Function of the DEMETER DNA glycosylase in the *Arabidopsis thaliana* male gametophyte

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In double fertilization, the vegetative cell of the male gametophyte (pollen) germinates and forms a pollen tube that brings to the female gametophyte two sperm cells that fertilize the egg and central cell to form the embryo and endosperm, respectively. The 5-methylcytosine DNA glycosylase DEMETER (DME), expressed in the central cell, is required for maternal allele demethylation and gene imprinting in the endosperm. By contrast, little is known about the function of DME in the male gametophyte. Here we show that reduced transmission of the paternal mutant *dme* allele in certain ecotypes reflects, at least in part, defective pollen germination. *DME* RNA is detected in pollen, but not in isolated sperm cells, suggesting that *DME* is expressed in the vegetative cell. Bisulfite sequencing experiments show that imprinted genes (*MEA* and *FWA*) and a repetitive element (*Mu1a*) are hypomethylated in the vegetative cell genome compared with the sperm genome, which is a process that requires DME. Moreover, we show that *MEA* and *FWA* RNA are detectable in pollen, but not in isolated sperm cells, suggesting that their expression occurs primarily in the vegetative cell. These results suggest that DME is active and demethylates similar genes and transposons in the genomes of the vegetative and central cells in the male and female gametophytes, respectively. Although the genome of the vegetative cell does not participate in double fertilization, its DME-mediated demethylation is important for male fertility and may contribute to the reconfiguration of the methylation landscape that occurs in the vegetative cell genome.

DNA demethylation | DNA methylation | transposon silencing | gene silencing | plant reproduction

Double fertilization is unique to flowering plants and underlies the distinctive cellular programming of epigenetic processes, such as plant gene imprinting, which are essential for plant reproduction (1). In the ovule, meiosis produces a haploid megaspore that undergoes three mitoses to form the female gametophyte with egg, central, synergid, and antipodal cells (2). In stamens, each haploid microspore undergoes an asymmetric mitosis to produce a large vegetative cell and a smaller generative cell (binucleate pollen), which have different fates. The generative cell, engulfed in the cytoplasm of the vegetative cell, undergoes a second mitosis to form two sperm cells. The three-cell male gametophyte (trinucleate pollen) dehydrates, matures, and is released from the stamen. Upon encountering specialized cells at the tip of the stigma of a receptive plant, the pollen grain rehydrates and the vegetative cell germinates, producing a pollen tube that grows to the ovules and transports two sperm cells to the female gametophyte where fertilization of egg and central cell generates the embryo and endosperm, respectively (3). The embryo and endosperm develop within the maternal seed coat, and together they comprise the seed. Endosperm, the site where most plant gene imprinting occurs, is a nutrient tissue, acquiring and storing resources from the maternal chalazal seed coat and underlying vasculature to nourish the embryo (4).

DNA methylation is a stable repressive epigenetic mark associated with gene and transposon silencing and gene imprinting in flowering plants (5). In *Arabidopsis*, a suite of DNA methyl-transferases, DNA METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3), and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), establish and maintain CG, CHG, and CHH DNA methylation (H = A, T, or C) (5). DNA glycosylase enzymes catalyze DNA demethylation by excising 5-methylcytosine. The cleavage of the *N*-glycosylic bond creates an abasic site, whereas the lyase activity nicks the DNA. An AP endonuclease generates a 3′-hydroxyl used by a DNA repair polymerase that inserts unmethylated cytosine. A DNA ligase seals the nick to complete the demethylation process (6).

The *Arabidopsis* DEMETER (DME) DNA glycosylase is primarily expressed in the central cell before fertilization (7) and is required for maternal allele DNA demethylation in the endosperm that establishes gene imprinting, which is the differential expression of alleles of the same gene depending on its parent of origin (8). For example, DME is required for demethylation of maternal alleles of the imprinted *MEDEA* (*MEA*) and *FERTILIZATION INDEPENDENT SEED 2* (*FIS2*) Polycomb-group protein genes (9, 10) and the *FLOWERING WAGENINGEN* (*FWA*) homeo-domain transcription factor gene (11). Maternal DME activity also profoundly reconfigures the endosperm methylation landscape and results in the demethylation of repeated sequences and transposable elements (12, 13).

DME was identified by mutations that cause maternal effects on seed viability (7). Inheriting a loss-of-function maternal *dme* allele results in inviable, aborted seeds—that is, *DME/dme-1* and *DME/dme-2* plants, either self-pollinated or pollinated with wild-type pollen, produce a 1:1 ratio of viable:inviable seeds, and all of the viable seeds are wild type (7). The inability to transmit a maternal mutant *dme* allele is a fully penetrant phenotype observed in all *Arabidopsis* ecotypes tested (L*er*, Col-*gl*, WS) (7). Similarly, functional maternal *MEA* and *FIS2* alleles, which are regulated by DME, are required for seed viability (14–16).

Mutations in the *DME* gene also influence pollen function in certain ecotypes. In the L*er* ecotype, *dme-1* and *dme-2* paternal alleles are efficiently transmitted to the next generation. That is, when *DME/dme-1* or *DME/dme-2* heterozygous plants were self-pollinated, or when their pollen was used to pollinate a wild-type plant, viable wild-type and heterozygous *DME/dme* F1 progeny were detected in equal frequency (7). However, when *DME/dme-1* or *DME/dme-2* heterozygous plants in the Col-*gl* ecotype were self-pollinated, only ~15% of the viable F1 progeny were heterozygous (17). These results suggest that DME functions in the male gametophyte in the Col-*gl* ecotype.

Here we report our analysis of DME function in the *Arabidopsis* male gametophyte. We found that DME is required for

normal pollen germination in the Col ecotype, but is dispensable in the Ler ecotype. We detected *DME* expression in Col and L*er* pollen, but not in sperm cells, suggesting by the process of elimination that *DME* is primarily expressed in the vegetative cell. We isolated sperm cells and vegetative cell nuclei by FACS (18) and analyzed cytosine methylation in each cell type using locus-specific bisulphite sequencing. We found that DME is required for demethylation of the *MEA* and *FWA* genes, and a *Mu1a* transposon, in the vegetative cell nucleus in Col and L*er* pollen. These findings support the view that DME is active and demethylates similar genes and transposons in the genomes of the vegetative and central cells of the male and female gametophytes, respectively. The vegetative cell genome does not participate in double fertilization. Hence, DME activity in the vegetative cell, unlike its activity in the central cell, does not regulate gene imprinting in the endosperm. However, DME activity is important for male fertility in certain ecotypes and may contribute to the reconfiguration of the methylation landscape that occurs in the vegetative cell genome (19).

Results

Genetic and Morphological Analysis of *dme* Mutant Pollen. We pollinated wild-type plants with heterozygous *DME/dme* pollen donors and measured the efficiency of paternal transmission of four independently isolated *dme* mutant alleles, *dme-1*, *dme-2*, *dme-6* (GK-252E03) (20), and *dme-7* (SALK_107538). For all mutant alleles, transmission was significantly lower than the predicted 50% (Table 1). Very low levels of seed abortion, less than 1%, were observed among the progeny of these crosses, which is consistent with previous reports showing that inheriting a paternal mutant *dme* allele does not affect seed viability and rules out a sperm fertility defect (7). Thus, in the Col ecotype, four independently isolated *dme* mutant alleles are transmitted paternally at a reduced level compared with wild type.

