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Imprinting of the *MEA* Polycomb Gene Is Controlled by Antagonism between MET1 Methyltransferase and DME Glycosylase

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Summary

The *MEA* Polycomb gene is imprinted in the *Arabidopsis* endosperm. DME DNA glycosylase activates maternal *MEA* allele expression in the central cell of the female gametophyte, the progenitor of the endosperm. Maternal mutant *dme* or *mea* alleles result in seed abortion. We identified mutations that suppress *dme* seed abortion and found that they reside in the *MET1* methyltransferase gene, which maintains cytosine methylation. Seeds with maternal *dme* and *met1* alleles survive, indicating that suppression occurs in the female gametophyte. Suppression requires a maternal wild-type *MEA* allele, suggesting that *MET1* functions upstream of, or at, *MEA*. DME activates whereas MET1 suppresses maternal *MEA::GFP* allele expression in the central cell. MET1 is required for DNA methylation of three regions in the *MEA* promoter in seeds. Our data suggest that imprinting is controlled in the female gametophyte by antagonism between the two DNA-modifying enzymes, MET1 methyltransferase and DME DNA glycosylase.

Introduction

Imprinting results in genes being expressed or silenced according to their parental origin (Ferguson-Smith and Surani, 2001; Reik and Walter, 2001). Imprinting occurs in mammals and plants and plays an important role in the reproductive strategies of both groups (Moore, 2001). In mammals, many of the imprinted genes control prenatal growth (Tycko and Morison, 2002); they are expressed in the extraembryonic membranes that serve as a conduit for the flow of nutrients from the mother to the embryo (Reik and Walter, 2001). In plants, the endosperm performs a similar function and is also a critical site for gene imprinting (Martienssen, 1998; Moore, 2001). Although some imprinted genes are essential for plant reproduction (Gehring et al., 2003), little is known about how imprinting is initiated and maintained in plants.

In mammals, one of the mechanisms of gene imprinting involves differential 5-cytosine methylation of alleles during gametogenesis that is then transmitted to the embryo (Ferguson-Smith and Surani, 2001; Li, 2002; Reik and Walter, 2001). In plants, DNA methylation is also responsible, at least in part, for many epigenetic phenomena (Martienssen and Colot, 2001). These include transcriptional silencing of transposons, transgenes, and pathogen DNA, as well as the silencing of genes that control flowering time, floral organ identity, fertility, and leaf morphology (Finnegan et al., 1996; Jacobsen et al., 2000; Kakutani et al., 1996; Miura et al., 2001; Soppe et al., 2000). DNA methyltransferases have been identified that establish and maintain patterns of symmetric (CpG and CpNpG) and asymmetric (CpNpN) cytosine methylation in the plant genome (Cao and Jacobsen, 2002a, 2002b; Finnegan and Dennis, 1993; Lindroth et al., 2001). This methylation is intimately related to histone modifications, chromatin remodeling, and the accessibility of DNA to transcription factors (Jackson et al., 2002; Johnson et al., 2002; Martienssen and Colot, 2001; Soppe et al., 2002). Genetic crosses between plants with wild-type and hypomethylated genomes suggest that DNA methylation is necessary for endosperm development and seed viability (Adams et al., 2000). However, the role that DNA methylation plays in the imprinting of specific genes has not yet been established.

The endosperm and embryo of flowering plants are derived from two fertilization events that occur in the female gametophyte. In *Arabidopsis*, a haploid megaspore undergoes three mitotic divisions to form an eight-nucleus, seven-cell female gametophyte containing the egg, central, synergid, and antipodal cells; the fusion of two haploid nuclei makes the nucleus of the central cell diploid. Fertilization of the egg cell by a sperm cell gives rise to a diploid embryo that ultimately generates the organs, tissues, and meristems of the plant. Fertilization of the central cell by a second sperm cell generates the triploid endosperm that supports embryo or seedling growth and development by producing storage proteins, lipids, and starch, and by mediating the transfer of maternal-derived nutrients to be absorbed by the embryo (Brown et al., 1999).

The *MEDEA* (*MEA*) gene is imprinted in the *Arabidopsis* endosperm. Only the maternal *MEA* allele is expressed (Kinoshita et al., 1999; Luo et al., 2000; Vielle-Calzada et al., 1999). *MEA* encodes a SET domain Polycomb group protein (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999). Polycomb group proteins repress gene transcription by remodeling chromatin at specific regions within the genome (Francis and Kingston, 2001). *MEA* prevents the onset of central cell proliferation prior to fertilization, represses endosperm growth after fertilization, and represses gene expression in the female

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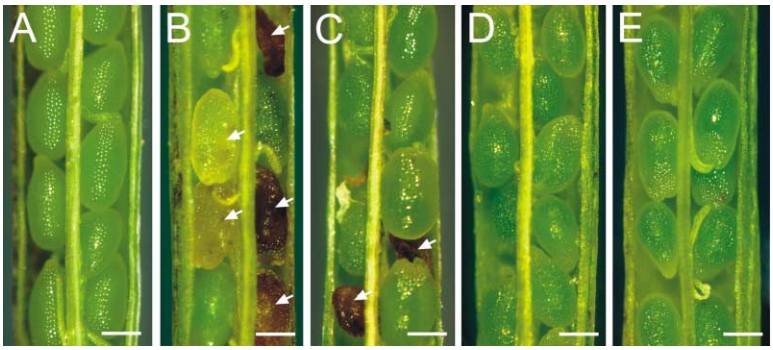

Figure 1. Effect of *dme* and *dme* Suppressor Mutations on Seed Viability

Siliques were dissected and photographed 14 days after self-pollination. The scale bars represent 0.5 mm. Arrows indicate aborted seeds. Siliques shown in (D) and (E) were F_1 progeny from a self-pollinated plant heterozygous for *DME/dme-1* and heterozygous for the *dme* suppressor (line 1424 described in Experimental Procedures).

(A) Wild-type silique.

(B) Heterozygous *DME/dme-1* silique.

(C) Silique is heterozygous for *DME/dme-1* and heterozygous for the *dme* suppressor mutation.

(D) Silique is heterozygous for *DME/dme-1* and homozygous for the *dme* suppressor mutation.

(E) Silique is homozygous for the *dme* suppressor mutation.

gametophyte and seed (Chaudhury et al., 1997; Kiyosue et al., 1999; Kohler et al., 2003). Because *MEA* is an essential imprinted gene, loss-of-function alleles have parent-of-origin effects on seed viability. A seed that inherits a mutant maternal *mea* allele aborts regardless of the genotype of the silent paternal allele (Chaudhury et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999).

The *DEMETER* (*DME*) gene is necessary for maternal *MEA* allele expression in the *Arabidopsis* central cell and endosperm (Choi et al., 2002). As a result, seed viability depends only on the maternal *DME* allele, and seed abortion results from maternal inheritance of a mutant *dme* allele regardless of the genotype of the paternal *DME* allele. *DME* is primarily expressed in the central cell of the female gametophyte where it is required to activate expression of the maternal *MEA* allele. *MEA* expression persists after the central cell is fertilized to form the endosperm, even though *DME* does not. Ectopic *DME* expression in cauline leaves and in endosperm activates *MEA* and paternal *MEA* allele expression, respectively, suggesting that differential expression of DME in maternal (expressed) and paternal (not expressed) reproductive organs is responsible, at least in part, for imprinting *MEA* in the endosperm.

DME encodes a large protein with DNA glycosylase and nuclear localization domains (Choi et al., 2002). Most DNA glycosylases function in a base excision DNA repair pathway that excises damaged, modified, or mispaired bases, nicks the DNA, and replaces the abasic sites with normal bases (Bruner et al., 2000; Jiricny, 2002). Ectopic expression of *DME* in cauline leaves causes single-stranded breaks in the *MEA* promoter, consistent with its DNA glycosylase function and with the view that DME acts directly on *MEA* (Choi et al., 2002). Mutagenesis of a conserved aspartic acid to asparagine in the putative DME glycosylase catalytic site abolishes the ability of the mutated *DME* transgene to complement a *dme* mutation (Y.C. and R.L.F., unpublished results). This further supports the idea that DME is a DNA glycosylase. The mechanism used by DME to regulate the transcription of the maternal *MEA* allele transcription is unknown.

