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DEMETER, a DNA Glycosylase Domain Protein, Is Required for Endosperm Gene Imprinting and Seed Viability in *Arabidopsis*

Yeonhee Choi,[1] Mary Gehring,[1] Lianna Johnson,[2] Mike Hannon,[1] John J. Harada,[3] Robert B. Goldberg,[2] Steven E. Jacobsen,[2,4] and Robert L. Fischer[1,5]

[1]Department of Plant and Microbial Biology
University of California, Berkeley
Berkeley, California 94720

[2]Department of Molecular, Cell, and Developmental Biology
University of California, Los Angeles
Los Angeles, California 90095

[3]Section of Plant Biology
Division of Biological Sciences
University of California, Davis
Davis, California 95616

[4]Molecular Biology Institute
University of California, Los Angeles
Los Angeles, California 90095

Summary

We isolated mutations in *Arabidopsis* to understand how the female gametophyte controls embryo and endosperm development. For the *DEMETER* (*DME*) gene, seed viability depends only on the maternal allele. *DME* encodes a large protein with DNA glycosylase and nuclear localization domains. *DME* is expressed primarily in the central cell of the female gametophyte, the progenitor of the endosperm. *DME* is required for maternal allele expression of the imprinted *MEDEA* (*MEA*) Polycomb gene in the central cell and endosperm. Ectopic *DME* expression in endosperm activates expression of the normally silenced paternal *MEA* allele. In leaf, ectopic *DME* expression induces *MEA* and nicks the *MEA* promoter. Thus, a DNA glycosylase activates maternal expression of an imprinted gene in the central cell.

Introduction

In flowering plants, the female gametophyte is the progenitor of the embryo and endosperm. Much is understood about female gametophyte morphology, and genes necessary for female gametophyte development and function have been identified (Drews et al., 1998). However, little is known about the molecular and genetic processes taking place in the female gametophyte that affect subsequent embryo and endosperm development.

The female gametophyte is formed within the ovule. In *Arabidopsis*, a haploid spore undergoes three mitotic divisions to form an 8-nucleus, 7-cell female gametophyte containing the egg, central, synergid, and antipodal cells. Before fertilization, a diploid nucleus is formed in the central cell by the fusion of two haploid nuclei. The endosperm is derived from fertilization of the central cell by a sperm cell; fertilization of the adjacent egg cell by a second sperm cell gives rise to the embryo (Brown et al., 1999). Thus, double fertilization generates a seed with a triploid endosperm and diploid embryo. The embryo generates organs (axis and cotyledon), tissues (protoderm, procambium, and ground meristem), and meristems (shoot and root). The fertilized central cell replicates to form a syncytium, and following cellularization, produces storage proteins, lipids, and starch, and mediates the transfer of nutrients from maternal tissues to be absorbed by the embryo (Brown et al., 1999).

To gain insights into the maternal control of embryo and endosperm development, mutations in a small number of genes have been identified where seed viability depends upon the genotype of the maternal allele. For example, these studies have shown that the female gametophyte provides the embryo with an MCM-related protein, PROLIFERA (PRL), necessary for cytokinesis (Springer and Holding, 2002). Also, wild-type maternal alleles encoding Polycomb group proteins are necessary for proper female gametophyte and seed development. *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) encode SET-domain, WD domain, and zinc finger Polycomb group proteins (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999; Ohad et al., 1999; Birve et al., 2001). Polycomb group proteins repress gene transcription by forming complexes that remodel chromatin structure at specific regions within the genome (Francis and Kingston, 2001). One function of *MEA*, *FIE*, and *FIS2* is to prevent the onset of central cell proliferation and endosperm development prior to fertilization and to repress endosperm growth and development after fertilization (Kiyosue et al., 1999; Vinkenoog et al., 2000). Thus, to date, no genes have been discovered that function primarily prior to fertilization in the female gametophyte to control processes essential for subsequent embryo and endosperm development after fertilization.

Because only the maternal allele is required for seed viability, loss-of-function *mea*, *fie*, *fis2*, and *prl* mutations cause parent-of-origin effects on seed viability. For example, inheritance of a mutant maternal *mea* allele results in seed abortion, even when the paternal *MEA* allele is wild-type. By contrast, inheritance of a mutant paternal *mea* allele has no detectable effect on seed viability. The parent-of-origin effects of *mea* mutations are due, at least in part, to its being an imprinted gene in the endosperm (Kinoshita et al., 1999; Vielle-Calzada et al., 1999; Luo et al., 2000). Only the maternal *MEA* allele is expressed throughout endosperm development while the paternal allele is silenced (Kinoshita et al., 1999). In this regard, *MEA* is distinct, as the *FIE* and *PRL* genes are not imprinted throughout seed development (Yadegari et al., 2000; Springer and Holding, 2002). It is unknown how *MEA* imprinted gene expression is regulated in the endosperm.

We isolated mutations, named *demeter* (*dme*), causing parent-of-origin effects on seed viability to understand how the female gametophyte controls embryo and endosperm development. We found that seed viability depended solely on the maternal *DME* allele. *DME* encodes a 1729 amino acid polypeptide that contains a

[5]Correspondence: rfischer@uclink4.berkeley.edu

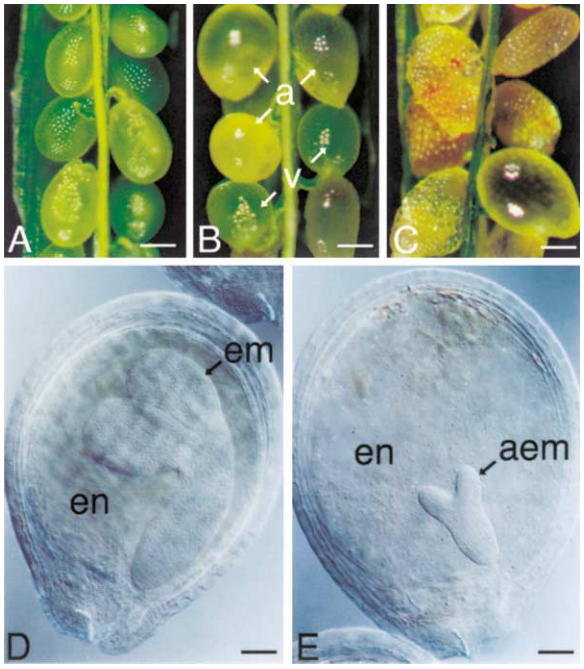

Figure 1. Effect of the *dme-1* Mutation on Embryo and Endosperm Development

(A) Wild-type silique harvested 9 days after pollination.
(B) Heterozygous *DME/dme-1* silique harvested 9 days after pollination.
(C) Homozygous *dme-1* silique harvested 9 days after pollination.
(D) Viable seed obtained from silique in (B).
(E) Aborted seed obtained from silique in (B).
Bars, 0.5 mm (A–C) and 0.1 mm (D and E). a, aborted seed; aem, aborted embryo; em, embryo; en, endosperm; v, viable seed.