We also measured the maternal transmission of *dme-6* and *dme-7* alleles. We found that approximately equal numbers of viable and nonviable F1 seeds are generated when heterozygous *DME/dme-6* or *DME/dme-7* plants were pollinated with wild-type pollen or were self-pollinated (Table S1). Moreover, in a population of progeny from selfed *DME/dme-6* plants, we detected 116 heterozygotes and no *dme-6* homozygotes. Thus, the maternal *dme-6* and *dme-7* alleles, like *dme-1* and *dme-2* (7), are not transmitted in genetic crosses.

Mutations in the *DME* gene do not cause obvious alterations in mature pollen morphology or viability. Mature pollen isolated from wild-type, *DME/dme-6*, or *DME/dme-7* inflorescences are indistinguishable and contain one vegetative cell nucleus and two sperm cells (Fig. 1 *A* and *B* and Table 2). To measure pollen viability, we performed Alexander staining of pollen in wild-type (Fig. 1*C*) and *DME/dme* heterozygous (Fig. 1*D*) stamens. Red-stained pollen grains are viable, whereas green-stained pollen grains are not (21). No significant difference in viability was detected in pollen from wild-type (97.5% viable, n = 631), *DME/dme-1* (99%, n = 145), or *DME/dme-2* (99%, n = 771) stamens.

We investigated whether *dme* mutations affect pollen tube growth in vivo. Ten days after pollinating wild-type Col-0 plants
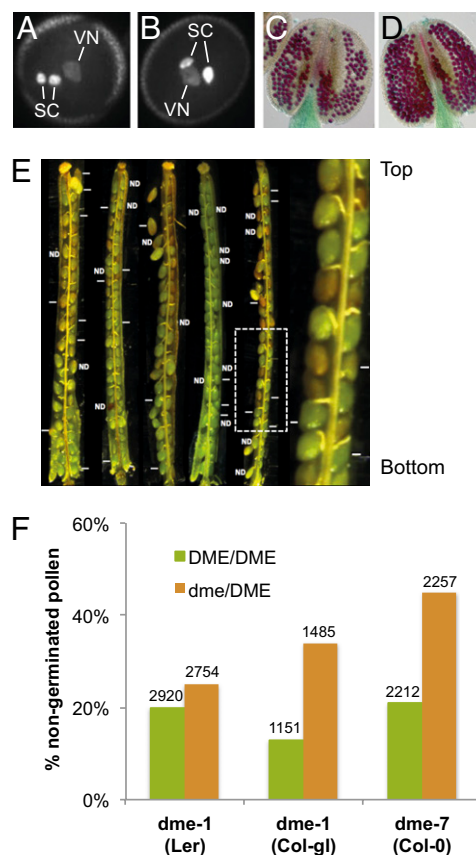

Fig. 1. Analysis of *dme* mutant pollen. (*A* and *B*) DAPI-stained pollen grain from a Col-0 wild-type (*A*) and *DME/dme-6* (*B*) open flower. Isolation of pollen is described in Table 2. Images were taken with 63× magnification. Z-stacks of 30 layers, covering a range of 12 μm, were recorded and subsequently processed to get maximal intensity projection images. SC, sperm cell; VN, vegetative cell nucleus. (*C* and *D*) Alexander stained pollen grains from Col-*gl* wild-type (*C*) and *DME/dme-1* (*D*) stamens. (*E*) Individual seeds in 10 d after pollination (DAP) siliques generated by pollinating a wild-type (Col-0) plant with pollen from a *DME/dme-7* plant. (*Right*) Area surrounded by a white box is enlarged to more easily visualize seeds. –, *DME/dme* seeds. ND, genotype not determined; no symbol, wild-type seeds. (*F*) Percentage of pollen that does not germinate. Numbers above the bars indicate the number of pollen inspected. Genotypes and ecotypes of parent plants are indicated.

with pollen from *DME/dme-6* or *DME/dme-7* plants, we determined the position and genotype of each seed in the silique (Fig. 1*E*). If mutant pollen tubes grow more slowly than wild type, we would expect more wild-type than *DME/dme* mutant seeds in the top half of the siliques. However, *DME/dme* seeds were distributed randomly in siliques (Fig. S1), suggesting that *dme* mutations do not impact pollen tube growth.

We measured the ability of pollen grains to germinate and form a pollen tube on solid media (Fig. S2). We found a significant increase in the percentage of nongerminating pollen harvested

Table 1. Reduced paternal *dme* allele transmission in the Col ecotype

Maternal parent	Paternal parent	Parent ecotype	Wild-type F1	Heterozygous F1	*dme* transmission, %	P
Wild type	*DME/dme-1*	Col-*gl*	494	81	14.1	<0.0001
Wild type	*DME/dme-2*	Col-*gl*	89	16	15.2	<0.0001
Wild type	*DME/dme-6*	Col-0	168	46	21.5	<0.0001
Wild type	*DME/dme-7*	Col-0	296	36	10.8	<0.0001

For crosses with *dme-1* and *dme-2*, F1 genotype was determined by PCR and by selection in plates with BASTA herbicide (*SI Materials and Methods*). For crosses with *dme-6* and *dme-7*, F1 seed genotypes were determined by PCR using primers described in Table S2. P, probability of null hypothesis (1:1 inheritance of wild-type and *dme* paternal alleles in the F1 generation) calculated by χ^2 test.

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Table 2. Morphology of wild-type and *dme* mutant pollen

Genotype of parent	Trinuclear pollen grains	Binuclear pollen grains	Microspores	Morphological abnormalities
Wild type	555	2	0	nd
DME/dme-6	536	2	0	nd
DME/dme-7	553	0	0	nd

All lines were Col-0 ecotype. A total of 15–20 open flowers were harvested into a microcentrifuge tube containing 100 µL of buffer [9% sucrose; 50 mM Tris·HCl (pH 7.5); 0.1% Triton X-100] and vortexed for 5 s. Flowers parts were removed from the tube and the pollen suspension was centrifuged for 2 min at 5,400 × *g* at room temperature. The pollen pellet was suspended in 10 µL of the same buffer and applied onto a microscope slide. DAPI was added to a final concentration of 1 µg/mL. Pollen were analyzed using an inverted microscope (Axio Imager; Zeiss) equipped with a spinning disk unit from Perkin Elmer (UltraView VoX). nd, not detected.

from *DME/dme-1* (Col-*gl*) and *DME/dme-7* (Col-0) compared with pollen isolated from their wild-type siblings (Fig. 1*F*). Moreover, the percentage of nongerminating pollen isolated from *DME/dme-1* plants in the L*er* ecotype, which suppresses the defect in transmission of the paternal mutant *dme-1* allele (7) (Table 1), was reduced compared with pollen from *DME/dme-1* Col-*gl* plants, and was similar to wild-type L*er* siblings (Fig. 1*F*). Taken together, these data suggest that reduced transmission of paternal *dme* mutant alleles in the Col ecotype is due, at least in part, to their diminished ability to germinate and form a pollen tube. However, these results do not rule out the possibility that *dme* mutant pollen tubes have a reduced ability to be attracted to an ovule (22).

DME Is Expressed During Male Gametogenesis. Previously, *DME* expression was not detected in pollen (7, 23). However, using improved methods to isolate and amplify pollen RNA, we were able to detect and analyze *DME* expression in pollen to understand the mechanism for reduced transmission and germination of *dme* mutant pollen.