We isolated four mutations that suppress *dme*-mediated seed abortion to understand how *MEA* gene imprinting is regulated. Map-based cloning revealed that all four mutations represented distinct lesions in the *MET1* gene (*met1-5* to *met1-8*). *MET1*, an *Arabidopsis* ortholog of the mammalian Dnmt1 methyltransferase gene, maintains cytosine methylation at CpG sites (Finnegan and Dennis, 1993; Kishimoto et al., 2001; Lindroth et al., 2001) and indirectly influences methylation at CpNpG and CpNpN sites (Cao and Jacobsen, 2002a). Inheritance of a maternal *met1* mutant allele by a female gametophyte was sufficient for complete suppression of *dme*-mediated seed abortion, whereas inheritance of a paternal *met1* mutant allele had little or no effect. Suppression of *dme* by *met1* mutations requires a maternal wild-type *MEA* allele, suggesting that *met1* mutations act upstream of *MEA* to rescue *dme* seed viability. Maternal *MEA::GFP* allele transcription in the central cell and endosperm, prevented by a maternal *dme* mutant allele, is fully restored when maternal *dme* and *met1* mutant alleles are inherited together. Bisulfite sequencing experiments revealed three regions of cytosine methylation in the *MEA* promoter that are hypomethylated in *met1* mutant seeds. These results suggest that DNA methylation plays an important role in the control of *MEA* imprinting and seed viability, and that these processes are controlled by antagonism between MET1 and DME enzymes in the female gametophyte.

Results

Identification of Mutations that Suppress *dme*-Mediated Seed Abortion

We mutagenized *DME/dme* heterozygous seed and identified four mutant lines that suppressed *dme*-mediated seed abortion (see Experimental Procedures). Whereas seeds from wild-type plants rarely abort (Figure 1A), self-pollinated heterozygous *DME/dme-1* siliques (Figure 1B) have a 1:1 segregation ratio of viable and nonviable seeds (272:250, $\chi^2 = 0.9$, $P > 0.4$) because inheritance of a maternal mutant *dme* allele is sufficient to cause seed abortion (Choi et al., 2002). By contrast, plants heterozygous for *DME/dme-1* and heterozygous

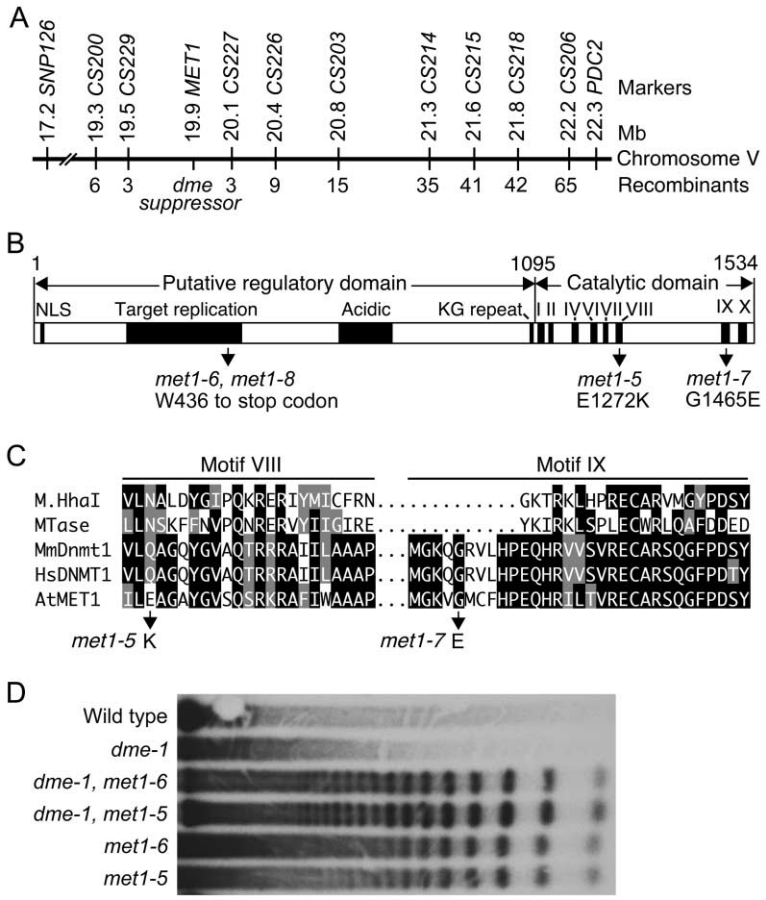

Figure 2. *dme* Suppressor Mutations Reside in the *MET1* Gene

(A) Map-based cloning of a *dme* suppressor mutant allele. The position of the *MET1* gene relative to SSLP molecular markers, and the number of recombinants between the *dme* suppressor (*met1-5*) and molecular markers, are shown.

(B) Position of *met1* alleles relative to conserved domains in the MET1 protein. The MET1 amino-terminal regulatory domain includes a nuclear localization signal (NLS), a sequence for targeting MET1 enzyme to DNA replication foci, a plant-specific acidic region of glutamic and aspartic acid residues, and lysine/glycine repeats by which the regulatory domain is fused to the catalytic domain. The catalytic domain has eight of ten conserved motifs found in prokaryotic DNA methyltransferases (Posfai et al., 1989). The codon (UGG) for tryptophan at position 436 was mutated to a stop codon in the *met1-6* (UAG) and *met1-8* (UGA) mutant alleles. *met1-5* and *met1-7* missense mutations altered the amino acid sequence in conserved motifs VIII and IX, respectively.

(C) Comparison of motif VIII and motif IX domains among DNA methyltransferases. The positions of the *met1-5* and *met1-7* mutations in the conserved motifs are shown. M. HhaI, *Haemophilus haemolyticus* methylase (GenBank accession number P05102); MTase, *Bacillus subtilis* phages ϕ3T DNA methyltransferase (accession number CTBPPT); MmDnmt1, *Mus musculus* DNA methyltransferase 1 (accession number P13864); HsDNMT1, *Homo sapiens* DNA methyltransferase 1 (accession number NP_001370); AtMET1, *Arabidopsis thaliana* DNA methyltransferase 1 (accession numbers AT5G49160 and AF139372).

(D) The *met1-5* and *met1-6* mutations result in genome hypomethylation. DNA was isolated from seedlings and digested with HpaII, blotted, and hybridized to a probe complementary to the 180 base pair centromere repeats (Kankel et al., 2003). Seedlings were homozygous for the indicated mutant alleles.

for its suppressor have a 3:1 segregation ratio of viable and nonviable seeds (Figure 1C; 184:63, $\chi^2 = 0.03$, $P > 0.85$). Seed abortion was completely suppressed in plants heterozygous for *DME/dme-1* and homozygous for its suppressor mutation (Figure 1D; 2 aborted seeds among 231checked), as well as in control plants homozygous for the wild-type *DME* allele and the *dme* suppressor (Figure 1E; 1 aborted seed among 135 checked). Mapping experiments showed that the *dme* suppressor mutations are near the bottom of chromosome 5 (Figure 2A) and are therefore genetically unlinked to *DME*, which is located near the top of chromosome 5. These results show that second-site suppressor mutations compensated for loss-of-function mutations in the maternal *dme* allele and restored seed viability.

dme Suppressor Mutations Are Loss-of-Function *met1* Alleles

High-resolution gene mapping experiments showed that a *dme* suppressor mutation resides in a 0.6 Mb region spanning the *MET1* gene (Figure 2A). MET1 is an *Arabidopsis* ortholog of mammalian Dnmt1 methyltransferase, which maintains methylation at CpG sites (Finnegan and Kovac, 2000). Certain phenotypes associated with homozygous *dme* suppressor plants (e.g., late flowering and abnormal patterning of floral organs) were similar to those observed in transgenic plants bearing an antisense *MET1* gene (Finnegan et al., 1996; Ronemus et al., 1996), suggesting that *dme* suppressor mutations might reside in the *MET1* gene. We determined the sequence of the *MET1* gene in all four mutant lines and found that each line harbored a lesion in the *MET1* gene (Figure 2B). These new *met1* alleles are distinct from those previously reported (Kankel et al., 2003; Saze et al., 2003), and are designated *met1-5* to *met1-8*.

In eukaryotes, MET1 and MET1-related proteins have an amino-terminal putative regulatory domain and a carboxy-terminal catalytic domain (Finnegan and Kovac, 2000; Posfai et al., 1989). The *met1-6* and *met1-8* alleles represent base pair changes that generate a stop codon at amino acid 436 in the MET1 polypeptide (Figure 2B). These alleles are likely to be null alleles, as the truncated polypeptide encoded by the *met1-6* and *met1-8* alleles lacks a large portion of the putative regulatory domain as well as the entire catalytic domain. The *met1-5* and *met1-7* alleles have base pair changes that alter single amino acids residing in catalytic domain motifs that are conserved in prokaryotic and eukaryotic cytosine 5-methyltransferases (Figures 2B and 2C). It is likely that these amino acids are critical for MET1 enzyme activity,

as *met1-5* and *met1-7* suppress *dme*-mediated seed abortion to the same extent as null alleles *met1-6* and *met1-8* (data not shown).