Figure 2. Developmental Abnormalities in Homozygous *dme-1* Mutant Plants

Light micrographs of homozygous *dme-1* and wild-type flowers and floral organs are shown. The percentage of wild-type (n = 105) and *dme-1* (n = 138) flowers with reduced number of petals and sepals was 0% and 3%; increased number of petals and sepals was 2% and 14%; stamens with fused filaments or petal-like anthers was 0% and 9%.
(A) Wild-type flower.
(B) *dme-1* flower with 2 sepals and 2 petals.
(C) *dme-1* flower with 7 sepals and 7 petals.
(D) Wild-type stamen.
(E) Stamens from *dme-1* flower. Arrows point to anthers on a fused filament.
(F) Petal-like stamens from *dme-1* flowers. a, anther-like region; p, petal-like region.
(G) Wild-type gynoecium.
(H) *dme-1* flower with 2 gynoecia.
(I) *dme-1* flower with unfused carpels. Arrow points to large stigma associated with unfused carpels.
Bars in (A–C), (H–I) represent 0.5 mm. Bars in (D–G) represent 0.1 mm.

DNA glycosylase domain and a highly basic region with a nuclear localization signal. *DME*, primarily expressed in the central cell, is required for maternal allele expression of *MEA* in the central cell and the endosperm. When *DME* was ectopically expressed in the endosperm, the expression of the normally silenced paternal *MEA* allele was detected. Ectopic *DME* expression in the leaf activated *MEA* expression and generated nicks in the *MEA* promoter DNA. These results suggest DME is a DNA glycosylase that controls maternal expression of an imprinted maternal gene in the central cell, a process that is essential for subsequent embryo and endosperm viability.

Results

Only the Maternal *DME* Allele Is Necessary for Seed Viability

Whereas seeds from wild-type plants rarely abort (Figure 1A), self-pollinated heterozygous *DME/dme-1* siliques (Figure 1B) have approximately equal numbers of viable and nonviable seeds (552:569, 1:1, $\chi^2 = 0.26$, $P > 0.7$). Likewise, *DME/dme-1* plants pollinated with wild-type pollen produced siliques with approximately equal numbers (148:147, 1:1, $\chi^2 = 0.003$, $P > 0.95$) of viable seeds with normal embryos (Figure 1D) and nonviable seeds with enlarged endosperm and aborted embryos (Figure 1E). All F_1 viable seeds from this cross were homozygous wild-type *DME*. Thus, inheritance of a *dme-1* mutant allele by the female gametophyte resulted in embryo and endosperm abortion even when a wild-type paternal *DME* allele was inherited. When the reciprocal cross was performed, siliques had no aborted F_1 seed and F_1 plants displayed a 1:1 segregation of the wild-type and *DME/dme-1* genotype (173:142, $\chi^2 = 3.1$, $P > 0.1$). Thus, seed viability depends only upon the presence of a wild-type maternal *DME* allele, and the paternal allele is expendable.

DME Prevents Sporadic Developmental Abnormalities

As described below, *dme-1* is a weak allele, allowing for rare mutant maternal allele transmission and the formation of homozygous *dme-1* plants. Homozygous *dme-1* plants generated normal rosette leaves, an inflorescence, and produced siliques containing nearly all (98%) aborted seeds (Figure 1C). However, we occasionally observed developmental abnormalities in mutant plants. For example, the *Arabidopsis* flower (Figure 2A) is normally composed of four sepals, four petals, six pollen-bearing stamens (Figure 2D), and two ovule-bearing carpels that form the gynoecium (Figure 2G). By contrast, homozygous *dme-1* plants sporadically formed individual flowers with reduced (Figure 2B) or increased (Figure 2C) petal and sepal number. We observed flowers with fused stamen filaments (Figure 2E), petal-like anthers (Figure 2F), two gynoecia (Figure 2H),

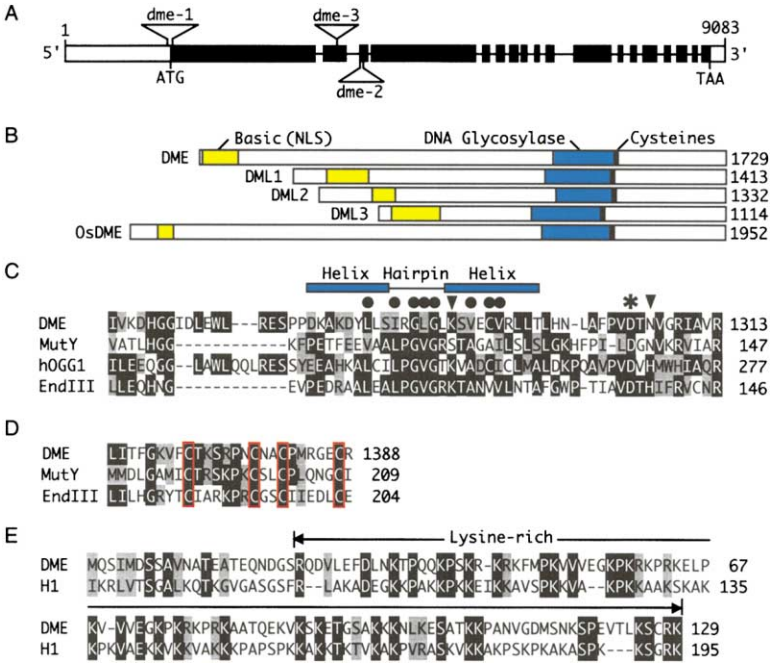

Figure 3. *DME* Gene and Protein Structure

(A) The *DME* gene. Transcription begins at 1 and ends at 9083. *dme-1* T-DNA is associated with a 177 base pair deletion. *dme-2* T-DNA is associated with a 48 base pair deletion. *dme-3* T-DNA is associated with 12 base pairs of unknown origin. Black box, translated exon; white box, untranslated exon; line, intron.

(B) Conservation of DNA glycosylase domain, cysteine cluster, and lysine-rich region in *Arabidopsis DME* gene family and rice (*Oryza sativa*, Os). GenBank Accession numbers for *DME*, *DML1*, *DML2*, *DML3*, and *OsDME* are AF521596, At2g36490, At3g10010, At4g34060, and BAB16489.1. EST for *OsDME* is AU056357. Compared to DME, percent amino acid sequence identity was 62% over 569 DML1 residues, 55% over 554 DML2 residues, 45% over 519 DML3 residues, and 47% over 1114 OsDME residues. Predicted polypeptide size is shown on the right.

(C) Comparison of the DME helix-hairpin-helix domain to DNA glycosylases. MutY, *E. coli* monofunctional adenine glycosylase; hOGG, human 8-oxoguanine bifunctional DNA glycosylase; and EndIII, *E. coli* bifunctional endonuclease III. Conserved aspartic acid is indicated with an asterisk. Lysine and histidine residues conserved in bifunctional glycosylases are indicated with triangles. Position of helices and hairpin determined with the Jpred (http://jura.ebi.ac.uk:8888/) program.

(D) Comparison of DME to DNA glycosylases with a $(4Fe\text{-}4S)^{2+}$ cluster. Conserved cysteines are in red boxes.