Pollen was isolated from inflorescences containing flower buds at all stages as described in *Materials and Methods*. This procedure yields pure pollen at the trinucleate stage containing two sperm cells and a vegetative cell. Previously, we developed a simple method to disrupt pollen grains and fractionate vegetative cell nuclei and sperm nuclei using FACS based on differences in their nucleic acids content (*Materials and Methods*) (18). RNA was isolated from pollen and cell-sorted sperm. We detected the ubiquitously expressed *TUBULIN BETA CHAIN 4* (*TUB4*) gene in both pollen and the cell-sorted sperm (Fig. 2*A* and Fig. S3*A*). We also detected *DUO1* RNA in both pollen and sperm cells (Fig. S3*B*), which is consistent with DUO1 transcription factor protein accumulating specifically in sperm cells (24). These control experiments suggest that at least a fraction of the cell-sorted sperm had maintained their cytoplasm.

We detected *DME* RNA in pollen, but not in cell-sorted sperm, of wild-type ecotypes Col-0, L*er*, and WS (Fig. 2*A* and Fig. S3*A*). This finding suggests, by the process of elimination, that *DME* is expressed in the vegetative cell.

Previous attempts to detect *DME::GUS* reporter gene expression in pollen were unsuccessful, whereas *DME::GUS* expression was visualized in the central cell of the ovule (7). It is possible that the level of endogenous *DME* expression in pollen is significantly lower than in the ovule, and can only be detected by sensitive RT-PCR methods. In support of this idea, quantitative RT-PCR experiments show that the level of *DME* RNA in pollen is ~1,000-fold lower than in ovules (Fig. S3*C*). Alternatively, important *cis*-acting DNA sequences required for *DME* expression in pollen may not have been present on the *DME::GUS* transgene.
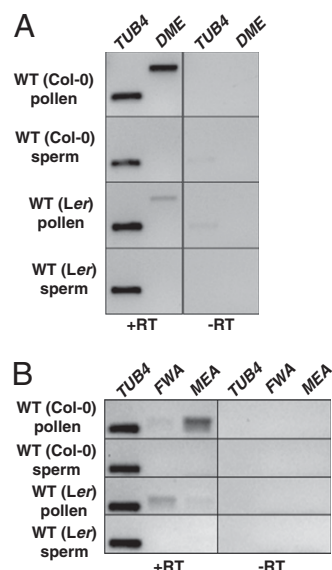
Fig. 2. Expression of *DME*, *MEA*, and *FWA* in pollen and sperm cells. Pollen was isolated from wild-type (WT) Col-0 and L*er* inflorescences as described in *Materials and Methods*. Pollen was disrupted and FACS was used to isolate the freed sperm cells. *DME* (*A*) and *FWA* and *MEA* (*B*) transcripts were analyzed by the RT-PCR (+RT). *TUB4* RNA, expressed in both pollen and sperm cells, was used as a control for loading equivalent amounts of cDNA on gels. We tested for amplifying contaminating DNA by performing PCR without the reverse transcriptase step (–RT).

DME is necessary for the activation of *MEA* and *FWA* promoters in the central cell of the female gametophyte (7, 11). By RT-PCR experiments, we detected *FWA* and *MEA* transcripts in mature pollen but not in sperm cells of wild-type plants in the Col-0, L*er*, and WS ecotypes (Fig. 2*B* and Fig. S3*D*). This finding suggests, by the process of elimination, that *MEA* and *FWA* are expressed in the vegetative cell.

The ROS1, DML2, and DML3 DNA glycosylases are related to DME and demethylate DNA in sporophytic tissues (6). By RT-PCR experiments, we detected their respective RNAs at a lower level than *DME* RNA in pollen, and none of their RNAs were detected in sperm (Fig. S3*B*). These experiments suggest that among the family of DNA glycosylases that demethylate DNA, *DME* is expressed at the highest level in the vegetative cell.

DNA Demethylation of *MEA*, *FWA*, and *Mu1a* in the Vegetative Cell Genome Requires DME. Because *DME*, *MEA*, and *FWA* are expressed in the vegetative cell, we investigated whether DME might demethylate and activate *MEA* and *FWA* expression in the vegetative cell genome. We also investigated DNA methylation at a *Mu1a* transposon, which has been shown to be hypomethylated in vegetative cell nuclei compared with sperm nuclei, and is transcribed in the vegetative cell (19). Pollen was harvested from wild-type and *DME/dme* mutant plants in different ecotype backgrounds; nuclei from sperm and vegetative cells were purified by FACS (*Materials and Methods*) (18); genomic DNAs were isolated; and bisulfite sequencing procedures were used to measure the levels of cytosine methylation at the *MEA*, *FWA*, and *Mu1a* loci.

The *MEA* gene is flanked by CG DNA methylation in sporophytic (e.g., leaf) genomes 5′ of the gene at −500 bp, and 3′ of the gene at seven 182-bp direct repeats in the Col-*gl*, RLD, WS, and L*er* ecotypes, with one exception: L*er* consistently has lower levels of methylation at −500 bp (9, 25) (Fig. S4). *DME* expression in the central cell of the female gametophyte is necessary for DNA demethylation at −500 bp and the 3′ direct repeats in the endosperm (9). We measured their DNA methylation in the male gametophyte by bisulfite sequencing using primers that span these

flanking regions, termed 5′-MEA and 3′-MEA, respectively. We detected a high level of CG methylation at 5′-MEA and 3′-MEA in the wild-type (Col-0) sperm genome similar to that reported in sporophytic tissues (residues indicated with an asterisk in Fig. 3A). By contrast, this methylation was absent from 5′-MEA and 3′-MEA in the wild-type (Col-0) vegetative cell genome (Fig. 3A). Thus, the 5′-MEA and 3′-MEA regions are hypomethylated in the vegetative cell genome compared with the sperm genome. Similar methylation profiles of the 5′-MEA and 3′-MEA regions were detected in vegetative cell and sperm genomes in the WS ecotype (Fig. S5).

We next determined whether DME was required for hypomethylation of 5′-MEA and 3′-MEA in the vegetative cell genome. DME/dme-6 (Col-0) plants are predicted to generate equal numbers of wild-type and dme-6 pollen. We detected two classes of bisulfite-sequenced clones in approximately equal numbers (Fig. 3B) from the vegetative cell genome. One class is methylated at 5′-MEA and 3′-MEA similar to the level detected in the sperm genome, and might represent vegetative cell genomes isolated

from mutant dme-6 pollen. The other class is unmethylated at 5′-MEA and 3′-MEA, and might represent vegetative cell genomes isolated from wild-type pollen. A similar set of DNA methylation profiles was obtained in the vegetative cell and sperm genome from wild-type and dme-2 mutant Ler plants (Fig. 3 C and D). As predicted from analysis of MEA DNA methylation in endosperm and sporophytic tissues (9), the 5′-MEA sequences at −500 bp were not methylated in the Ler vegetative cell and sperm genomes (Fig. 3C). At the 3′-MEA region, the wild-type (Ler) vegetative cell genome was hypomethylated compared with the sperm genome (Fig. 3C). In vegetative cell, genomes from pollen harvested from DME/dme-2 (Ler) plants, two classes of clones, methylated and unmethylated, were obtained in approximately equal numbers (Fig. 3D). Taken together, these results suggest that DME is required for DNA demethylation in regions flanking the MEA gene in the vegetative cell genome.