Plants homozygous for mutations in the *MET1* gene display DNA hypomethylation (Kankel et al., 2003; Saze et al., 2003). As shown in Figure 2D, the 180 base pair repeated centromere DNA from wild-type and homozygous *dme-1* mutant plants is highly methylated and cannot be cleaved by the methylation-sensitive restriction endonuclease HpaII. By contrast, these centromeric repeats are hypomethylated in the genome of homozygous *met1-5* or *met1-6* plants, as well as in plants homozygous for both *dme-1* and *met1* alleles (Figure 2D). Thus, full suppression of *dme*-mediated seed abortion is associated with missense and nonsense mutations that cause DNA hypomethylation.

Distinct Developmental Abnormalities in Plants with Mutant *dme* and *met1* Alleles

Both *DME* and *MET1* are required for stable, reproducible patterns of floral and vegetative development (Choi et al., 2002; Finnegan and Kovac, 2000). Homozygous *dme-1* or *met1* plants, as well as antisense *MET1* transgenic plants, display sporadic developmental abnormalities (Choi et al., 2002; Finnegan et al., 1996; Kakutani et al., 1996; Kankel et al., 2003; Ronemus et al., 1996). Plants homozygous for both *dme-1* and *met1-6* mutant alleles have distinctive sporadic phenotypes. For example, homozygous *dme-1 met1-6* mutant siliques were sometimes distended (Figure 3A), and ovules appeared to be converted to leaf-like organs (Figure 3B) or carpel-like organs tipped with stigmatic papillae and connected by a funiculus to the placenta (Figure 3C). Sometimes a single flower (Figure 3D) or influorescence shoot (Figure 3E) emerged from a homozygous *dme-1 met1-6* silique. In the extreme cases, the pattern of producing flowers in siliques was reiterated multiple times (Figure 3F). These sporadic phenotypes increased in frequency with each generation, were detected in about 15% of the F_3 homozygous *dme-1 met1-6* plants, and were not observed in control homozygous F_3 *dme-1* or F_3 *met1-6* plants. Analysis of subsequent generations was not possible because F_3 homozygous *dme-1 met1-6* plants are sterile. These distinct mutant phenotypes suggest a genetic interaction between DME and MET1 is necessary to generate stable, reproducible patterns of floral and vegetative development.

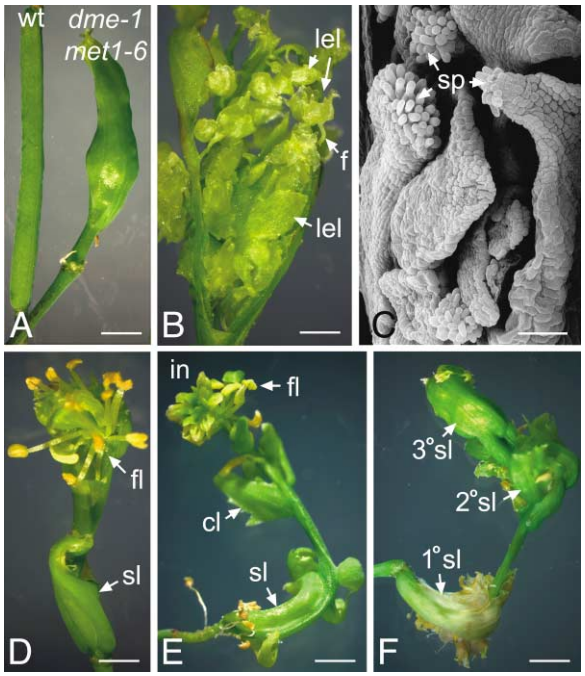

Figure 3. Developmental Abnormalities in Homozygous *dme-1 met1-6* Plants

Heterozygous *DME/dme-1 MET1/met1-6* plants were self-pollinated, F_1 homozygous *dme-1 met1-6* plants were self-pollinated, and phenotypes of F_2 homozygous *dme-1 met1-6* plants were analyzed.

(A) Siliques from wild-type and homozygous *dme-1 met1-6* plants. wt, wild-type. The scale bar represents 2 mm.

(B) Dissected homozygous *dme-1 met1-6* silique showing ovule converted to leaf-like (lel) structures connected by a funiculus (f) to the placenta. The scale bar represents 0.5 mm.

(C) Scanning electron micrograph of homozygous *dme-1 met1-6* ovules converted to leaf-like structures with stigmatic papilla (sp). The scale bar represents 50 μm.

(D) Flower (fl) emerging from a mature homozygous *dme-1 met1-6* silique (sl). The scale bar represents 2 mm.

(E) Influorescence shoot (in) with cauline leaves (cl) and flowers (fl) emerging from a mature *dme-1 met1-6* silique (sl). The scale bar represents 2 mm.

(F) Generation of primary (1°sl), secondary (2°sl), and tertiary (3°sl) siliques in a *dme-1 met1-6* homozygous plant. The scale bar represents 2 mm.

Maternal *met1* Allele Suppresses *dme*-Mediated Seed Abortion

Inheritance of a maternal *DME* allele is vital for seed viability, while a paternal *DME* allele is dispensable (Choi et al., 2002). As a result, *DME/dme-1* heterozygous plants pollinated with wild-type pollen produce siliques with a 1:1 segregation ratio of viable and aborted seeds (Choi et al., 2002) and essentially all of the viable F_1 progeny inherit a maternal wild-type *DME* allele (Table 1). By contrast, *DME/dme-1 MET1/met1-6* plants pollinated with wild-type pollen generate siliques with a 3:1 ratio of viable to aborted seeds (140:44, $\chi^2 = 0.1$, $P > 0.8$). All viable F_1 progeny that inherited a maternal mutant *dme-1* allele also inherited a maternal mutant *met1-6* allele (Table 1), indicating that *dme-1 met1-6* female gametophytes pollinated with wild-type pollen produce viable seed. We observed a 1:1:1 segregation ratio of viable progeny with *dme-1 met1-6*, *DME met1-6*, and *DME MET1* maternal alleles (Table 1; $\chi^2 = 4.8$, $P > 0.1$), showing that pollinated *dme-1 met1-6* and *DME met1-6* female gametophytes produced equal numbers of viable seeds. Thus, *met1-6* is a fully penetrant suppressor of *dme* in the female gametophyte.

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), also causes genome hypomethylation. When *DME/dme-1 DDM1/ddm1-2* plants were self-pollinated, we observed siliques with a 1:1 ratio of viable to aborted seeds (742:716, $\chi^2 = 0.46$, $P = 0.5$), suggesting that the *ddm1-2* mutation did not suppress *dme*-mediated seed abortion. These results show that the genetic

Table 1. Effect of a Maternal *met1* Allele on Transmission of Maternal *dme* and *mea* Mutant Alleles

Genetic Cross		Maternal Alleles of Viable F_1 Seedlings				
Maternal Parent	Paternal Parent	N[a]	%	Genotype		
DME/dme-1	Wild-type	94	1	*dme-1*		
			99	*DME*		
DME/dme-1, *MET1/met1-6*	Wild-type	86	25	*dme-1*	*met1-6*	
			0	*dme-1*	*MET1*	
			30	*DME*	*met1-6*	
			45	*DME*	*MET1*	
MEA/mea-3	Wild-type	89	6	*mea-3*		
			94	*MEA*		
MEA/mea-3, MET1/met1-6	Wild-type	64	2		*met1-6*	*mea-3*
			0		*MET1*	*mea-3*
			45		*met1-6*	*MEA*
			53		*MET1*	*MEA*
DME/dme-1, MEA/mea-3, MET1/met1-6	Wild-type	68	0	*dme-1*	*met1-6*	*mea-3*
			0	*dme-1*	*MET1*	*mea-3*
			0	*DME*	*met1-6*	*mea-3*
			0	*DME*	*MET1*	*mea-3*
			24	*dme-1*	*met1-6*	*MEA*
			0	*dme-1*	*MET1*	*MEA*
			41	*DME*	*met1-6*	*MEA*
			35	*DME*	*MET1*	*MEA*

[a] Number of F_1 seedlings checked.

interaction between the *met1* and *dme* mutations is a specific one.