(E) Comparison of DME to *Xenopus laevis* histone H1 (GenBank Accession number P22844).

and improperly fused carpels (Figure 2I). Sporadic abnormalities in leaf and stem morphology were also detected (data not shown). Thus, in *Arabidopsis*, the *DME* gene is required for stable, reproducible patterns of floral and vegetative development. Preliminary efforts to transmit these developmental abnormalities to subsequent generations have not been successful, suggesting that the lesions responsible for the defects are not stable, or that they did not occur in cells that give rise to gametes in *Arabidopsis* (Irish and Sussex, 1992).

Cloning the *DME* Gene

Three mutant T-DNA alleles *dme-1*, *dme-2*, and *dme-3* were obtained (Figure 3A). Each T-DNA cosegregated with the seed abortion phenotype (data not shown). To isolate the *DME* gene, plant DNA flanking the *dme-2* T-DNA was isolated and used to clone the wild-type *DME* gene and cDNA. We rescued the *dme* seed abortion phenotype by introducing a transgene composed of 3.4 kb of 5′-flanking *DME* genomic sequence ligated to a full-length *DME* cDNA (see Experimental Procedures). The *dme-2* and *dme-3* alleles are probably null alleles as their respective T-DNAs inserted into the middle portion of the *DME* gene. The weak *dme-1* allele was created by insertion of the T-DNA at the boundary of the 5′-untranslated region. Low-level transcription from within the *dme-1* T-DNA was detected (data not shown) that would produce a slightly truncated (46 amino acids) DME polypeptide.

DME Encodes a DNA Glycosylase Domain Protein

The *DME* cDNA encodes a 1,729 amino acid protein. A conserved domain search of NCBI databases revealed a 201 amino acid domain (Figure 3B; amino acids 1167–1368) related to the helix-hairpin-helix superfamily of base excision DNA repair proteins (Pfam score of $3e^{-15}$, http://www.sanger.ac.uk/Software/Pfam/). The hallmark of the base excision DNA glycosylase superfamily is a helix-hairpin-helix structural element followed by a glycine/proline-rich loop and a conserved aspartic acid (Krokan et al., 1997; Bruner et al., 2000; Scharer and Jiricny, 2001) all of which are present in DME (Figure 3C). Very highly conserved glycines (G1282 and G1284) are present within the conserved DME hairpin. A conserved aspartic acid (position 1304) present in all DNA glycosylases is distal to the helix-hairpin-helix domain and serves as the electron donor in the base excision reaction. There are two classes of DNA glycosylases. Bifunctional glycosylases couple base excision (DNA glycosylase activity) with 3′-phosphodiester bond breakage (DNA nicking activity). Monofunctional enzymes have DNA glycosylase activity and an AP (apurinic or apyrimidinic) endonuclease is responsible for nicking the DNA (Bruner et al., 2000; Jiricny, 2002). DME is predicted to be a member of the monofunctional class of DNA glycosylases (e.g., MutY and AlkA) where the conserved aspartic acid deprotonates a water molecule, which then displaces the damaged or mismatched base by nucleophilic attack at the anomeric center. Like all monofunctional DNA glycosylases, DME lacks a histidine (position 1306) essential for bifunctional DNA glycosylases (EndoIII and hOGG1) and like monofunctional MutY has asparagine at this position (Figure 3C). DME also has four conserved cysteine residues (Figure 3D) adjacent to the DNA glycosylase domain (Figure 3B) that function to hold a $(4Fe\text{-}4S)^{2+}$ cluster in place. This

cluster, found in many DNA glycosylases, is thought to play a role in DNA binding. Thus, *DME* encodes each of the amino acid residues essential for DNA glycosylase activity.

DME also encodes an amino-terminal 129 amino acids that are highly basic and are related to the carboxy-terminal domain of a *Xenopus laevis* H1 linker histone (31% identity, Figure 3E) that binds linker DNA in chromatin (Kasinsky et al., 2001). It is possible that this basic region of DME facilitates interactions with DNA or chromatin. A bipartite nuclear localization signal is in the basic region (amino acids 43–78).

Three additional *DME-like* (*DML*) genes in the *Arabidopsis* genome, *DML1*, *DML2*, and *DML3*, encode a family of related high molecular weight DNA glycosylase domain proteins (Figure 3B). The structure and organization of the DNA glycosylase domain, the conserved cysteine residues, and the nuclear localization signal are all conserved. Moreover, a highly related gene, *OsDME* (Figure 3B), is expressed in rice suggesting that DME structure and function has been conserved during flowering plant evolution.

Pattern of *DME* RNA Accumulation and Promoter Activity

Measurement of *DME* RNA levels by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) procedures showed that the *DME* RNA is most abundant in immature flower buds (Figure 4A). As flowers mature, *DME* RNA decreases to a low level (Figure 4A). Analysis of dissected flowers revealed that *DME* RNA was abundant in the ovule-bearing carpels and not detectable in pollen-bearing stamens (Figure 4A). *DME* RNA was detected in developing (stage 12) and mature (stage 14) ovules (Figure 4A). However, after fertilization, the level of *DME* RNA dramatically decreased in developing seeds (Figure 4A). These results show that high level *DME* expression is specifically associated with maternal reproductive structures prior to fertilization.

To visualize *DME* gene expression, we transformed *Arabidopsis* plants with two chimeric genes, each with 2.3 kb of 5′-flanking *DME* sequences, 1.9 kb of sequences encoding 148 amino acids of *DME* spanning the putative nuclear localization signal, ligated to the β-*glucuronidase (DME::GUS)* reporter gene (Jefferson et al., 1987) or to the *GREEN FLUORESCENT PROTEIN* (*DME::GFP*) reporter gene (Niwa et al., 1999). Multiple independently isolated lines displayed the same pattern of reporter gene expression. As shown in Figure 4B, GUS staining was detected in the two unfused polar nuclei in the central cell, which will form the diploid nucleus of the central cell, as well as in the synergid cells. The polar nuclei and the synergid cells derive from the third of the haploid mitoses that generate the female gametophyte and are thus closely related in time and space. Later in the development of the mature unfertilized female gametophyte, when the polar nuclei had fused to form the central cell nucleus, GUS staining was primarily detected in the central cell (Figure 4C). No GUS staining was detected in developing (data not shown) or in mature anthers and pollen grains (Figure 4D). Thus, *DME* promoter activity is associated with female gametophyte development, consistent with the expression of the endogenous *DME* gene (Figure 4A). The *DME::GFP* reporter gene showed a similar pattern of expression (Figure 4E). After fertilization, *DME::GFP* promoter activity rapidly decreased. GFP fluorescence was no longer detected prior to the first division of the primary endosperm nucleus (Figure 4F). Nor was GFP fluorescence detected during subsequent endosperm or embryo development (Figures 4G and 4H). These results are consistent with RT-PCR analysis of endogenous *DME* gene expression in prefertilization ovules and developing seed (Figure 4A). These results show *DME* promoter activity is detectable before fertilization in the female gametophyte, primarily in cells leading to the formation of the central cell. Finally, the GFP and GUS used in the construction of reporter transgenes lack any subcellular localization sequences (Niwa et al., 1999). Hence, localization of GUS activity and GFP fluorescence to nuclei (Figures 4B, 4C, and 4E) is due to *DME*-encoded nuclear localization sequences.