FWA gene expression is silenced by CG DNA methylation at short interspersed element (SINE)-related direct repeats, which
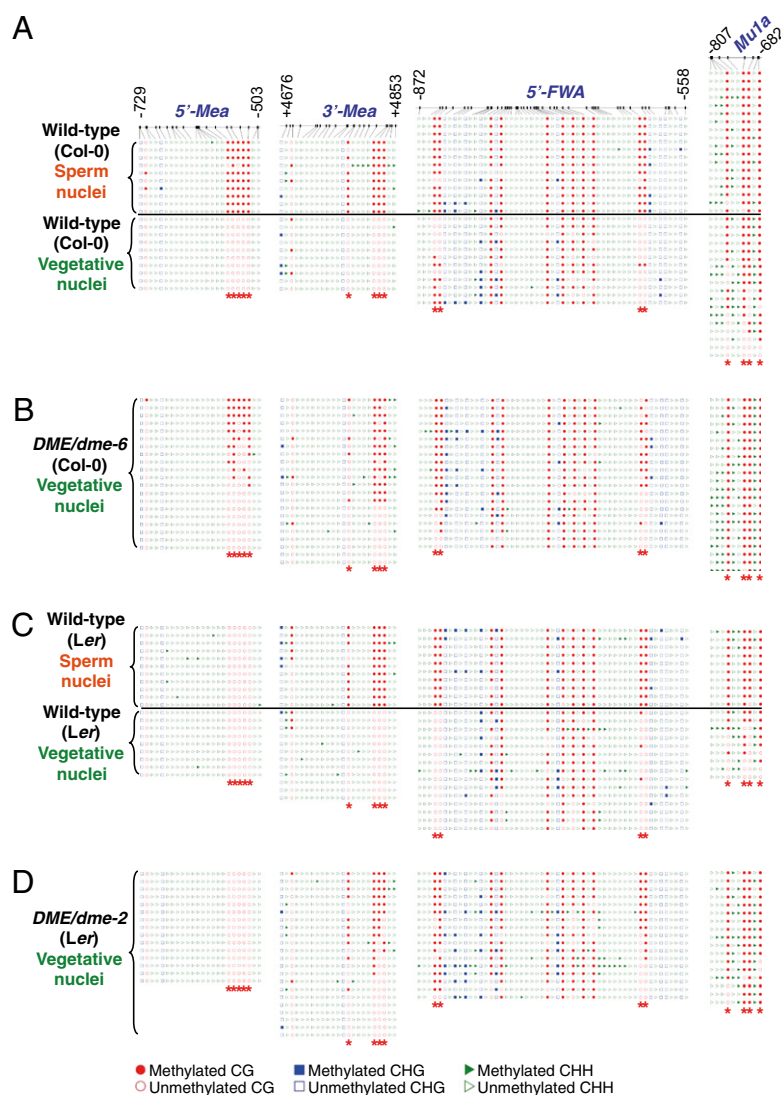

Fig. 3. DNA methylation profiles of MEA, FWA, and Mu1a. Bisulfite sequencing analysis of cytosine methylation is shown at 5′-MEA, 3′-MEA, 5′-FWA, and Mu1A (AT4g08680) in sorted sperm cells and vegetative cell nuclei of pollen from wild-type Col-0 (A) and Ler (C) plants, and in sorted vegetative cell nuclei from DME/dme-6 Col-0 (B) and DME/dme-2 Ler (D) plants. (A Top) Numbers from the start codon. Asterisks in red indicate cytosine residues referred to in the text. 5-methylcytosines in the CG, CHG, and CHH contexts were analyzed and displayed using CyMATE (32). Primers for bisulfite sequencing are shown in Table S2. Primers for 5′-MEA, 3′-MEA, and 5′-FWA are identical with those used previously to measure MEA and FWA DNA methylation (9, 11). Primers used to measure Mu1a DNA methylation partially overlap with those used previously, with the 3′ three CG base pairs identical with the 5′ three CG base pairs shown in Slotkin et al. (19).

are located 5′ of the *FWA* gene, in wild-type (Col-0, L*er*, and WS) sporophytic tissues (26). *DME* expression in the central cell of the female gametophyte is necessary for DNA demethylation at the SINE-related repeats, termed 5′-*FWA*, which results in expression of the maternal *FWA* allele in the endosperm (11). By bisulfite sequencing using primers that span these SINE-related repeats, we have analyzed *FWA* DNA methylation in the vegetative cell and sperm genomes. We detected a high level of methylation in wild-type sperm genomes in the Col-0, L*er*, and WS ecotypes (residues denoted with asterisks in Fig. 3 *A* and *C* and Fig. S5). By contrast, significantly less methylation at these sites was observed in wild-type vegetative cell genomes from Col-0, L*er*, and WS (Fig. 3 *A* and *C* and Fig. S5). Restoration of methylation was detected in approximately half of the vegetative cell genome clones in pollen harvested from *DME/dme-6* Col-0 (Fig. 3*B*) and *DME/dme-2* L*er* (Fig. 3*D*) plants. Although the number of demethylated cytosines we detected are fewer in number than those flanking maternal *FWA* alleles in the endosperm (11), these results suggest that DME mediates DNA demethylation of certain 5-methylcytosine residues within the SINE-related repeats that flank the *FWA* gene.

Genome-wide analyses showed that maternal DME fractionally (about 10%) reduces CG methylation at transposons in the endosperm (13). To investigate the role of paternal DME in transposon demethylation, we measured DNA methylation of a *Mu1a* transposon in the male gametophyte. In wild-type Col-0 and L*er* sperm cells, four *Mu1a* CG residues were uniformly methylated (residues indicated with an asterisk in Fig. 3 *A* and *C*). By contrast, CG methylation at these sites was fractionally decreased in the vegetative cell genome in the Col-0 (Fig. 3*A*) and L*er* (Fig. 3*C*) ecotypes. Moreover, DNA methylation at the four *Mu1A* CG residues was elevated in clones from vegetative cell genomes obtained from *DME/dme-6* Col-0 (Fig. 3*B*) and *DME/dme-2* L*er* pollen (Fig. 3*D*). Taken together, these results are consistent with DNA demethylation of the *Mu1A* transposon that is mediated by DME.

We detected *ROS1*, *DML2*, and *DML3* RNA in pollen, which was not detected in sperm cells (Fig. S3*B*), suggesting that these DNA glycosylases might be active in the vegetative cell. To test this idea we measured DNA methylation at the 3′-*MEA* and 5′-*FWA* regions in the vegetative cell and sperm genomes from pollen (Col-0) isolated from homozygous single *ros1*, *dml2*, *dml3* mutants, and triple-mutant (termed *rdd*) stamens. However, we could not detect alterations in the DNA methylation profiles in the mutant sperm or vegetative cell genomes (residues indicated with an asterisk in Fig. S6), which resembled DNA methylation profiles observed in wild-type Col-0 (Fig. 3*A*). Thus, DME is the primary DNA glycosylase that affects demethylation at these sites. This is consistent with there being no detectable reproductive phenotypes associated with *rdd* mutant plants (27).

Discussion

The DME DNA glycosylase plays multiple critical roles in maternal aspects of plant reproduction. Here we identify a paternal role for DME in plant reproduction. Paternal mutant *dme* alleles (Col-*gl* and Col-0 ecotypes) are inherited less efficiently than wild type (Fig. 1 and Table 1). We detected *DME* expression in pollen, but not in sperm, suggesting by the process of elimination that it is expressed in the vegetative cell of the male gametophyte (Fig. 2). Paternal DME is active because it is necessary for the demethylation of the *MEA* and *FWA* genes, and the *Mu1a* transposon, in the vegetative cell genome (Fig. 3). Taken together, these data show that DME functions in the male gametophyte.