Suppression of *dme* by *met1-6* Requires a Maternal Wild-Type *MEA* Allele

DME functions upstream of *MEA* to control seed viability (Choi et al., 2002). Like *DME*, inheritance of a maternal *MEA* allele is needed for seed viability while a paternal *MEA* allele is dispensable (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999). As a result, *MEA/mea-3* heterozygous plants pollinated with wild-type pollen produce siliques with a 1:1 segregation ratio of viable and aborted seed (88:84, $\chi^2 = 0.1$, $P > 0.8$), and most viable F_1 progeny inherit a maternal wild-type *MEA* allele (Table 1).

Do *met1* mutations function upstream of, at the level of, or downstream of *MEA* to suppress *dme*-mediated seed abortion? We addressed this question genetically by determining whether *met1* mutations suppress *mea*-mediated seed abortion. If downstream, a *met1* mutation would be expected to suppress *mea*-mediated seed abortion, whereas no suppression would be expected if *met1* functions at the level of *MEA* or upstream of *MEA*. To distinguish between these alternatives, *MEA/mea-3 MET1/met1-6* heterozygous plants were pollinated with wild-type pollen and the percentage of seed abortion and genotypes of F_1 progeny were analyzed. Siliques had a 1:1 segregation ratio of viable and aborted seeds (107:84, $\chi^2 = 2.7$, $P > 0.1$) and essentially all of the viable F_1 progeny inherited a maternal wild-type *MEA* allele (Table 1). Thus, a maternal *met1-6* allele does not suppress *mea*-mediated seed abortion, suggesting *MET1* functions either at the level of *MEA* or upstream of *MEA*.

If *MET1* functions upstream of, or at, *MEA*, then a wild-type *MEA* allele should be necessary for *met1* suppression of *dme*-mediated seed abortion. To test this hypothesis, *DME/dme-1 MET1/met1-6 MEA/mea-3* heterozygous plants were pollinated with wild-type pollen and the genotypes of viable F_1 progeny were determined. All viable F_1 progeny that inherited a maternal mutant *dme-1* allele also inherited a mutant *met1-6* allele and a wild-type *MEA* allele (Table 1). Thus, a wild-type maternal *MEA* allele is required for suppression of *dme* by *met1-6* in the female gametophyte. These results indicate that *met1* functions upstream of, or at, *MEA* in the female gametophyte to rescue the seed abortion caused by a maternal mutant *dme* allele.

DME and *MET1* Antagonism Regulates *MEA* Gene Expression

DME is necessary for *MEA* RNA accumulation (Choi et al., 2002) and *MEA* RNA, present in wild-type flowers, is not detected in homozygous *dme-1* flowers (Figure 4). Suppression of *dme* by *met1* mutations might be due, at least in part, to restoration of *MEA* gene expression. To test this idea, we isolated RNA from homozygous mutant *dme-1 met1-6* flowers and measured the

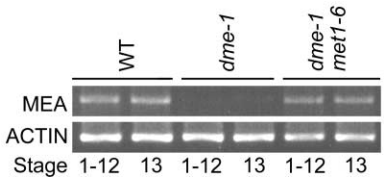

Figure 4. *MET1* and *DME* Genes Antagonistically Regulate *MEA* RNA Accumulation

RNA was isolated from developing floral buds (stage 1–12) and open flowers (stage 13). The approximate level of *MEA* RNA was determined by semiquantitative RT-PCR as described (Choi et al., 2002). Floral stages are as described (Bowman, 1994). Plants were homozygous for the indicated mutant alleles. WT, wild-type.

level of *MEA* RNA using reverse transcriptase polymerase chain reaction (RT-PCR) procedures. We found that the level of *MEA* RNA in *dme-1 met1-6* flowers was similar to that in wild-type (Figure 4). This result shows that MET1 is necessary for suppression of *MEA* expression in a *dme* mutant genetic background.

To understand the spatial and temporal control of *MEA* gene expression by *MET1* and *DME* during ovule and seed development, we analyzed the effect of *met1* and *dme-1* mutations on transcription of a *MEA::GFP* transgene consisting of 4.2 kb of *MEA* 5′-flanking sequences ligated to the *GFP* reporter gene (Choi et al., 2002). Essentially all (>99%) prefertilization ovules from control transgenic plants homozygous for the *MEA::GFP* transgene displayed strong fluorescence in the central cell nucleus and cytoplasm prior to fertilization. In a plant homozygous for the *MEA::GFP* transgene and heterozygous for *DME /dme-1*, we detected a 1:1 segregation ratio of fluorescent and nonfluorescent ovules (164:149, $\chi^2 = 0.7$, $P > 0.4$), suggesting that female gametophytes inheriting the *dme-1* mutant allele did not express the *MEA::GFP* transgene (Figure 5A). By contrast, in a plant homozygous for the *MEA::GFP* transgene, *DME/dme-1*, and *MET1/met1-6*, a 3:1 segregation ratio of fluorescent and nonfluorescent ovules (253:99, $\chi^2 = 1.8$, $P > 0.25$) was observed, suggesting that female gametophytes inheriting *dme* and *met1* mutant alleles expressed the *MEA::GFP* transgene (Figure 5B). To verify this hypothesis, we examined ovules from plants homozygous for the *MEA::GFP* transgene, *dme-1*, and *met1-6*. We found that essentially all ovules contained fluorescent central cells (Figure 5C; 241 fluorescent among 245 checked). These experiments reveal that *MET1* represses *MEA* promoter activity in a *dme* mutant central cell.

Activation of the *MEA::GFP* transgene by DME in the central cell is sufficient for postfertilization transcription of the *MEA::GFP* transgene in the endosperm, when DME is no longer expressed (Choi et al., 2002). As a result, we observed a 1:1 segregation of fluorescent and nonfluorescent seeds 24 hr (Figure 5D; 92:105, $\chi^2 = 0.9$, $P > 0.4$) and 90 hr (Figure 5G; 165:151, $\chi^2 = 0.6$, $P > 0.5$) after plants homozygous for the *MEA::GFP* transgene and heterozygous for *DME/dme-1* were pollinated with wild-type pollen. To determine whether activation of a *MEA::GFP* transgene in a *dme met* mutant female gametophyte likewise persists after fertilization, we pollinated flowers homozygous for the *MEA::GFP* transgene, and heterozygous for *DME/dme-1, MET1/met1-6* with wild-type pollen and observed GFP fluorescence in the endosperm of F_1 seeds. We observed a 3:1 segregation of fluorescent and nonfluorescent seeds 24 hr (Figure 5E; 253:99, $\chi^2 = 1.8$, $P > 0.25$) and 90 hr (Figure 5H; 304:123, $\chi^2 = 3.3$, $P > 0.08$) after pollination, showing that *MEA::GFP* transcription persists in the endosperm after fertilization of *dme met1* female gametophytes. Thus, prefertilization activation of *MEA* promoter activity in a *dme met1* central cell is not suppressed postfertilization by a wild-type paternal *MET1* allele. Consistent with this conclusion, essentially all F_1 seeds fluoresced 24 hr (Figure 5F; 157 fluorescent among 161 checked) and 90 hr (Figure 5I; 207 fluorescent among 209 checked) after pollination of flowers homozygous for the *MEA::GFP* transgene, *dme-1*, and *met1-6*. These results show that two DNA-modifying enzymes, DME glycosylase and MET1 methyltransferase, antagonistically regulate *MEA* expression in the central cell and endosperm.

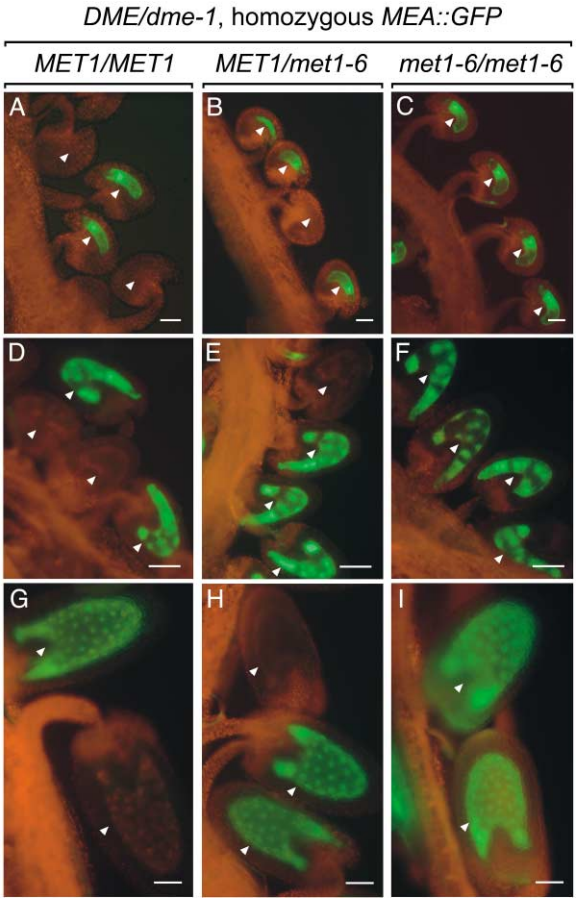

Figure 5. *MET1* and *DME* Genes Antagonistically Regulate *MEA* Promoter Activity

The GFP and chlorophyll fluorescence was converted to green and red, respectively.