DME Regulates *MEA* Expression

Seed viability depends on wild-type maternal *DME*, *MEA*, *FIE*, and *FIS2* alleles that are expressed in the female gametophyte. However, *DME* is distinct in two ways; only *DME* encodes a DNA glycosylase domain protein, and only *DME* is not expressed after fertilization in the embryo and endosperm. One possibility is that the *DME* DNA glycosylase gene controls seed development in a pathway that does not include the *MEA*, *FIE*, and *FIS2* Polycomb group genes. Alternatively, *DME* and the Polycomb group genes may be part of the same pathway. For example, *DME* may be necessary for expression or activity of *MEA*, *FIE*, or *FIS2*. To understand the relationship between the *DME* DNA glycosylase gene and the *MEA*, *FIE*, and *FIS2* Polycomb group genes, we measured their respective RNA levels in wild-type and mutant genetic backgrounds. *MEA, FIE*, and *FIS2* RNAs accumulated in developing and mature wild-type flowers. *MEA* RNA was not detected in homozygous *dme-1* flowers, whereas *FIE* and *FIS2* RNA accumulation was not significantly affected by the *dme-1* mutation (Figure 5A), suggesting that *DME* is required for floral *MEA* expression. Moreover, homozygous *mea* plants accumulate normal levels of floral *DME* RNA (Figure 5B), demonstrating that *MEA* is not required for *DME* expression. Thus, *DME* is necessary for *MEA* gene expression prior to fertilization.

To understand the spatial and temporal control of *MEA* gene expression by *DME* during ovule and seed development, we observed the effect of the *dme-1* mutation on transcription of a *MEA::GFP* transgene. A single locus of the *MEA::GFP* transgene consisting of approximately 4.2 kb of *MEA* 5′-flanking sequences ligated to the *GFP* reporter gene was introduced into wild-type *DME*/*DME* plants. Approximately one-half of prefertilization ovules from transgenic plants hemizygous for the *MEA::GFP* transgene displayed strong fluorescence in the central cell nucleus and cytoplasm prior to fertilization (data not shown), consistent with Mendelian inheritance of the *MEA::GFP* transgene by one-half of the female gametophytes. In a plant hemizygous for the *MEA::GFP* transgene and heterozygous *DME /dme-1*, one-fourth of the prefertilization female gametophytes are predicted to inherit both the wild-type *DME* allele and the *MEA::GFP* transgene, whereas one-fourth will

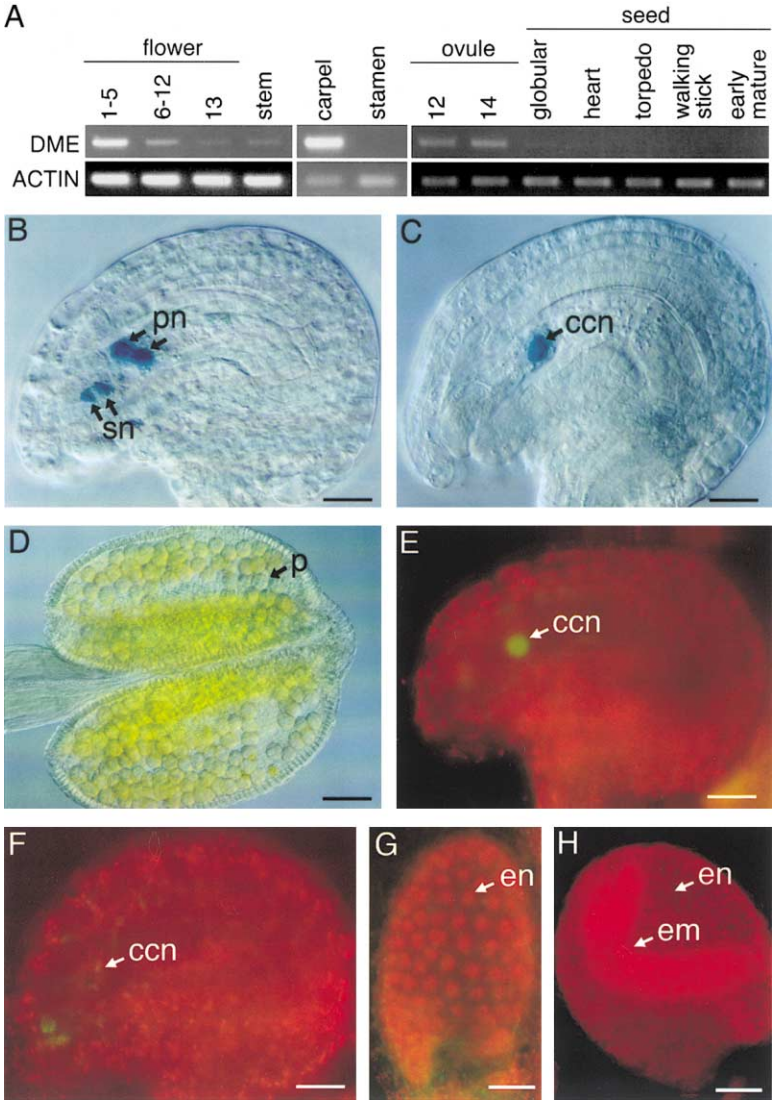

Figure 4. Regulation of *DME* RNA Accumulation and Promoter Activity

(A) Expression of *DME* in wild-type flowers, floral organs, ovules, and seeds. Carpels and stamens were dissected from stage 12 flowers. Ovules were isolated from stage 12 and 14 flowers. Seeds were isolated with embryos at the indicated stages. Upper lane designations refer to tissues used for total RNA isolation. Floral stages are as described (Bowman, 1994). Left-hand side designations refer to the gene specific primers used to amplify RNA by RT-PCR. (B)–(D) are light micrographs of ovules and stamens from transformed plants homozygous for a *DME::GUS* transgene. (E)–(H) are fluorescence micrographs of transformed plants homozygous for a *DME::GFP* transgene. GFP and chlorophyll florescence was converted to green and red, respectively. ccn, central cell nucleus; en, endosperm; em, embryo; p, pollen; pn, polar nucleus; sn, synergid cell nucleus.

(B) Unfertilized ovule prior to fusion of polar nuclei. Bar represents 0.01 mm.

(C) Mature unfertilized ovule after fusion of polar nuclei. Bar represents 0.01 mm.

(D) Mature stamen showing anther and pollen. Bar represents 0.005 mm.

(E) Mature unfertilized ovule after fusion of polar nuclei. Bar represents 0.01 mm.

(F) Seed 8 hr after pollination with wild-type pollen. Bar represents 0.01 mm.

(G) Seed 90 hr after pollination with wild-type pollen. Bar represents 0.3 mm.

