein bound to PVDF membranes. Affinity-purified MEA antibodies were tested for their ability to bind specifically to HIS-MEA and GST-MEA proteins produced in Escherichia.

Generation of *FIE*::*GFP* and *FIE*::*GUS* Transgenic Plants

The *GREEN FLUORESCENT PROTEIN* (*GFP*) gene used in the construction of reporter transgenes is the synthetic variant S65T (sGFP[S65T]) and lacks any subcellular localization sequences (Heim et al., 1995; Chiu et al., 1996; Niwa et al., 1999). The complete coding sequence of sGFP(S65T) and a polyadenylation signal from the nopaline synthase gene (nos3′) were excised together from a donor plasmid (CaMV35S-sGFP[S65T]-nos3′) by use of BamHI and EcoRI

and were cloned into the corresponding sites of the *Agrobacterium tumefaciens* binary vector pBI101.1 (Jefferson et al., 1987), replacing the β-glucuronidase–nos3′ reporter cassette (pBI-GFP[S65T]; M. Pastuglia and R.L. Fischer, unpublished data). The *FIE*::*GFP* transgenes used in this study were created by PCR amplification of two sets of *FIE* upstream sequences, restriction modification of the ends, and direct cloning into pBI-GFP(S65T). A genomic clone (p12Cla) was used as a template to amplify ~1.3 kb of *FIE* 5′ flanking sequences in a PCR reaction with a genome-based primer, RY013 (5′-CCCGGGATCCCTTCGACATTCGATATTCG-3′), and a plasmid-based primer, T3 (5′-AATTAACCCTCACTAAAGGG-3′). The insert of p12Cla is identical to the genomic cosmid pCLD36, which was previously shown to complement the *fie* mutation (Ohad et al., 1999). Another genomic clone (Cer2) was used as a template to amplify ~4.9 kb of *FIE* 5′ flanking sequences in a PCR reaction with two genome-based primers, RY013 and RY014 (5′-ACTAGTCGACGGTGGCTTGTGAAGATGGTTAG-3′). The 1.3- and 4.9-kb amplification products were restriction-digested with BamHI and with BamHI plus SalI, respectively, and then cloned into the corresponding sites upstream of the sGFP(S65T) cassette in pBI-GFP(S65T). Therefore, the resulting binary constructs contained variable 5′ sites positioned at −1273 bp (*1.3kbFIE*::*GFP*) and −4942 bp (*4.9kbFIE*::*GFP*) relative to the putative start codon, and both contained a fixed 3′ site at position +9 bp fused in frame to *sGFP(S65T)*. The *FIE*::β-*GLUCURONIDASE* (*GUS*) transgenes were created similarly by PCR amplification of 1.3 kb of *FIE* 5′ flanking sequences in a PCR reaction using p12Cla as a template and primers RY013-G (5′-CCCGGATCCTTCGACATTCGATATTCG-3′) and T3, followed by restriction modification of the ends and direct cloning into pBI101.2 (Jefferson et al., 1987). Agrobacterium GV3101 pMP90 strains (Koncz and Schell, 1986) containing the binary vectors were then used to transform (Century et al., 1997) three Arabidopsis ecotypes, Landsberg *erecta* (L*er*), Columbia-0 (Col-0), and Columbia-PRL, *gl1* (Col-PRL, *gl1*), to generate 10 to 50 independent kanamycin-resistant T_1 plants for each *FIE*::*GFP* and *FIE*::*GUS* construct.