(A–C) Fluorescence micrographs of stage 12 ovules. Arrows point to central cells. The genotype of the plant is shown above the fluorescence micrographs. The scale bars represent 0.04 mm.

(D–F) Fluorescence micrographs of seeds photographed 24 hr after a cross with wild-type pollen. The genotype of the pistil donor is shown above the fluorescence micrographs. The scale bars represent 0.16 mm.

(G–I) Fluorescence micrographs of seeds photographed 90 hr after a cross with wild-type pollen. The genotype of the pistil donor is shown above the fluorescence micrograph. The scale bars represent 0.32 mm.

MET1 Is Necessary for Cytosine Methylation in the *MEA* Promoter

What is the mechanism by which MET1 suppresses *MEA* gene transcription in a *dme* mutant central cell? We previously did not detect 5-methylcytosine residues in 2 kb of *MEA* 5′-flanking sequences from L*er* (Landsberg *erecta* ecotype) seeds or leaves, suggesting that DNA methylation does not play a direct role in the regulation of maternal *MEA* allele gene expression (Choi et al., 2002). However, the involvement of MET1 in the control of maternal *MEA* allele expression (Figure 4) prompted

us to examine the entire 4.2 kb *MEA* promoter that regulates expression of the *MEA::GFP* transgene (Figure 5), and to compare the patterns of methylation in wild-type and *met1* genetic backgrounds.

Because DNA methylation is often associated with genes that are not expressed, we initially analyzed DNA isolated from stamens (Columbia *glabrous* [Col *gl*] ecotype), an organ where *MEA* expression is not detected (data not shown). Using bisulfite sequencing methods (see Experimental Procedures), we identified three regions with significant cytosine methylation at −0.5 kb (−585 to −521), −3 kb (−3099 to −3071), and −4 kb (−4235 to −3800) relative to the translation start site of *MEA*. In wild-type seeds (Col *gl* ecotype), DNA sequence analysis of approximately 20 top strand and 20 bottom strand clones revealed clusters of eight, four, and five methylated CpG sites in the −4 kb, −3 kb, and −0.5 kb regions, respectively (Figures 6A and 6C). In addition, the −4 kb region contained six CpNpG and 28 CpNpN methylated sites on the top strand, and four CpNpG and 46 CpNpN methylated sites on the bottom strand (Figure 6B; Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/5/6/891/DC1). Similar results were obtained when wild-type seeds were isolated from L*er* ecotype plants, except that the level of CpG methylation at −0.5 kb was reduced to approximately 10%, which may explain why it was not detected previously (Choi et al., 2002). These results show that the 4.2 kb *MEA* promoter contains three regions with cytosine methylation.

To determine whether MET1 is responsible for maintaining cytosine methylation in the *MEA* promoter, we measured the level of cytosine methylation in *met1-6* mutant seeds (Col *gl* ecotype). We found that CpG methylation in the −4 kb, −3 kb, and −0.5 kb regions was dramatically reduced to 8%, 1%, and 0.7% in homozygous *met1-6* seeds compared with 81%, 21%, and 61% in wild-type Col *gl*, respectively (Figure 6A). Methylation at CpNpG and asymmetric CpNpN sites was also substantially reduced in *met1-6* mutant seeds (Figure 6B). A similar reduction in cytosine methylation was observed in plants homozygous for the *met1-5* allele (data not shown). Thus, *MET1* is necessary to maintain cytosine methylation at the three distinct sites in the *MEA* promoter.

Discussion

We isolated mutations that suppress *dme*-mediated seed abortion to understand how *MEA* imprinting is regulated. All mutations resided in the *MET1* methyltransferase gene that maintains cytosine methylation. Suppression requires a maternal wild-type *MEA* allele, suggesting that *MET1* functions upstream of, or at, *MEA*. DME activates whereas MET1 suppresses *MEA* gene expression. Three regions in the *MEA* promoter are hypomethylated in *met1* mutant seeds. Our analyses suggest a mechanism for the regulation of imprinted genes that are maternally expressed and paternally silenced in the endosperm. In the central cell of the female gametophyte, the MET1 methyltransferase represses *MEA* gene transcription, but expression of *DME* DNA glycosylase specifically in the central cell overcomes *MET1*-mediated silencing and activates the maternal *MEA* allele expression that persists during endosperm development.

Control of *MEA* Imprinting and Seed Viability in the Female Gametophyte by Antagonists DME and MET1

Like DME DNA glycosylase, MET1 methyltransferase functions in the female gametophyte. This conclusion is based upon data showing that inheritance of maternal mutant *met1* allele by the female gametophyte is sufficient to rescue maternal *MEA* expression in the central cell and endosperm of *dme* mutant plants (Figure 5) and to restore seed viability (Figure 1; Table 1). In the genetic crosses shown in Figure 5 and Table 1, the paternal parents were wild-type, and the maternal heterozygous *met1* parents were derived from mutagenized plants that were never homozygous for *met1* mutant alleles. Because rescue does not require that either parent be homozygous for a mutant *met1*allele, these data strongly suggest that MET1 methyltransferase, like DME DNA glycosylase (Choi et al., 2002), functions in the female gametophyte to control *MEA* imprinting and seed viability. This hypothesis is consistent with *MET1* being necessary for epigenetic inheritance during plant gametogenesis (Saze et al., 2003) and suggests that genes in the central cell, as well as in the egg, are epigenetically modified by *MET1*.

In the maternal parent, MET1 methyltransferase functions at, or upstream of, *MEA* and controls imprinting and seed viability. This is based upon our demonstration that rescue of *dme*-mediated seed abortion by the maternal *met1* allele requires a wild-type maternal *MEA* allele (Table 1). MET1 methyltransferase may suppress maternal *MEA* allele expression by directly methylating the *MEA* promoter. This idea is supported by the fact that MET1 methyltransferase is responsible for maintaining cytosine methylation in three regions of the *MEA* promoter (Figure 6). Alternatively, it is also possible that MET1 methylates, and thereby suppresses, an unknown gene that in turn activates maternal *MEA* expression. In either case, we propose that passive postmeiotic demethylation associated with mitoses during *met1* mutant female gametophyte development allows the maternal *MEA* allele to be expressed in the absence of DME DNA glycosylase activity (Figure 5).

After fertilization, MET1 may be relatively unimportant for control of the expression of the maternal *MEA* allele. This is because the postfertilization expression of *MEA* is stably maintained (Figure 5), even though the MET1 methyltransferase is expressed (M.G. and R.L.F., unpublished results) and its antagonist, DME DNA glycosylase, is not expressed at that time. Thus, wild-type *MET1* alleles cannot reestablish silencing of the maternal *MEA* allele in the endosperm (Figure 5), suggesting that epigenetic modification of the maternal *MEA* allele by DME DNA glycosylase cannot be reversed by MET1 methyltransferase in the endosperm.