(H) Seed at the walking stick stage of embryo development. Bar represents 0.6 mm.

inherit the mutant *dme-1* allele along with the *MEA::GFP* transgene. We found that approximately one-fourth of the prefertilization ovules displayed GFP fluorescence in their central cells (153:396, fluorescent:dark, 1:3, $\chi^2 = 2.4$, $P > 0.15$), suggesting that female gametophytes inheriting the *dme-1* mutant allele did not express the *MEA::GFP* transgene (Figure 5C). Similar results were obtained in an independently isolated transgenic line where the same *MEA* 5′-flanking sequences were ligated to a *GUS* reporter (81:279, blue central cell:colorless central cell, 1:3, $\chi^2 = 1.2$, $P > 0.37$). Thus, a wild-type *DME* allele is necessary for transcription of the *MEA::GFP* and *MEA::GUS* transgenes in the central cell of the female gametophyte prior to fertilization.

Normally, the maternal *MEA* allele is expressed after fertilization in the endosperm (Kinoshita et al., 1999) at a time when *DME* is not expressed (Figure 4). We determined the effect of the maternal mutant *dme-1* allele on postfertilization expression of the maternal *MEA::GFP* transgene to see if expression of *DME* in the central cell of the female gametophyte prior to fertilization was necessary for postfertilization maternal *MEA::GFP* allele transcription during endosperm development. Flowers hemizygous for the *MEA::GFP* transgene and heterozygous *DME/dme-1* were pollinated with wild-type nontransgenic pollen. We observed approximately one-fourth seeds with GFP fluorescence (123:332, fluorescent:dark, 1:3, $\chi^2 = 0.95$, $P > 0.4$) in endosperm cells at 24 hr (Figure 5D) and 90 hr (Figures 5E and 5F) after pollination. This result suggests that female gametophytes that inherited the *dme-1* mutant allele did not express the *MEA::GFP* transgene in the endosperm after fertilization. Thus, the maternal wild-type *DME* allele, expressed prior to fertilization in the female gametophyte, is necessary for transcription of the maternal *MEA::GFP* transgene after fertilization during endosperm development. These results are consistent with the model that *DME* controls maternal *MEA* allele expression in the endosperm, and that the parent-of-origin effects of *dme* mutations on seed viability are due, at least in part, to a failure to express the maternal *MEA* allele during female gametophyte and early seed development.

To determine if *DME* is sufficient for *MEA* gene expression, we generated *CaMV::DME* transgenic plants where transcription of *DME* is under the control of the cauli-

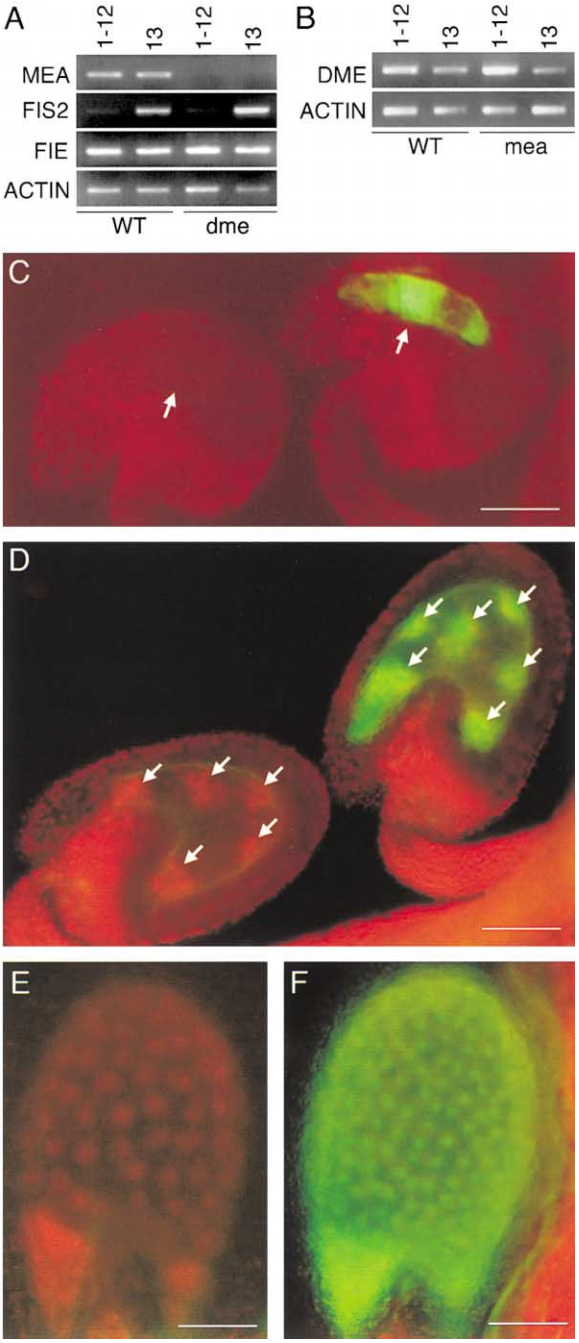

Figure 5. *DME* Controls *MEA* RNA Accumulation and Promoter Activity

For (A) and (B), RNA was isolated from developing floral buds (stage 1-12) and open flowers (stage 13). Floral stages are as described (Bowman, 1994). Left-hand side designations refer to the gene specific primers used to amplify RNA by RT-PCR. Lower lane designations specify the genotype; WT, wild-type; dme, homozygous third generation *dme-1*; mea, homozygous *mea-3*.

(A) *MEA* RNA does not accumulate in *dme-1* flower buds.

(B) *DME* RNA accumulation is the same in wild-type and *mea-3* flower buds. For (C)–(F), fluorescence micrographs were taken from *DME / dme-1* plants hemizygous for a *MEA::GFP* transgene. GFP and chlorophyll fluorescence was converted to green and red, respectively.

(C) Unfertilized ovules from stage 12 flowers. Arrows point to central cell nuclei. Bar represents 0.04 mm.

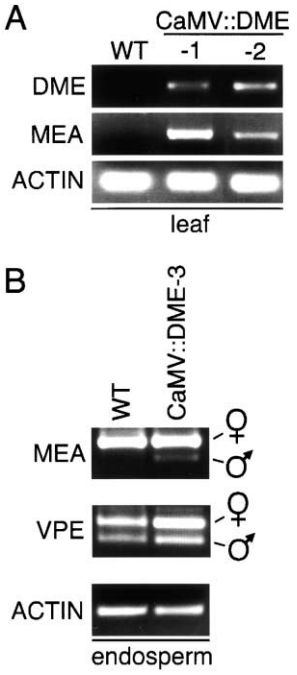

Figure 6. Ectopic *DME* Expression Activates *MEA* Gene Transcription

CaMV::DME-1, CaMV::DME-2, and CaMV::DME-3 represent three independently isolated transgenic lines. WT, wild-type; leaf, cauline leaf.

(A) *DME* is sufficient for *MEA* expression in the leaf.