Mutant *dme* pollen (Col-*gl* and Col-0) germinates and forms a pollen tube on solid media less efficiently than wild-type pollen. This result is consistent with our data suggesting that DME is expressed in the vegetative cell, whose primary role in reproduction is to germinate and form a pollen tube that brings the sperm cells to the female gametophyte for double fertilization. The reason for the decrease in *dme* mutant transmission and pollen germination in the Col ecotype is unknown. DME regulates the expression of multiple regulatory genes that are imprinted in the endosperm and whose mutant paternal transmission has yet to be tested (8), which represent potential candidates for DME-induced gene expression in the vegetative cell. Why the defect in paternal *dme* mutant allele transmission and pollen germination is suppressed in the L*er* ecotype is unknown. However, the Col and L*er* ecotypes differ genetically by over 400,000 single-nucleotide polymorphisms (8). The Col and L*er* genomes also differ epigenetically and have distinct DNA methylation profiles (28). It is possible that a key regulatory gene for pollen germination is demethylated by DME in the Col ecotype, whereas the regulatory gene is not methylated in the L*er* ecotype, and hence DME is not required.

The DME-mediated DNA demethylation at *MEA*, *FWA*, and *Mu1a* occurs in all ecotypes tested (Col, L*er*, and WS). These studies suggest that DME-mediated DNA demethylation is a general phenomenon that occurs in both female and male gametophyte accessory cells, the central cell, and the vegetative cell, respectively. A wild-type maternal *DME* allele is required for seed viability, imprinted gene expression in the endosperm, and the dramatic reconfiguration of the endosperm DNA methylation landscape (7–9, 13). Because the vegetative cell does not participate in double fertilization, DNA demethylation mediated by the paternal *DME* allele in the male gametophyte does not influence the regulation of endosperm gene imprinting. However, it has been shown that transposable elements in the vegetative cell genome are hypomethylated relative to the sperm genome, and that this process involves down-regulation of the heterochromatin remodeler *DECREASE IN DNA METHYLATION 1* (*DDM1*). Increased transposon expression in the vegetative cell results in the generation of siRNAs that likely silence transposable elements in the sperm cell genome (19). Maternal DME, expressed in the central cell, is necessary for the dramatic reconfiguration of DNA methylation that occurs in the endosperm that includes genome-wide demethylation of transposons and repeated sequences (13). Our locus-specific bisulfite sequencing experiments suggest that DME may have similar target specificity in the vegetative cell of the male gametophyte. Understanding whether paternal *DME* plays a significant role in demethylating transposons in the vegetative cell genome awaits the analysis of genome-wide DNA methylation profiles of wild-type and *dme* mutant vegetative cell and sperm cell genomes.

Materials and Methods

Plant Lines. We carried out all analyses using wild-type *Arabidopsis thaliana* ecotypes Col-0, Col-*gl*, L*er*, and WS. We used the following alleles: *ros1* (SAIL_1249_A01), *dml-2* (SALK_121317), *dml-3* (SALK_056440), *ros-1 dml-2 dml-3* (27), *dme-1* and *dme-2* (7), *dme-6* (GK-252E03), and *dme-7* (SALK107538). The *dme-6* has a transfer DNA (T-DNA) insertion with a *tp-sul* gene, which confers resistance to sulfadiazine. The *dme-2* mutant allele is due to insertion of a pSKI015 T-DNA (29) with a *BAR* gene, which confers resistance to glufosinate ammonium herbicide (BASTA; Crescent Chemical Co.). For bisulfite sequencing experiments, pollen was collected from ~3,000 *DME/dme-6* and *DME/dme-2* self-pollinated plants. *DME/dme-6* heterozygous plants were selected on Murashige and Skoog media containing 5.25 mg/L sulfadiazine. *DME/dme-2* heterozygous plants were selected by spraying seedlings with BASTA (120 mg/L) at the age of 10, 14, and 17 d after germination.

Pollen Isolation. We isolated pollen as described previously (30) with modifications as described in Schoft et al. (18). Inflorescences from ~1,000 plants were cut and collected in a beaker; 300 mL of 9% sucrose was added, and the beaker was shaken for 1 min. The pollen suspension was then filtered through a 100-μm nylon mesh. Pollen grains were precipitated in 250-mL centrifuge tubes using a Beckman Coulter Avanti J-26 XP centrifuge with the J-10 rotor (1,387 × *g*, 10 min, 4 °C). To purify pollen, the pellet was resuspended in buffer A [1 M sorbitol, 7% ficol PM 400, 20% glycerol, 5 mM MgAc, 3 mM CaCl₂, 5 mM EGTA, 50 mM Tris·HCl (pH 7.5), 2% Triton X-100] and

filtered through a 40-μm nylon mesh. The supernatant was added back to the beaker containing the inflorescences, and the pollen harvesting procedure was repeated once again. The resulting two fractions of pollen suspension were pooled, concentrated in a 15-mL Falcon tube by centrifugation (800 × *g*, 10 min, 4 °C), and then precipitated in a 1.5-mL centrifuge tube (5,100 × *g*, 5 min). This procedure yielded 100–150 μL of pollen. DAPI-stained pollen (*n* = 587) were visualized under the microscope, and 97% were trinuclear with two sperm cells and one vegetative cell nucleus, 1.5% were binuclear with a generative cell and vegetative cell, and 1.5% were microspores.

Extraction of Vegetative Cell Nuclei and Sperm Cells from Pollen. As described previously (18), we disrupted the rigid cell wall of mature pollen grains by vortexing pollen in the presence of glass beads. This procedure leaves the nuclei intact. Fifty-microliter aliquots of pollen suspension were loaded onto 1.8 g of acid-washed glass beads (0.4–0.6 mm, Sartorius) in 2-mL centrifuge tubes and subjected to vortexing using a Retsch MM 400 ball mill for 1.5 min at a frequency of 15 Hz. Subsequently, holes were cut at the bottom of the tubes using a needle (0.45 mm), and the crude vegetative cell nuclei and sperm cells were collected in 1.5-mL centrifuge tubes by centrifugation (800 × *g*, 10 min, 4 °C). The suspensions were pooled, and a DAPI-stained aliquot was inspected by fluorescence microscopy to evaluate the efficiency of pollen extraction and the quality of the nuclei.

Fractionation of Vegetative Cell Nuclei and Sperm by Fluorescence Activated Cell Sorting (FACS). Pollen extract was diluted with an equal volume of buffer B [15 mM Tris·HCl (pH 7.5), 2 mM Na₂EDTA, 0.5 mM spermine·4HCl, 80 mM KCl, 20 mM NaCl, 2% Triton X-100]. The sample (~250 μL) was filtered through a 35-μm nylon mesh and aliquoted into FACS tubes and incubated with 5 μL of SYBR Green I (Roche 11988131001) for 5 min on ice. We separated vegetative cell nuclei from sperm using FACSAria (BD Biosciences), based on differences in their SYBR Green I staining. The sperm and vegetative cell nuclei fractions were collected and digested with proteinase K, and DNA was purified by phenol extraction for bisulphite sequencing. DNA samples from several nuclei sorting experiments were pooled and concentrated with Amicon Ultra-4 (Ultracel-50k) centrifugal filter devices (Millipore).

Bisulfite Sequencing Analysis of DNA Cytosine Methylation. Bisulfite conversion of genomic DNA extracted from sorted sperm and vegetative cell nuclei of wild-type and mutant *Arabidopsis* plants was performed using EpiTect

Bisulfite Kit (Qiagen) according to the manufacturer's suggestions. PCR primers and thermocycling conditions for gene amplification are in *SI Materials and Methods*. PCR products were cloned into the pGEMTeasy vector (Promega), and individual clones were sequenced. DNA cytosine methylation in the CG, CHG, and CHH contexts was analyzed and displayed using CyMATE (31).