Models for the Antagonistic Interaction between MET1 and DME

The discovery of an antagonistic relationship between MET1 and DME has provided important information about DME function. In the absence of DME DNA glycosylase activity in a *dme* mutant female gametophyte,

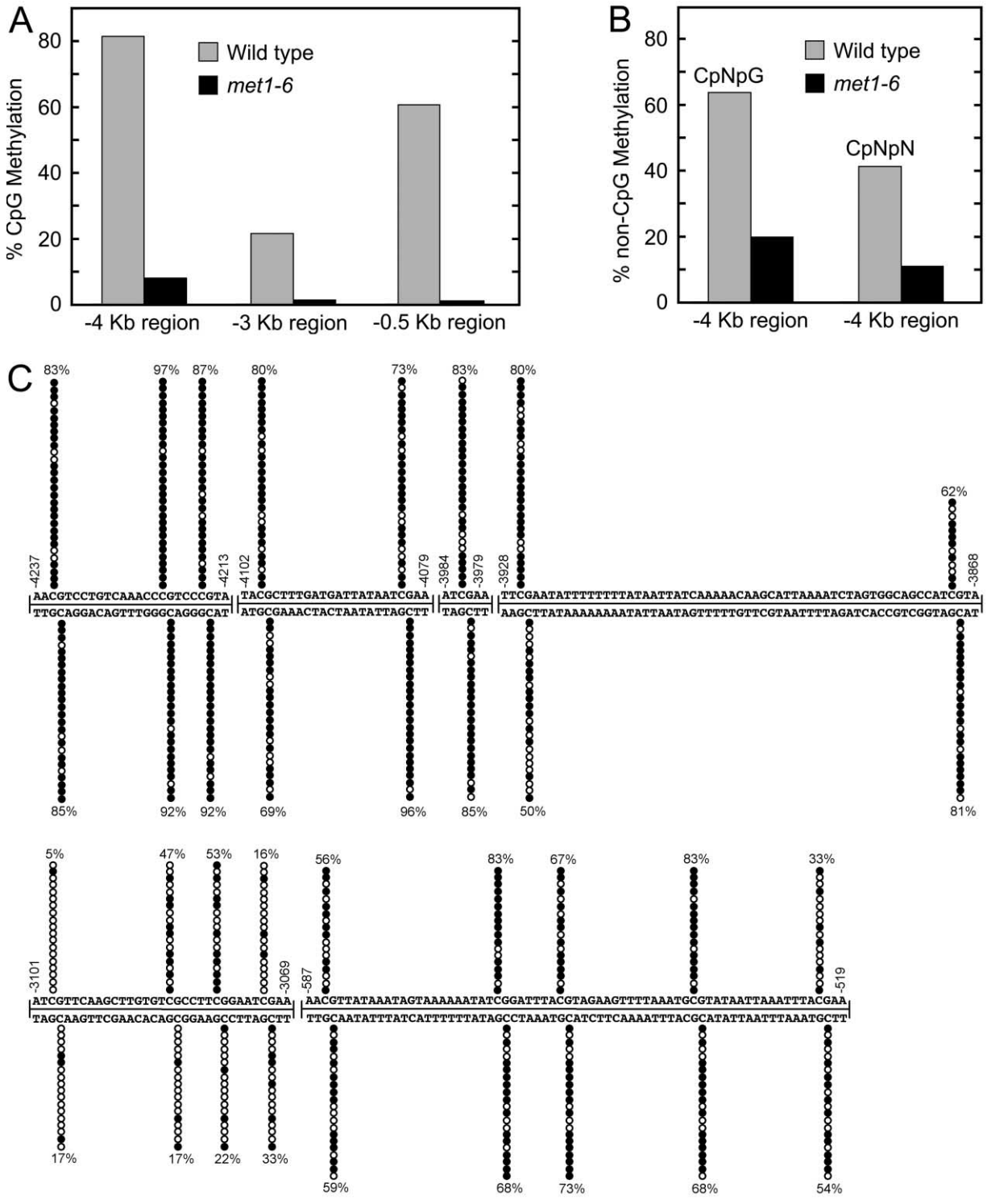

Figure 6. Pattern of DNA Methylation in the *MEA* Promoter

Percentage of CpG (A) and non-CpG (B) methylation in three regions of the *MEA* promoter isolated from wild-type and *met1-6* mutant seeds. (C) shows converted and nonconverted CpG sites in the sequenced clones in the three regions. Methylated and unmethylated cytosines are indicated by black and white circles, respectively. Number of sequences is relative to the translation start site of *MEA*.

MET1 methyltransferase maintains suppression of the maternal *MEA* allele (Figure 5) by maintaining patterns of DNA methylation. Thus, in wild-type plants, an essential function of DME is to overcome MET1 methyltransferase activity in the central cell of the female gametophyte.

How does DME overcome MET1-mediated DNA methylation and activate maternal *MEA* allele transcription in the central cell? One model is that DME DNA glycosylase excises 5-methylcytosine. Completion of the base excision DNA repair process would result in insertion of

a cytosine into the abasic site created by excision of 5-methylcytosine by DME. In support of this model, other related mammalian DNA glycosylases have been shown to excise 5-methylcytosine from the genome (Jost et al., 2001). Moreover, *ROS1*, the gene most closely related to *DME* in the *Arabidopsis* genome, represses DNA methylation-mediated transgene silencing in vivo and functions to excise 5-methylcytosine in vitro (Gong et al., 2002). Alternatively, DME may use a more indirect mechanism to overcome MET1-mediated silencing of the maternal *MEA* allele. For example, DNA nicking associated with the base excision DNA repair process may facilitate nucleosome sliding and alter chromatin structure, allowing access of transcription factors to activate *MEA* gene transcription or preventing maintenance of *MEA* promoter methylation by MET1. This possibility is consistent with the broad pattern of nicks in the *MEA* promoter induced in vivo by DME (Choi et al., 2002).

DME acts as an antagonist to MET1 in the central cell to control endosperm imprinting and seed viability. Because chromosomes inherited by the endosperm are not transmitted to progeny, DME- and MET1-based epigenetic modification of maternal alleles in the central cell need not be reset at the next generation. Thus, the imprinting mechanism in plants regulated by two DNA-modifying enzymes, MET1 methyltransferase and DME glycosylase, is fundamentally different from that in mammals, where epigenetic modification including CpG methylation of imprinted genes is reset at every generation.

Experimental Procedures

Isolation of Mutations that Suppress *dme*

Mutant *dme* alleles are not transmitted maternally (Choi et al., 2002), and in the Col *gl* ecotype are transmitted paternally at a reduced level. Thus, 15% of the progeny from self-pollinated *DME/dme* plants inherit the mutant *dme* allele, instead of the expected 75%. To isolate *dme* suppressors we selected lines with increased transmission of the *dme* mutant allele. Measuring transmission rate was facilitated by the fact that *dme-1* and *dme-2* mutant alleles (Choi et al., 2002) are due to insertion of a pSKI015 T-DNA (Weigel et al., 2000) with a BAR gene, which confers resistance to glufosinate ammonium herbicide (Basta; Crescent Chemical Co.). M_1 seeds from *DME/dme-1* or *DME/dme-2* self-pollinated plants were treated with ethylmethanesulfonate (EMS; Ohad et al., 1996). Approximately 8,000 M_1 plants were grown and M_2 seeds from four consecutive siliques were separately harvested, germinated, and the number of 7-day-old seedlings counted. Seedlings were sprayed with Basta and 4 days later the number of Basta-resistant seedlings was counted. When the percentage of Basta-resistant M_2 seedlings significantly exceeded 15%, the percentage of M_3 seed abortion in self-pollinated M_2 siliques was determined. Four putative *dme* suppressor lines (212, 1424, 6683, and 7598) were identified with a 3:1 segregation ratio of viable and aborted seeds. Lines were crossed to wild-type (Col *gl*) six times to remove additional mutations.

Cloning of *dme* Suppressors

Line 212 was crossed to L*er*, F_1 plants were self-pollinated, and the percentage of seed abortion was determined in 50 F_2 plants. DNA from F_2 plants was isolated and the position of mutation 212 was mapped relative to molecular markers *SNP126* (17.2 Mb) and *PDC2* (22.3 Mb). This procedure was used to map lines 1424, 6683, and 7598. A population of approximately 600 F2 plants was used to map 212 between markers *CS229* (19.5 Mb) and *CS227* (20.1 Mb), a region that spans the *METI* gene (19.9 Mb). By DNA sequencing, we identified a lesion in the *METI* gene from homozygous 212 (*met1-5*), 1424 (*met1-6*), 6683 (*met1-7*), and 7598 (*met1-8*) plants. Primers for molecular markers are *CS200* 5′-TGACAAACCATTTTATTTCATCG-3′ and 5′-TGAGAGAAATCGCAGCCC-3′; *CS229* 5′-TTCTAGAGAAAAGTGGCTCACG-3′ and 5′-TTGTAATCTGAATTAGCATATCATG-3′; *CS227* 5′-AAAAAGACTTTTTCGACAAATCA-3′ and 5′-GTGGCAGCCGCTGTAAAT-3′; *CS226* 5′-AGGGTAGCTTCGGTTCGG-3′ and 5′-ATGCATGGGAATTGTGGG-3′, *CS203* 5′-CTGTCAAGTGTCAACAATCACC-3′ and 5′-AGAATCTCAAACCCGTTATTCG-3′; *CS214* 5′-CCTGCAAGTAAGGCCCAA-3′ and 5′-TCGCCATTGCAACTTTCA-3′; *CS215* 5′-TTGTTGCTCTTCAAATTTCTCG-3′ and 5′-GAGAGTGAAATCTCTCTTGAAACG-3′; *CS218* 5′-TTTGGCATCATCGCTCAA-3′ and 5′-ACCCTTTCGAAATTCCGC-3′; *CS206* 5′-TGCCATCGCAAAAACTT-3′ and 5′-TCTCAATACCCTCCCAATCG-3′.