(B) A *CaMV::DME* transgene activates paternal *MEA* allele gene expression in the endosperm. Endosperm and embryos were dissected from F_1 seeds obtained 7 days after wild-type (Columbia *gl* ecotype) plants or *CaMV::DME* (Columbia *gl* ecotype) were pollinated with RLD ecotype pollen. Control biallelic expression of the *VACUOLAR PROCESSING ENZYME* (*VPE*) gene is shown. For the actin control, no attempt was made to distinguish between maternal and paternal alleles.

flower mosaic virus (*CaMV*) promoter (Rogers et al., 1987). Whereas *DME* and *MEA* gene expression were not detectable in wild-type leaf, both *DME* and *MEA* RNAs were present in leaves from independently isolated *CaMV::DME* transgenic lines (Figure 6A). Thus, *DME* is sufficient to activate *MEA* gene expression in the leaf.

The mechanism for paternal *MEA* allele silencing in the endosperm is not known. It is possible that restriction of *DME* expression to the female gametophyte prevents activation of paternal *MEA* allele transcription in the endosperm. To test this hypothesis, we used wild-type pollen (RLD ecotype) to pollinate control wild-type (Columbia *gl* ecotype) plants and *CaMV::DME* transgenic (Columbia *gl* ecotype) plants (Figure 6B). We isolated and dissected F_1 seeds, isolated RNA from the endosperm (plus maternal seed coat), and measured the level of maternal and paternal *MEA* RNA using ecotype-spe-

(D) Seeds 24 hr after pollination with wild-type pollen. Arrows point to endosperm nuclear-cytoplasmic domains. Bar represents 0.08 mm.

(E and F) Seeds 90 hr after pollination with wild-type pollen. Bar represents 0.2 mm.

cific restriction endonuclease polymorphisms (Kinoshita et al., 1999). Whereas only the maternal *MEA* allele was detected in control wild-type endosperm, both maternal and paternal *MEA* expression was detected in the *CaMV::DME* endosperm (Figure 6B). The paternal *MEA* allele in *CaMV::DME* endosperm was expressed at a lower level compared to the paternal allele of a control nonimprinted gene (Figure 6B). The lower expression may reflect inefficient endosperm expression of the *CaMV::DME* transgene. Or, additional mechanisms may control *MEA* expression in the endosperm. However, these results are consistent with the hypothesis that the restricted pattern of *DME* gene expression is responsible, at least in part, for the silencing of the paternal *MEA* allele in wild-type endosperm.

DME Expression Results in Nicks in the *MEA* Promoter

The amino acid sequence of DME (Figure 3C) suggests it is a monofunctional DNA glycosylase that generates an abasic site by carrying out a base excision reaction. The next step in DNA repair is single-stranded DNA cleavage (nicking) 5′ to the abasic site by an AP endonuclease (Bruner et al., 2000; Jiricny, 2002). If DME acts directly on *MEA::GFP* to regulate its expression (Figure 5), the abasic residues should generate sites for AP endonuclease nicking in the 4.2 kb *MEA* promoter region. To test this hypothesis, we devised a sensitive PCR-based procedure to localize nicks produced in vivo. As shown in Figure 7A, to identify nicks on the sense-strand of the *MEA* promoter, a *MEA*-specific primer (MEA sense-R1) was used to initiate first-strand DNA synthesis. Nicks would cause termination of synthesis at specific sites; if there were no nicks, termination would occur randomly. DNA from the first-strand synthesis reaction was then purified and tailed with terminal deoxynucleotidyl transferase and dCTP. A linker with G residues at its 3′ end (linkerGGG) and a second nested *MEA*-specific primer (MEA sense-R2) were used to amplify DNAs by PCR. DNA products from the first PCR amplification were diluted 100-fold into multiple aliquots, and the linker and a third nested MEA-specific primer (MEA sense-R3) were used for a second PCR amplification. PCR products were then analyzed by agarose gel electrophoresis.

It is not possible to isolate DNA and determine the pattern of nicks in the *MEA* promoter in wild-type and *dme* mutant central cells because they are embedded within the female gametophyte and ovule. Because ectopic expression of a *CaMV::DME* transgene induces *MEA* expression in leaves (Figure 6A), we compared the pattern of nicks in the *MEA* promoter isolated from readily accessible wild-type and *CaMV::DME* leaves to test the hypothesis that DME directly regulates *MEA*. Specific DNA bands were detected when template DNAs were isolated from *CaMV::DME* leaves (Figure 7B). Repetition of PCR reactions produced different size DNA bands and DNA sequence analysis verified that these DNA bands were derived from the *MEA* promoter. The fact that different size bands were synthesized in multiple PCR reactions indicates there is a stochastic element to PCR sampling of the *CaMV::DME*-induced nicks, suggesting the molecules with nicks at different

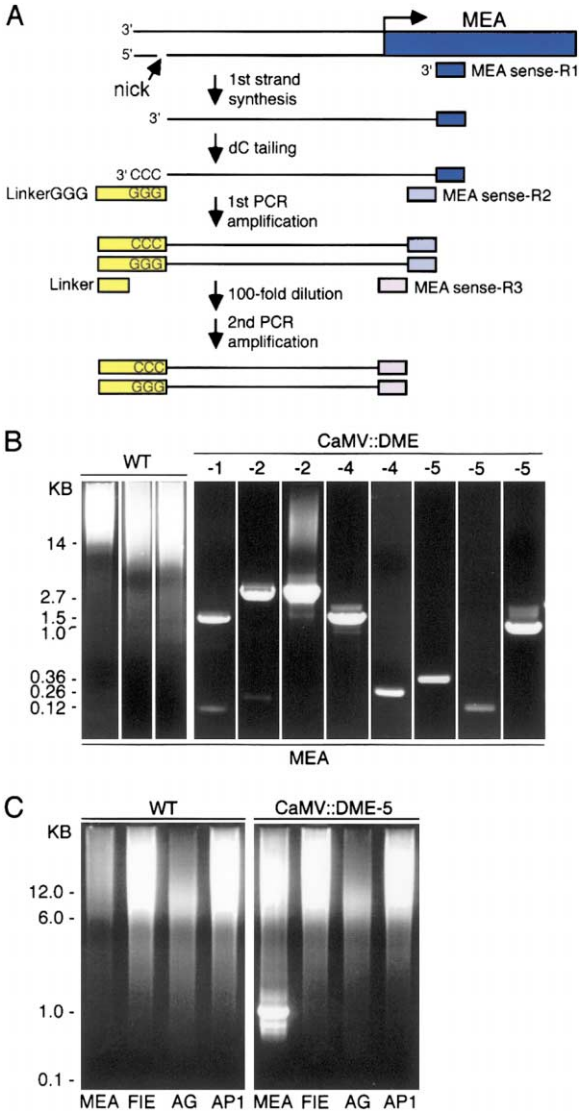

Figure 7. Ectopic *DME* Expression Generates Nicks in the *MEA* Promoter

(A) PCR-based strategy for detecting nicks in the sense-strand of the *MEA* promoter.

(B) PCR products from the sense-strand of the *MEA* promoter. PCR products obtained with DNA templates isolated from leaves from wild-type (WT) and CaMV::DME-1, CaMV::DME-2, CaMV::DME-4, and CaMV::DME-5 independent transgenic lines. Amplifications of multiple aliquots from the first PCR amplification of wild-type and *CaMV::DME* templates are shown as described in the text.