Analysis of Pollen RNA. Approximately 30 μL of mature pollen were frozen in liquid N₂ and ground to a fine powder with mortar and pestle. Total RNA was then isolated using RNeasy Plant Mini Kit (Qiagen) according to the manual, followed by two successive digests with DNase I (Roche) [32 μL RNA, 24 μL MgCl₂ (25 mM stock), 2 μL NaAc (3 M stock, pH 5.2), 1 μL RNase inhibitor, and 1 μL DNase; 30 min at 37 °C] and purification with RNeasy MinElute Cleanup Kit (Qiagen) after each digest to completely remove genomic DNA. The reverse transcription was done with SuperScript III Reverse Transcriptase (Invitrogen) with poly(dT) primers and ~0.5 μg of total RNA according to the manual. PCR was performed with 1 μL of a 1:5 dilution of the respective cDNAs using Maxima Hotstart Taq DNA polymerase (Fermentas). Total RNA from sorted sperm cells of wild-type *Arabidopsis* plants was isolated using RNeasy Plus Micro Kit (Qiagen). cDNA was prepared using Message Booster Kit (Epicentre Biotechnologies). Primers and cycling conditions for PCR reactions are listed in Table S2. Negative control reactions (−RT) for testing DNA contamination lacked reverse transcriptase. PCR and RT-PCR amplification conditions, as well as primers used, are shown in Table S2. *DME*, *MEA*, and *FWA* RNAs amplified by these procedures were 458, 157, and 154 bp, respectively. These amplified DNAs were excised from gels and sequenced to validate their identity.

Analysis of Pollen Phenotypes. Methods for pollen germination assays, pollen viability assays, and paternal transmission of *dme* mutant alleles are described in *SI Materials and Methods*.

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PLANT BIOLOGY

Supporting Information

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SI Materials and Methods

Pollen Germination Assays. Pollen germination assay on solid medium for pollen isolated from Col-gl, *DME/dme-1* Col-gl, L*er*, and *DME/dme-1* L*er* was performed as previously described (1). For measuring the germination of pollen from Col-0 and *DME/dme-7* Col-0, pollen was germinated from five flowers of the same plant, the germination efficiency for each flower was calculated, and the highest value was considered to be the pollen germination potential. Three mutant and three wild-type sibling plants were used for each experiment, which was repeated five times. A pollen grain was classified as germinated if the pollen tube length was equal to or greater than the pollen grain diameter.

Paternal Transmission of *dme* Mutant Alleles. Measuring transmission rate of *dme-1* and *dme-2* mutant alleles, as well as the primers for detecting the *BAR* gene in pSKI015 T-DNA of mutant plants, are as described (2). For measuring paternal transmission of *dme-6* and *dme-7* alleles, DNA was isolated from individual

seeds and PCR reactions were performed using primers and conditions described in Table S2.

Pollen Viability Assays. For Alexander staining for pollen viability, stamens from mature flowers were placed on a microscopic slide. A few drops of Alexander stain buffer (95% ethanol, 10 mL; Malachite green (1% in 95% ethanol), 1 mL; Fuchsin acid (1% in water), 5 mL; Orange G (1% in water), 0.5 mL; phenol, 5 g; chloral hydrate, 5 g; glacial acetic acid, 2 mL; glycerol, 25 mL; distilled water, 50 mL) were added. Stained pollen grains were observed with a Zeiss Axio Imager A1 microscope equipped with differential contrast interference microscopy optics. The cytoplasm of a viable pollen grain is stained red purple; the cytoplasm of an aborted pollen grain is green (3).

Real-Time qRT-PCR. RNA levels were quantified by real-time qRT-PCR (CFX96; Bio-Rad) and data were analyzed with the CFX Manager Software (Bio-Rad). Primer sequences for DME transcripts are described in Choi et al. (4).

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3. Alexander MP (1969) Differential staining of aborted and nonaborted pollen. *Stain Technol* 44:117–122.
4. Choi YH, et al. (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis. *Cell* 110:33–42.
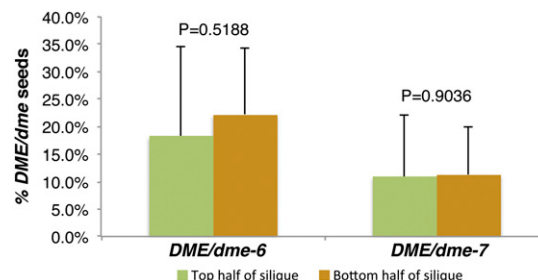
Fig. S1. Mutant *dme* alleles do not impact in vivo pollen tube elongation. Five crosses between *DME/dme-6* or *DME/dme-7* heterozygous males and wild-type (Col-0) females were carried out. Ten days after pollination, the position in the silique and genotype of individual F1 seeds was determined using PCR primers and conditions in Table S2. Random position of heterozygous seeds in the siliques was tested using a two-tailed *t* test. Columns represent the percentage of the *DME/dme* seeds located in top (green) and bottom (orange) half of the siliques. Error bars indicate SD. High *P* values above the bars indicate no significant difference between the number of heterozygous seeds in the top or bottom halves of the siliques.
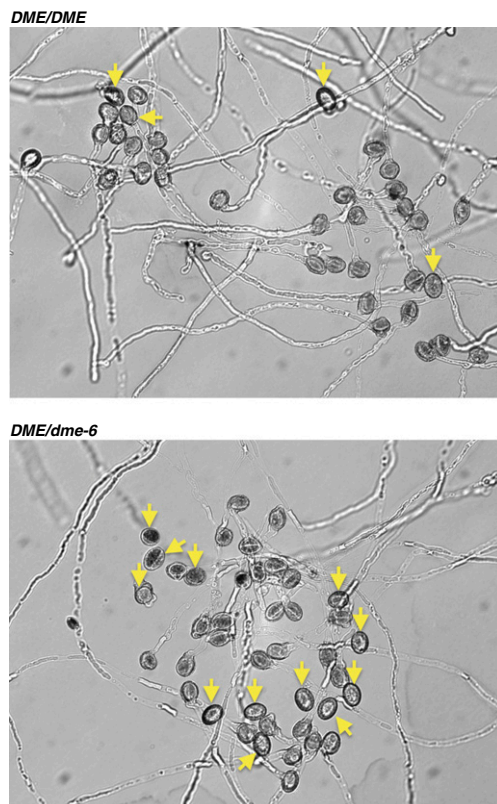
DME/DME

DME/dme-6

Fig. S2. Bright-field images of germinated and nongerminated pollen harvested from wild-type and *DME/dme-6* mutant sibling plants. Images were taken with 20× magnification. Nongerminated pollen grains are marked by an arrow.
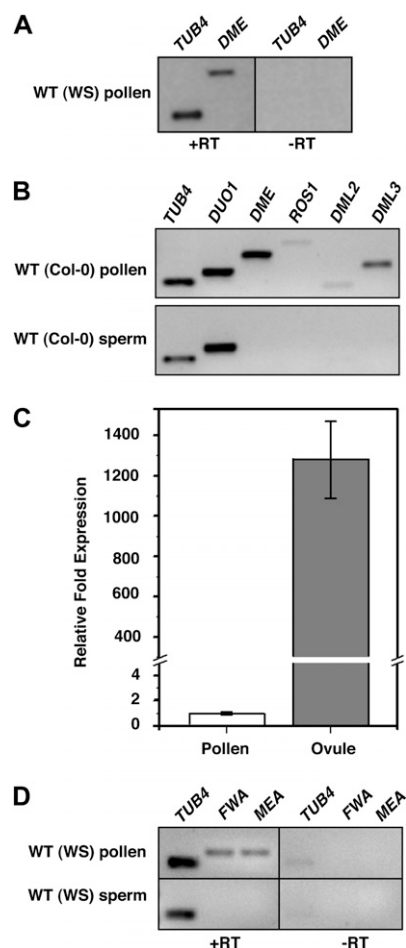
Fig. S3. Analysis of gene expression in pollen, sperm cells, and ovules. (*A*) RT-PCR analysis of *DME* RNA in pollen of wild-type ecotype WS (+RT). Negative controls for testing DNA contamination lacked reverse transcriptase (−RT). (*B*) RT-PCR analysis of *DME*, *ROS1*, *DML2*, and *DML3* transcripts in pollen and sperm cells of wild-type ecotype Col-0. (*C*) Real-time qRT-PCR analysis of *DME* RNA level in wild-type pollen isolated from stage-14 open flowers (Col-0) and un-fertilized ovules from stage-13 ovules (L*er*) (1). Values are plotted relative to expression of *DME* in pollen, which was set at 1.0, and represent the average of triplicate measurements ± SD. (*D*) RT-PCR analysis of *FWA* and *MEA* RNA in pollen and sperm cells of wild-type ecotype WS. *TUB4* RNA, expressed in both pollen and sperm cells, was used as a control for loading equivalent amounts of cDNA on gels. Negative controls for testing DNA contamination lacked RT (−RT).