Plant Materials

To prevent accumulation of epigenetic abnormalities, homozygous *dme-1* and *met1-6* plants were generated from self-pollinated heterozygous *DME/dme-1 MET1/met1-6* plants. To determine plant genotype, DNA was isolated, PCR amplified, and when necessary digested with restriction endonucleases. The *dme-1* allele was detected by amplifying the BAR gene (*BAR-F* 5′-ATCTACCATGAGCCCAGAAC-3′ and *BAR-R* 5′-GTCATCAGATCTCGGTGACG-3′). The *DME* allele was detected by PCR amplification across the T-DNA insertion site (*SKB-6* 5′-CACTGAGATTAATTCTTCAGACTCGTG-3′ and *SKES2.5* 5′-TTCAGACTCAGAGTCACCTTGC-3′). The *MET1* and *met1-6* alleles were distinguished by amplification with dCAPs (Neff et al., 1998) primers (*1424dBglII* 5′-TGTGACTGAGAACCGCTGTCAGGATCGTTTAAAGAGATC-3′ and *1424F* 5′-CGTACTATAAGACCTCCGAAG-3′) followed by digestion with BglII. *MEA* and *mea-3* alleles were distinguished as described (Yadegari et al., 2000).

Microscopy

Scanning electron microscopy (Bowman, 1994) and GFP fluorescence microscopy (Yadegari et al., 2000) were performed as described.

Bisulfite Genomic DNA Sequencing

Stamens were collected from wild-type Col *gl* open flowers. Late heart and torpedo stage seeds were isolated from Col *gl* wild-type plants. Heterozygous *met1-6* Col *gl* plants were self-pollinated, homozygous *met1-6* F_1 progeny were identified, self-pollinated, and late heart and torpedo stage homozygous *met1-6* seeds were isolated. DNA (0.3–0.7 μg) was digested with the restriction enzymes XhoI, NdeI, and PstI or HindIII in a 20 μl reaction, boiled 2 min, placed on ice for 1 min, and treated with 2.2 μl of fresh 3 M NaOH at 37°C for 15 min. The rest of the treatment was as described (Jacobsen et al., 2000) except that the DNA was treated with 208 μl sodium bisulfite solution, and the bisulfite conversion was at 55°C for 15 min and 95°C for 30 s for 30 cycles. Two microliters of 50 μl of bisulfite-treated DNA was used in each PCR reaction. PCR reactions were 50 μl with 400–600 nM primers and 0.5 μl Ex Taq DNA polymerase and 1× dNTPs (Takara). PCR conditions were 95°C 5 min, 5 cycles of 95°C 15 s, 60°C 3 min, 72°C 3 min followed by 10 cycles of 95°C 15 s, 60°C 1 min, 72°C 2 min then 30 cycles of 95°C 15 s, 50°C 1 min 30 s, 72°C 2 min, and finally 72°C for 5 min. For some reactions a 50°C annealing temperature was used for all cycles.

The bottom strand of the *MEA* promoter from −4248 to −1 (relative to the translation start site) was sequenced in Col *gl* stamens, a tissue where *DME* and *MEA* are not expressed. The promoter was amplified as 14 overlapping segments. Primer pairs are: mea3979F 5′-CTARATTTTAATTTRCRRTRTACCRC-3′ and mea4510R 5′-GGTTAYTAYATGTTGGTAATAATAAG-3′; mea4445F 5′-CATTAAAATCTARTRRCARCCATCRTAAATAART-3′ and mea4879R 5′-TGGGAAGAGAYTGTTGYTTGAATGAGA-3′; mea4800F 5′-CCAAACACACTTTCTTAAARCTTTATATACATCTTTCT-3′ and mea5234R 5′-GAGAAYGATYYAGYAATGTATAGATGGG-3′; mea5212F 5′-CATTCCCATCTATACATTTRCTRRATCRTTC-3′ and mea5582R 5′-TYYAAAYGTATYTGAAGGTTAYGTTTAA-3′; mea5487F 5′-CTTTTRRTCTAATRTRRTRRTRRARRCTAA-3′ and mea6106R 5′-TTYGTTATAAATYYTTGTGTTAAAAYGTAAAT-3′; mea6020F 5′-CATTTARTTAACRTTATAAARARTAAAAA-3′ and mea6244R 5′-GTGTTTGAYYATTAYATGGATAAAGTT-3′; mea6167F 5′-TAATATTATRTACAACACACATTTAATCTT-3′ and mea6424R 5′-TAAAAAYATGTYYAAAYTTATGGTAATGAAAAG-3′; mea6271F 5′-TCCATCTRCCRRCTRTRTTCATCRRTAAACC-3′ and mea6589R 5′-GAAAATGGGATGATAYTGTTTYTTGA

ATGTG-3′; mea6610F 5′-TCTTACATCCTCTRTTCCTTCACA-3′ and mea6812R 5′-GAAAGAGGAAAGATAGAGGGAGGA-3′; mea6790F 5′-CCTCCCTCTATCTTTCCTCT-3′ and mea6994R 5′-AGATGTAGA GATGGGAATGGAGAA-3′; mea6938F 5′-CCACARTCTCTCARRA AAACCARAATRCTCTRT-3′ and mea7386R 5′-TGTAATAYATAYAY YAGTTYAYAAAATTGAGA-3′; mea7320F 5′-CRRCRRATARACTTA ACCTCCCCATTCRT-3′ and mea7627R 5′-TGTGAYATATATAYGG GTTAAATTYYTAGYAAGA-3′; mea7529F 5′-TATTTRACATATTATAC TCATCTCTTRAAT-3′ and mea7935R 5′-GTYATTATATATATTAGT ATTYATTYYTAG-3′ and mea7871F 5′-TTCTTCCATATATRCATAAT ATATAARC-3′ and mea8396R 5′-GGATTTYATAAYYTAGTYAATTYA TATATG-3′.

PCR products were cloned into the TOPO TA cloning vector pCR2.1 (Invitrogen). Between four and seven individual clones were sequenced for each segment. Additional clones were sequenced from the three segments (mea3970F to mea4510R, mea5212F to mea5582R, and mea7529F to mea7935R) that showed nonconversion of a specific cytosine in two or more clones. The methylation status of the three segments on the top and bottom strand was determined in seeds. Top strand primers are: mea3970TF 5′-TGT GAAAGAYTAGATTTTAATTTGYGGTG-3′ and mea4455TR 5′-CCA CTARATTTTAATRCTTRTTTTTRATAATT-3′; mea4383TF 5′-GGAA GATTGTTAAATGTYAAATATTTAATT-3′ and mea4583TR 5′-AACA CARCCRRCTRATRRACCATCCTC-3′; mea5028TFc 5′-GGTTGATG TTGGAATTTTATATATAATTTTG-3′ and mea5337TRc 5′-CCACAAC TCTAAACCACATTAACATCAC-3′; and mea7520TFc 5′-GATGAT TATGTGTAAGATATTTGATATATT-3′ and mea7933TRc 5′-CATTA TATATTAATATTCATTCCTAACT-3′. For wild-type seeds, between 18 and 30 clones were sequenced for each strand. For *met1* seeds, between 12 and 23 clones were sequenced for each strand.

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References

Adams, S., Vinkenoog, R., Spielman, M., Dickinson, H.G., and Scott, R.J. (2000). Parent-of-origin effects on seed development in *Arabidopsis thaliana* require DNA methylation. Development *127*, 2493–2502.

Bowman, J.L. (1994). Flowers: introduction. In *Arabidopsis*: An Atlas of Morphology and Development, J.L. Bowman, ed. (New York: Springer-Verlag), pp. 135–145.

Brown, R.C., Lemmon, B.E., Nguyen, H., and Olsen, O.-A. (1999). Development of endosperm in *Arabidopsis thaliana*. Sex. Plant Reprod. *12*, 32–42.

Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000). Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. Nature *403*, 859–866.

Cao, X., and Jacobsen, S.E. (2002a). Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proc. Natl. Acad. Sci. USA *99*, 16491–16498.