(C) Comparison of PCR products from the sense-strand of the *MEA*, *FIE*, *AG*, and *AP1* 5′-flanking regions using wild-type and *CaMV::DME-5* DNA templates.

sites in the *MEA* promoter were present in very low concentration (Cavrois et al., 1995; Taberlet et al., 1996). By contrast, no discrete DNA bands were detected when wild-type template DNA was isolated from leaves which do not express *MEA* (Figure 7B). The diverse range of high molecular weight PCR products suggested that during the first-strand synthesis reaction the DNA polymerase terminated randomly. The same result was obtained when the entire procedure (i.e., first-strand synthesis, two PCR amplifications) was repeated three

times with wild-type DNA template with multiple aliquots used for the second PCR amplification (data not shown). In control PCR reactions using *MEA* sense-R3 and a primer located in the *MEA* promoter, PCR products were obtained with equal efficiency with wild-type and *CaMV::DME-1* DNA templates (data not shown). Finally, specific PCR products were not detected from the sense-strand of wild-type or *CaMV::DME* template DNA within the 5′-flanking regions of the control *FIE* gene and the floral homeotic (Meyerowitz and Clark, 1994) *AGAMOUS* (*AG*) or *APETALA1* (*AP1*) genes (Figure 7C). Thus, nicking, a property of base excision DNA repair, was detected in the sense-strand of *CaMV::DME* lines and not in the wild-type sense-strand within 14 kb of the start of *MEA* transcription.

Discussion

We isolated mutations in the *Arabidopsis DME* gene to understand how the female gametophyte influences embryo and endosperm development. We demonstrated that seed viability depends solely on the maternal allele and found that DME is a large protein with DNA glycosylase and nuclear localization domains. Transcribed primarily in the central cell, *DME* is necessary for activation of imprinted *MEA* expression in the central cell and the endosperm. Ectopic expression of *DME* results in expression of the normally silenced paternal *MEA* allele. When *DME* was expressed in the leaf, we observed *MEA* expression and in vivo nicking of the *MEA* promoter. We conclude from these results that DME is a DNA glycosylase that mediates imprinting in the central cell and that this process is required for seed viability.

A Model for the Control of *MEA* Gene Imprinting in the Endosperm

Our analysis of DME suggests a mechanism for the regulation of imprinted (maternally expressed, paternally silenced) genes in the endosperm. The reason that the maternal *MEA* allele, and not the paternal *MEA* allele, is expressed in the endosperm is because only the maternal *MEA* allele is accessible to DME in the central cell of the female gametophyte before fertilization. This model is based in part upon the highly restricted pattern of *DME* expression. *DME* is primarily expressed in the central cell of the female gametophyte, and its transcription is turned off soon after fertilization (Figure 4). *DME* RNA and promoter activity was not detected in the male gametophyte-producing stamens (Figure 4). Thus, only the maternal *MEA* allele and not the paternal *MEA* allele, is exposed to DME activity. The model is also based on experiments showing *DME* regulates *MEA* gene expression. *MEA* RNA and promoter activity was not detected in *dme* mutants (Figure 5), whereas ectopic *DME* expression in the leaf and endosperm activated *MEA* and paternal *MEA* allele expression, respectively (Figure 6). Finally, we found that ectopic expression of *DME* in leaf caused single-stranded breaks in the *MEA* promoter within 2 kb of the start of *MEA* gene transcription (Figure 7). We propose that DME might mark the maternal *MEA* allele in the female gametophyte, allowing sustained maternal *MEA* allele expression to occur in the endosperm after fertilization. The paternal allele is not marked and therefore not expressed during endosperm development.

A Role for DNA Glycosylases in Controlling Gene Imprinting and Seed Viability

DNA glycosylases represent a diverse array of small (200–300 amino acids), monomeric, structurally related DNA repair proteins that are highly conserved in evolution (Krokan et al., 1997; Scharer and Jiricny, 2001). These proteins excise mismatched or altered bases (e.g., oxidized, deaminated, alkylated, and methylated) by cleaving the N-glycosidic bond between the base and the sugar-phosphate backbone of the DNA. DNA glycosylases represented by DME, *E. coli* MutY and endonuclease III, human 8-oxoguanine DNA glycosylase and MBD4 have a conserved helix-hairpin-helix domain. Excision by monofunctional DNA glycosylases results in an abasic site that is mutagenic and must be removed. Single-strand cleavage 5′ to the abasic site by an AP endonuclease generates a 3′-hydroxyl used by a specialized DNA repair polymerase that inserts a single nucleotide and removes the abasic sugar-phosphate (Jiricny, 2002). A DNA ligase seals the nick to complete the repair process. It has been proposed that highly mutagenic oxidized, deaminated, or alkylated bases are associated with pathophysiologic processes such as cancer and aging in mammals. However, mice with mutations in DNA glycosylase genes do not display overt developmental abnormalities (Scharer and Jiricny, 2001). Thus, the role of DNA glycosylases in the control of development or tumor suppression is unknown.

The *Arabidopsis* genome encodes multiple small helix-hairpin-helix DNA glycosylases, some of which have been shown to function in DNA repair (Garcia-Ortiz et al., 2001). However, the DME protein is unique from other DNA glycosylases in several regards. First, the DME-predicted polypeptide is much larger than typical DNA glycosylases that function in DNA repair (Figure 3). Moreover, it has a highly basic region related to histone H1 (Figure 3). This basic region might enhance the ability of DME to interact with DNA or with other chromatin proteins. These unique molecular properties, coupled with the phenotypes of mutant *dme* plants (Figures 1 and 2), and its role in regulating *MEA* gene expression (Figure 5), suggest that any base excision activity of DME is probably not involved solely with DNA repair.

How might DME work to regulate the expression of *MEA*? One possibility is that DME modifies chromatin structure by excising 5-methylcytosine. Genomic imprinting in mammals reflects modifications in DNA methylation (Reik and Walter, 2001) and the sporadic developmental abnormalities observed in *dme* homozygous mutant plants (Figure 2) are reminiscent of genome methylation defective mutants in *Arabidopsis* (Kakutani et al., 1996). Other related DNA glycosylases have been shown to excise 5-methylcytosine from the genome (Jost et al., 2001). However, using bisulfite sequencing methods we have been unable to detect 5-methylcytosine residues in a 2 kb region sufficient for regulation of *MEA* gene expression by DME in seed or leaf from wild-type or mutant *dme* genetic backgrounds (see Experimental Procedures for details).

DME is predicted to be a monofunctional DNA glycosylase (Figure 3). Following base excision, the DNA is nicked 5′ to the abasic site by an AP endonuclease. Consistent with this prediction, we observed nicks on the sense-strand of the *MEA* promoter in multiple independently isolated *CaMV::DME* transgenic lines (Figure 7). The nicked DNA molecules may be very rare because only a fraction of the population of *MEA* genes in *CaMV::DME* leaves may be transcribed at any given time. Also, it is possible that the DME-induced nicks are quickly repaired, thereby lowering their concentration. These results strongly support the hypothesis that DME carries out a base excision reaction with subsequent nicking in the *MEA* promoter by an AP endonuclease, although it is formally possible that the nicking we observed is an indirect effect of the activation of *MEA* gene transcription.