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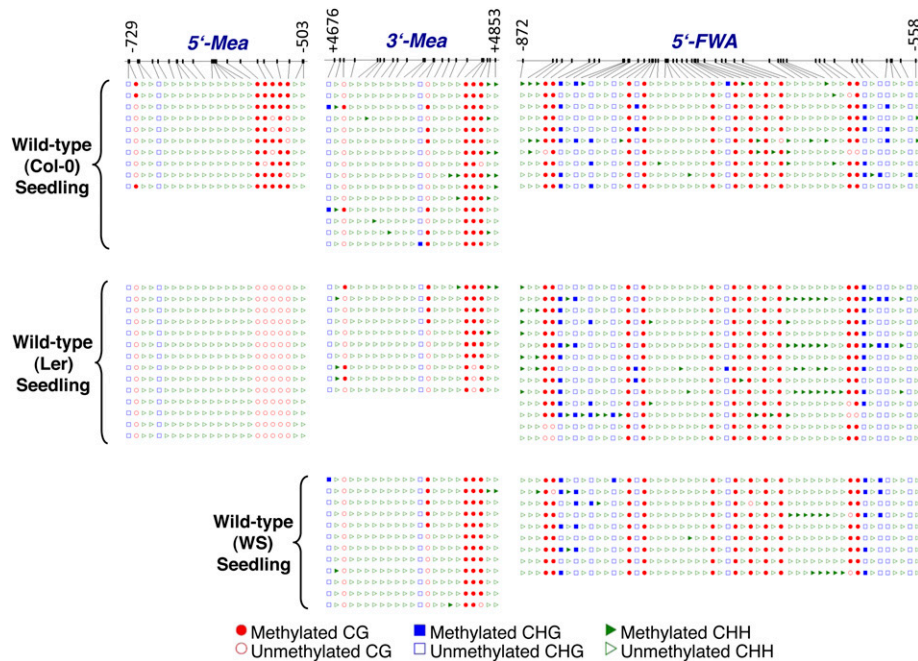
Fig. S4. DNA methylation at 5′-*MEA*, 3′-*MEA*, and 5′-*FWA* flanking regions in seedlings of wild-type plants in Col-0, L*er*, and WS ecotypes. Numbers are from the translation start site. 5-methylcytosines in the CG, CHG, and CHH (H = A, T, or C) contexts were analyzed and displayed using CyMATE (1). Asterisks in red indicate cytosine residues referred to in the text.

1. Hetzl J, Foerster AM, Raidl G, Mittelsten Scheid O (2007) CyMATE: A new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. *Plant J* 51:526–536.

Fig. S5. DNA methylation at 5′-*MEA,* 3′-*MEA,* and 5′-*FWA* flanking regions in sorted sperm and vegetative cell nuclei of wild-type plants in WS ecotype. Numbers are from the translation start site. Asterisks in red indicate cytosine residues referred to in the text. 5-methylcytosines in the CG, CHG, and CHH (H = A, T, or C) contexts were analyzed and displayed using CyMATE (1).

1. Hetzl J, Foerster AM, Raidl G, Mittelsten Scheid O (2007) CyMATE: A new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. *Plant J* 51:526–536.
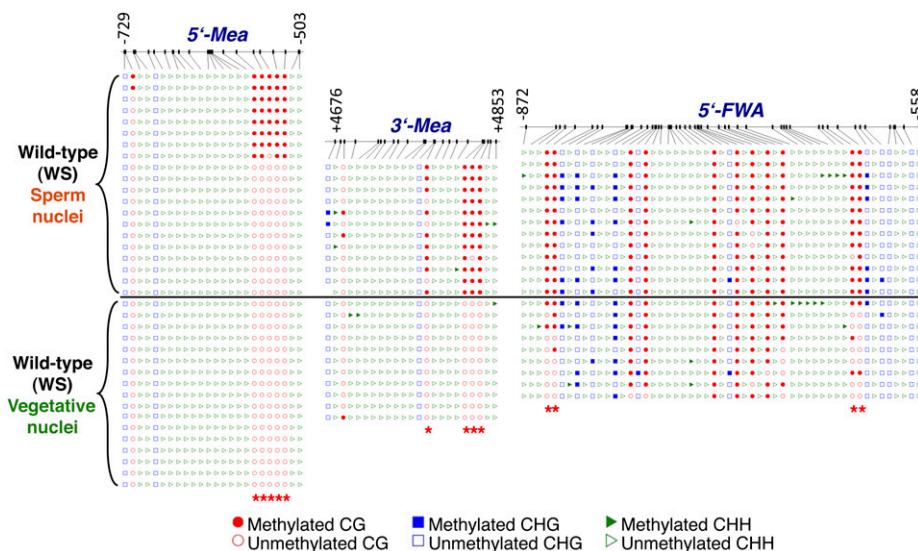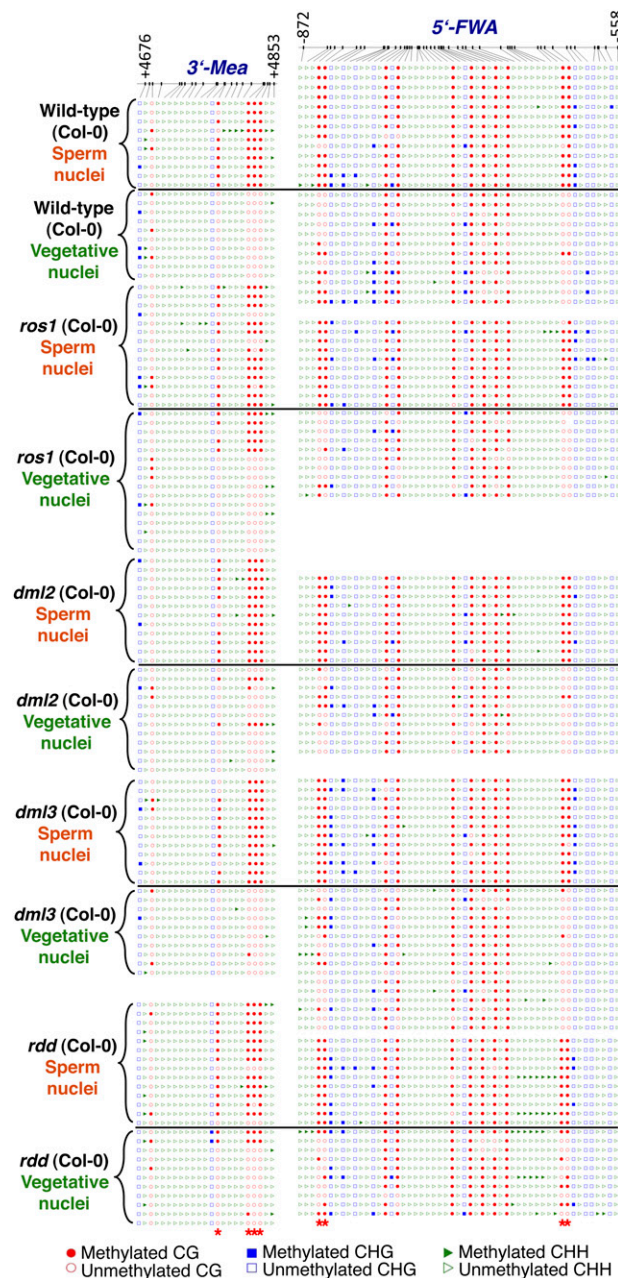
Fig. S6. DNA methylation at 3′-*MEA* and 5′-*FWA* flanking regions in sorted sperm and vegetative cell nuclei of wild-type, *ros1*, *dml2*, and *dml3* single-mutant plants, and *ros1*, *dml2*, and *dml3* triple (*rdd*)-mutant plants in the Col-0 ecotype. Numbers are from the translation start site. 5-methylcytosines in the CG, CHG, and CHH (H = A, T, or C) contexts were analyzed and displayed using CyMATE (1). Asterisks in red indicate cytosine residues referred to in the text.