Cao, X., and Jacobsen, S.E. (2002b). Role of the *Arabidopsis* DRM methyltransferases in de novo DNA methylation and gene silencing. Curr. Biol. *12*, 1138–1144.

Chaudhury, A.M., Luo, M., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *94*, 4223–4228.

Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J.J., Goldberg, R.B., Jacobsen, S.E., and Fischer, R.L. (2002). DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in *Arabidopsis*. Cell *110*, 33–42.

Ferguson-Smith, A.C., and Surani, M.A. (2001). Imprinting and the epigenetic asymmetry between parental genomes. Science *293*, 1086–1089.

Finnegan, E.J., and Dennis, E.S. (1993). Isolation and identification by sequence homology of a putative cytosine methyltransferase from *Arabidopsis thaliana*. Nucleic Acids Res. *21*, 2383–2388.

Finnegan, E.J., and Kovac, K.A. (2000). Plant DNA methyltransferases. Plant Mol. Biol. *43*, 189–201.

Finnegan, E.J., Peacock, W.J., and Dennis, E.S. (1996). Reduced DNA methylation in *Arabidopsis* results in abnormal plant development. Proc. Natl. Acad. Sci. USA *93*, 8449–8454.

Francis, N.J., and Kingston, R.E. (2001). Mechanisms of transcriptional memory. Nat. Rev. Mol. Cell Biol. *2*, 409–421.

Gehring, M., Choi, Y., and Fischer, R.L. (2003). Imprinting and seed development. Plant Cell, in press.

Gong, Z., Morales-Ruiz, T., Ariza, R.R., Roldan-Arjona, T., David, L., and Zhu, J.-J. (2002). ROS1, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. Cell *111*, 803–814.

Grossniklaus, U., Vielle-Calzada, J.-P., Hoeppner, M.A., and Gagliano, W.B. (1998). Maternal control of embryogenesis by *MEDEA*, a polycomb-group gene in *Arabidopsis*. Science *280*, 446–450.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556–560.

Jacobsen, S.E., Sakai, H., Finnegan, E.J., Cao, X., and Meyerowitz, E.M. (2000). Ectopic hypermethylation of flower-specific genes in *Arabidopsis*. Curr. Biol. *10*, 179–186.

Jeddeloh, J.A., Stokes, T.L., and Richards, E.J. (1999). Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nat. Genet. *22*, 94–97.

Jiricny, J. (2002). An APE that proofreads. Nature *415*, 593–594.

Johnson, L.M., Cao, X., and Jacobsen, S.E. (2002). Interplay between two epigenetic marks: DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. *12*, 1360–1367.

Jost, J.-P., Oakeley, E.J., Zhu, B., Benjamin, D., Thiry, S., Siegmann, M., and Jost, Y.-C. (2001). 5-Methylcytosine DNA glycosylase participates in the genome-wide loss of DNA methylation occurring during mouse myoblast differentiation. Nucleic Acids Res. *29*, 4452–4461.

Kakutani, T., Jeddeloh, J.A., Flowers, S.K., Munakata, K., and Richards, E.J. (1996). Developmental abnormalities and epimutations associated with DNA hypomethylation mutations. Proc. Natl. Acad. Sci. USA *93*, 12406–12411.

Kankel, M.W., Ramsey, D.E., Stokes, T.L., Flowers, S.K., Haag, J.R., Jeddeloh, J.A., Riddle, N.C., Verbsky, M.L., and Richards, E.J. (2003). *Arabidopsis* MET1 cytosine methyltransferase mutants. Genetics *163*, 1109–1122.

Kinoshita, T., Yadegari, R., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (1999). Imprinting of the *MEDEA* polycomb gene in the *Arabidopsis* endosperm. Plant Cell *11*, 1945–1952.

Kishimoto, N., Sakai, H., Jackson, J., Jacobsen, S.E., Meyerowitz, E.M., Dennis, E.S., and Finnegan, E.J. (2001). Site specificity of the *Arabidopsis* MET1 DNA methyltransferase demonstrated through hypermethylation of the SUPERMAN locus. Plant Mol. Biol. *46*, 171–183.

Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J., Goldberg, R.B., and Fischer, R.L. (1999). Control of fertilization-independent endosperm development by the *MEDEA* polycomb gene in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *96*, 4186–4191.

Kohler, C., Hennig, L., Spillane, C., Pien, S., Gruissem, W., and Grossniklaus, U. (2003). The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes Dev. *17*, 1540–1553.

Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat. Rev. Genet. *3*, 662–673.

Lindroth, A.M., Cao, X., Jackson, J.P., Zilberman, D., McCallum, C.M., Henikoff, S., and Jacobsen, S.E. (2001). Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. Science *292*, 2077–2080.

Luo, M., Bilodeau, P., Koltunow, A., Dennis, E.S., Peacock, W.J., and Chaudhury, A.M. (1999). Genes controlling fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *96*, 296–301.

Luo, M., Bilodeau, P., Dennis, E.S., Peacock, W.J., and Chaudhury, A. (2000). Expression and parent-of-origin effects for *FIS2*, *MEA*, and *FIE* in the endosperm and embryo of developing *Arabidopsis* seeds. Proc. Natl. Acad. Sci. USA *97*, 10637–10642.

Martienssen, R. (1998). Chromosomal imprinting in plants. Curr. Opin. Genet. Dev. *8*, 240–244.

Martienssen, R.A., and Colot, V. (2001). DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science *293*, 1070–1074.

Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., and Kakutani, T. (2001). Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature *411*, 212–214.

Moore, G. (2001). Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. Reproduction *122*, 185–193.

Neff, M.M., Neff, J.D., Chory, J., and Pepper, A.E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J. *14*, 387–392.

Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P., and Fischer, R.L. (1996). A mutation that allows endosperm development without fertilization. Proc. Natl. Acad. Sci. USA *93*, 5319–5324.

Posfai, L., Bhagwat, A.S., Posfai, G., and Roberts, R.J. (1989). Predictive motifs from cytosine methyltransferases. Nucleic Acids Res. *17*, 2421–2435.

Reik, W., and Walter, J. (2001). Genomic imprinting: parental influence on the genome. Nat. Rev. Genet. *2*, 21–32.

Ronemus, M.J., Galbiati, M., Ticknor, C., Chen, J., and Dellaporta, S.L. (1996). Demethylation-induced developmental pleiotropy in *Arabidopsis*. Science *273*, 654–657.

Saze, H., Scheid, O.M., and Paszkowski, J. (2003). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat. Genet. *34*, 65–69.

Soppe, W.J.J., Jacobsen, S.E., Alonso-Blanco, C., Jackson, J.P., Kakutani, T., Koornneef, M., and Peeters, A.J.M. (2000). The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. Mol. Cell *6*, 791–802.

Soppe, W.J.J., Jasencakova, Z., Andreas, H., Tetsuji, K., Armin, M., Huang, M.S., Jacobsen, S.E., Ingo, S., and Fransz, P.F. (2002). DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. EMBO J. *21*, 6549–6559.

Tycko, B., and Morison, I.M. (2002). Physiological functions of imprinted genes. J. Cell. Physiol. *192*, 245–258.

Vielle-Calzada, J.-P., Thomas, J., Spillane, C., Coluccio, A., Hoeppner, M.A., and Grossniklaus, U. (1999). Maintenance of genomic imprinting at the *Arabidopsis medea* locus requires zygotic DDM1 activity. Genes Dev. *13*, 2971–2982.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Frankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., et al. (2000). Activation tagging in *Arabidopsis*. Plant Physiol. *122*, 1003–1013.

Yadegari, R., Kinoshita, T., Lotan, O., Cohen, G., Katz, A., Choi, Y., Katz, A., Nakashima, K., Harada, J.J., Goldberg, R.B., et al. (2000). Mutations in the FIE and MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell *12*, 2367–2381.

Note Added in Proof

Recently it was shown that DME and MET1 regulate another gene, *FWA*, which is imprinted in the endosperm (Kinoshita et al., 2003). DME activates expression of the maternal *FWA* allele, whereas MET1 represses expression of the paternal *FWA* allele. Thus, control of maternal-specific expression by MET1 and DME may be a general mechanism for endosperm imprinting in *Arabidopsis*.

Kinoshita, T., Miura, A., Choi, Y., Kinoshita, Y., Cao, X., Jacobsen, S.E., Fischer, R.L., and Kakutani, T. (2003). A one-way control of *FWA* imprinting in *Arabidopsis* endosperm by DNA methylation. Science, in press.