Analysis of *FIE*::*GFP* Expression during Development and in Reciprocal Crosses

Approximately 22 and 10 independently transformed T_1 lines each for the *1.3kbFIE*::*GFP* and *4.9kbFIE*::*GFP* constructs, respectively, and ~30 independently transformed T_1 lines for the *1.3kbFIE*::*GUS* construct were used to determine the overall pattern of *FIE*::*GFP* and *FIE*::*GUS* expression during development. Four T_1 lines each for the *1.3kbFIE*::*GFP* and *4.9kbFIE*::*GFP* constructs were used to determine the overall pattern of *FIE*::*GFP* expression after reciprocal crosses. Subsequently, more detailed analysis of *FIE*::*GFP* gene expression was performed with T_2 sibling plants that were heterozygous or homozygous for a single *FIE*::*GFP* locus. For analysis of *FIE*::*GFP* and *FIE*::*GUS* expression in mature female gametophytes and to prepare pistils for reciprocal crosses, flowers were emasculated during stages 12A to 12C to prevent self-fertilization (Smyth et al., 1990; Christensen et al., 1997). Seed development was correlated to morphological changes occurring during endosperm and embryo development (Mansfield and Briarty, 1990a, 1990b, 1990c; Mansfield et al., 1990; Jurgens and Mayer, 1994). Intact unfertilized ovules and developing seeds from *FIE*::*GFP* transformants were excised, mounted in water, and observed immediately with a Zeiss Axioskop 2 fluorescence microscope (Carl Zeiss, Thornwood, NY) or an Olympus SZX12 fluorescence stereomicroscope (Olympus America, Melville, NY). The former was equipped with a fluorescein isothiocyanate (FITC) filter set (exciter 470/40, dichroic 505, emitter OG515LP; Chroma Technology, Inc., Brattleboro, VT) and a GFP filter set (wt-GFP Longpass Emission: exciter HQ450/50x, dichroic Q480LP, emitter HQ48LP; Chroma Technology), and the latter was equipped with two analogous filter sets (GFP Longpass: exciter 460 to 490, dichroic 505, emitter 510; GFP Bandpass: exciter 460 to 490, dichroic 505, emitter 510 to 550; Olympus America). Histochemical localization of GUS activity in *FIE*::*GUS* plants was performed on intact pistils and developing siliques, which were excised longitudinally, vacuum-infiltrated for 10 to 15 min on ice with staining solution (50 mM sodium phosphate buffer, pH 7.0, 0.2% Triton X-100, 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, and 1 mM X-gluc), and incubated for 12 to 16 hr at 37°C (Sessions et al., 1999). Individual ovules and developing seeds were then dissected out, mounted in 50 mM sodium phosphate buffer, pH 7.0, and observed with a Zeiss Axioskop microscope equipped with differential contrast interference microscopy optics. Images were captured on 35-mm slide film (Kodak Elite Chrome, ISO 100) or with an Optronics Color 3 charge-coupled device camera (Optronics, Goleta, CA) and Scion 1.62c image acquisition software (Scion, Frederick, MD). Image processing and reproduction were performed with Photoshop 5.0.2 (Adobe Systems, San Jose, CA).

Distinguishing L*er* and RLD *FIE* mRNAs in Dissected Seed

The two Arabidopsis ecotypes used in these experiments were L*er* and RLD (Hardtke et al., 1996). The DNA sequences for the L*er* and RLD *FIE* genes were 99.8% identical (data not shown) over a 442-bp region, and parent-of-origin effects on seed development of the *fie-1* mutation were observed in both the L*er* (Ohad et al., 1996) and RLD (data not shown) genetic backgrounds. Plants were selected for reciprocal crosses 1 week after bolting. Flowers were pollinated 2 days after removal of anthers. F_1 seed were harvested from siliques at 4, 6, 7, or 8 days after pollination. Seed were dissected into embryo and endosperm-plus-seed-coat components under a stereomicroscope. To confirm seed stage, seed were cleared and visualized with a Zeiss Axioskop microscope with Nomarski optics (Ohad et al., 1999). RNA was isolated as described previously (Kinoshita et al., 1999). cDNA synthesized with the primer *FIE-RTr* (5′-CTGTAATCAGGCAAACAGCC-3′) and amplified by PCR with primers *FIE-RTr* and *FIE-RTf* (5′-CTGTAATCAGGCAAACAGCC-3′) were as described by Kinoshita et al. (1999). To determine the ratio of RLD and L*er FIE* sequences, purified PCR products were digested with the MseI restriction endonuclease and subjected to electrophoresis on a 3% Metaphor agarose gel (FMC BioProducts, Rockland, ME).

Measuring the Effect of *ddm1-2* on Transmission of a Maternal *fie-1* or *mea-3* Mutant Allele

Heterozygous *mea-3* (Kiyosue et al., 1999) (backcrossed six times to the Col-0 ecotype) and *fie-1* (Ohad et al., 1999) (L*er* ecotype) were pollinated with pollen from homozygous *decrease in DNA methylation* (*ddm*)*1-2* (Col-0 ecotype) plants (Vongs et al., 1993). Progeny from these crosses were grown, DNA was isolated (Murray and Thompson, 1980), and genotypes were determined by using derived cleaved amplified polymorphic sequence primers (Neff et al., 1998). For *ddm1-2*, *DDM1f* (5′-CAGATCTCTACCCTCCTGT-3′) and *ddm1-2dRsa* (5′-TGAGCTACGAGCCATGGGTTTGTGAAACGTA-3′) prim-

ers were used to amplify DNA by PCR. Amplified fragments were digested with RsaI restriction endonuclease and subjected to agarose gel electrophoresis (3% Metaphor agarose). For *mea-3*, the *f644dXba* (5′-CATGCAACGACGGCAATGACGTCTATCAGCAAAATTCT-3′) and *UCB3SR85* (5′-CGAAGTGGATGTTTCGGAC-3′) primers were used; for *fie-1*, the *579dXba* (5′-CATTACTGCCATTGGTGTATCTCTTATTATCTA-3′) and *48S4* (5′-CACTGTTGACGTCAATGACTCGG-3′) primers were used. The amplified DNAs were digested with XbaI restriction enzyme and analyzed by agarose gel electrophoresis. PCR conditions were as described by Kinoshita et al. (1999).

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Mutations in the *FIE* and *MEA* Genes That Encode Interacting Polycomb Proteins Cause Parent-of-Origin Effects on Seed Development by Distinct Mechanisms

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