1. Hetzl J, Foerster AM, Raidl G, Mittelsten Scheid O (2007) CyMATE: A new tool for methylation analysis of plant genomic DNA after bisulphite sequencing. *Plant J* 51:526–536.

Table S1. Seed abortion caused by inheritance of maternal *dme-6* and *dme-7* alleles

Maternal parent	Paternal parent	Viable F1 seeds	Inviable F1 seeds	χ2 for 1:1 segregation	P
DME/dme-6	Wild type	391	389	0.005	>0.94
DME/dme-6	*DME/dme-6*	243	207	1.44	>0.23
DME/dme-7	*DME/dme-7*	158	159	0.003	>0.95

All parents were Col-0 ecotype. Siliques were opened and the number of viable seeds with green embryos, and inviable seeds with aborted embryos, were counted. *P*, probability that deviation from the expected 1:1 segregation is due to random chance, which was calculated by χ² test.

Table S2. PCR and RT-PCR primers

	Primer sequence (top strand)	
Primers for bisulfite sequencing		
Genomic region		Cycling protocol
DDM1	F: GAYGGGAAAAYGGAGAAAGATGY	95 °C 4 min, 35 × (95 °C 30 s, 52 °C 30 s, 72 °C 40 s),
	R: CTTTTTRRCTCTCACTTTCTATCCCAT	72 °C 10 min
5′-FWA (1)	F: GGTTYTATAYTAATATYAAAGAGTTATGGGYYGAAG	95 °C 4 min, 35 × (95 °C 30 s, 57 °C 30 s, 72 °C 40 s),
	R: CAAARTACTTTACACATAARCRAAAAACARACAAATC	72 °C 10 min
5′-MEA	F: TTGAATGTTTTTGAGATTTTTTTATTTTTATTTTYTATTT	95 °C 4 min, 35 × (95 °C 30 s, 50 °C 30 s, 72 °C 30 s),
	R: TATTCATTCCTARCTARTTTTCTATTTAARC	72 °C 10 min
3′-MEA (2)	F: AAAGTGGYTGTAGYTTAYGAAAGGTTTTA	95 °C 4 min, 35 × (95 °C 30 s, 52 °C 30 s, 72 °C 30 s),
	R: CTTAAAARATTTTCAACTCATTTTTTTTAAAARRTTT	72 °C 10 min
Mu1a	F: AAATATATTAATYYAAGATGTAYYAYAGGTYAAG	95 °C 4 min, 35 × (95 °C 30 s, 50 °C 30 s, 72 °C 30 s),
	R: CTATARRCARCCCTTTTAARATATCAC	72 °C 10 min
Primers for RT-PCR		
cDNA		Cycling protocol
TUB4 AT5G44340	F: ATCCCAAACAACGTCAAGTCC	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 30 s, 72 °C 30 s),
	R: CTCTCCGGCTGTAGCATCTTGGTAC	72 °C 5 min
	F: CCGGAGAGGAAGAGTACGAGGA	95 °C 4 min, 35 × (95 °C 20 s, 59 °C 20 s, 72 °C 20 s),
	R: GGGGAAAGAAGAAAGTGAATGCTGCT	72 °C 5 min
DME AT5G04560	F: GAAGTGTGGAGGGAAAGCGTCT	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 30 s, 72 °C 30 s),
	R: CAATGCGTTTGCTTTCTTCCAGTCA	72 °C 5 min
FWA AT4G25530	F: CACTGCGAAAGCCTCTCGACC	95 °C 4 min, 35 × (95 °C 20 s, 59 °C 20 s, 72 °C 20 s),
	R: GCCACTTGTCCACCGAAGGACTC	72 °C 5 min
MEA AT1G02580	F: GATCAGAGGATTGGTCTATTTGCGGAG	95 °C 4 min, 35 × (95 °C 20 s, 59 °C 20 s, 72 °C 20 s),
	R: GAGCTGGACGGGCTTCCTTAGAC	72 °C 5 min
DUO1 AT3G60460 (3)	F: AACGTCAAACCAATCCGTCAATCC	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 30 s, 72 °C 30 s),
	R: CGAACAATGGCTCAGAAGAATCAGC	72 °C 5 min
ROS1 AT2G36490	F: AGAAGAAATTCCTACCATCA	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 20 s, 72 °C 30 s),
	R: ACCGTTCTTCGAGGTAATTC	72 °C 5 min
DML2 AT3G10010	F: CATCAGAGGATCCTTGGGAA	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 20 s, 72 °C 30 s),
	R: TCGCCTCTCAAACCCTTCTA	72 °C 5 min
DML3 AT4G34060	F: GCGATTTGGCAACCAGGTGAAACATC	95 °C 4 min, 35 × (95 °C 30 s, 53 °C 20 s, 72 °C 30 s),
	R: CCCTGTCCAAAAGCAAAGTTCAATCCG	72 °C 5 min
Primers for genotyping dme-6 and dme-7 mutant alleles		
Allele		Cycling protocol
WT sibling of DME/dme-6	F: CTGGTTGTAGGAAACTCTGCG	95 °C 5 min, 40 × (95 °C 15 s, 55 °C 15 s, 72 °C 60 s),
	R: AACTGATGTGTCCAAACCAGC	72 °C 5 min
DME/dme-6	F: ATATTGACCATCATACTCATTGC	95 °C 5 min, 40 × (95 °C 15 s, 55 °C 15 s, 72 °C 45 s),
	R: AACTGATGTGTCCAAACCAGC	72 °C 5 min
WT sibling of DME/dme-7	F: TATTGTTGGGATCTCGTCAGG	95 °C 5 min, 40 × (95 °C 15 s, 55 °C 15 s, 72 °C 60 s),
	R: CTTGCTTTCCCTGTGAGTCAG	72 °C 5 min
DME/dme-7	F: TGGTTCACGTAGTGGGCCATCG	95 °C 5 min, 40 × (95 °C 15 s, 55 °C 15 s, 72 °C 45 s),
	R: CTTGCTTTCCCTGTGAGTCAG	72 °C 5 min

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