The AP endonuclease-mediated DNA nicking activity that follows DME base excision might catalyze nucleosome sliding, as has been demonstrated for nicks in linker DNA in vitro (Langst and Becker, 2001). The H1 linker histone-related region that is located at the amino terminus of DME might facilitate this process. Nucleosome sliding may allow transcription factors to activate *MEA* gene transcription by RNA polymerase. Once the nucleosome structure has been altered on the maternal *MEA* allele, it may be perpetuated after fertilization, allowing for continued maternal *MEA* allele transcription (Figure 5) in the absence of *DME* expression (Figure 4) in the endosperm.

It is unknown how DME is directed to sites in the *MEA* promoter. One possibility is that DME acts at modified base pairs. Alternatively, it has been shown that a mammalian thymine DNA glycosylase acts in a protein complex to remodel chromatin (Tini et al., 2002). Thus, DME might function in a protein complex that could provide promoter specificity for base excision/DNA nicking of the maternal genome. Finally, it is important to consider that nicks at specific sites in DNA might constitute an essential feature in the control of chromatin structure and gene expression.

Experimental Procedures

Plant Materials and Microscopy

Methods for growing plants, fixing tissues, photography, GUS activity localization, and GFP fluorescence microscopy are as previously described (Yadegari et al., 2000).

Mutagenesis

dme-1 and *dme-2* alleles were obtained by screening 5000 T1 plants (Columbia *gl*) mutagenized with activation T-DNA vector, pSKI015 for siliques with 50% seed abortion. The *dme-3* allele (Wassilewskija) was from the *Arabidopsis* Knockout Facility where mutagenesis was with a nonactivation T-DNA vector, pD991. Mutant lines were crossed to wild-type (Landsberg *er*) six times to remove additional mutations.

Molecular Cloning of *DME*

DNA from *DME/dme-2* plants was used to isolate *DME* sequences flanking the left and right border T-DNA regions. *DME* genomic clones were obtained from a wild-type (Columbia *gl*) genomic library. A cDNA library of floral mRNAs was used to obtain a cDNA clone with a 3′-poly A tail plus 2.7 kb of *DME* sequences (4169–6871). Using reagents and 5′- and 3′-RACE procedures from Clonetech and Gibco Bethesda Research, overlapping cDNA clones (1–2921 and 2279–4973) extending through the *DME* 5′-untranslated region were obtained. Using restriction enzyme sites, clones were ligated into a full-length *DME* cDNA (1–6871). To complement the *dme* mutation, *DME* 5′-flanking sequences (3424 base pairs) were ligated to the full-length *DME* cDNA and inserted into pBI-GFP(S65T). A single locus transgenic T_1 plant was crossed with *DME/dme-1* pollen generating F_1 progeny hemizygous for the transgene and *DME/dme-1*. In self-pollinated F_1 plants, a 3:1 ratio of viable to aborted F_2 seeds (329:121, 3:1, $\chi^2 = 0.86$, $P > 0.43$) was observed.

Analysis of RNA Accumulation

Total RNA was isolated and reverse transcriptase reactions and PCR reactions were carried out (Yadegari et al., 2000). Primers for amplifying *DME* were *cDNA-5* (CAGAAGTGTGGAGGGAAAGCGTCT GGC) and *SKEN-5* (GCAATGCGTTTGCTTTCTTCCAGTCATCT), for *FIE* were *cer1ns8517n* (CTGTAATCAGGCAAACAGCC) and *cer8191n* (TCAAGGTCTCAGGGAGTAGC), and for *FIS2* were *F2-f5/6* (TCAAG GTCTCAGGGAGTAG) and *F2-r7/8* (CTCTCTAGCCTTGTACCGCTT TGCATATAACTG). Primers for *MEA* in Figure 5A and Figure 6A were as described (Kiyosue et al., 1999). For measuring maternal and paternal *MEA* RNA accumulation, RNA was prepared and RT-PCR reactions were carried out as described (Kinoshita et al., 1999). All primer pairs spanned intron sequences so that amplification of RNA could be distinguished from amplification of any contaminating DNA.

Generation of Plants with Reporter Transgenes

Using BamHI and EcoRI, the sGFP(S65T) (Niwa et al., 1999) coding sequence was excised from CaMV35S-sGFP(S65T)-nos3′ and inserted into pBI101.1 (Jefferson et al., 1987), replacing β-glucuronidase-nos3′ to create pBI-GFP(S65T). A portion of the *DME* gene (2282 bp of 5′-flanking sequences plus 1922 bp of the first exon) was inserted into XbaI/BamHI sites of T-DNA pBI-GFP(S65T), introduced into *Agrobacterium*, and five transgenic lines (Columbia *gl*) obtained. For *DME::GUS*, the same sequences were inserted into pBI101.1 upstream of β-glucuronidase-nopaline synthase (Jefferson et al., 1987). For *CaMV::DME*, a full-length *DME* cDNA was inserted downstream of the *CaMV* promoter of vector pMD1. To construct *MEA::GFP* and *MEA::GUS* transgenes, clone 6–22 (Kiyosue et al., 1999) was used as template in a PCR reaction to amplify 4.2 kb of *MEA* 5′ sequences with primers *MEA*4105Sal (5′-TATTGTCGACCG TCCTGTCAAACCCGTCCCGT-3′) and *ME*A8323Xba (5′-ATATTCTA GACTTTTTTTCTCGTCTTCTCTGATGTTGGT-3′). The PCR product was digested with SalI and XbaI and inserted into pBI-GFP(S65T) and pBI101.2 creating the *MEA::GFP* and *MEA::GUS* transgenes with 4193 bp *MEA* 5′-flanking sequences and 26 bp 5′-untranslated sequences ligated to *GFP* and *GUS* reporter genes, respectively.

Bisulfite Sequencing

DNA was isolated from Landsberg *er* leaves, wild-type seeds, and homozygous *dme-1* seeds. Seeds were harvested 2–3 days postpollination. DNAs were treated with bisulfite and PCR products from −2080 to +1 were purified, cloned, and 15–20 clones sequenced (Jacobsen et al., 2000). The 2 kb region represents the overlap of a 7 kb transgene with approximately 2 kb of 5′-flanking sequences that was imprinted like the endogenous *MEA* gene (data not shown), and the *MEA::GFP* transgene (Figure 5).

Detection of DNA Nicks

First-strand DNA synthesis was with 0.5 ug genomic DNA template, *MEA* sense-R1 (5′-CTTCTCCATTAACCACTCGCCTCTT-3′) and 400 uM dNTPs using Ex Taq DNA polymerase from Takara at 95°C 5 min, 52°C 5 min, and 72°C 40 min. Single-stranded DNA was purified (Qiagen PCR Kit) and tailed with 200 uM dCTP and terminal deoxynucleotidyl transferase (TdT; Invitrogen). DNA was treated at 95°C for 3 min, chilled, 1 ul of TdT (10 units) added, and incubated at 37°C for 10 min. TdT was inactivated at 65°C for 10 min. The first PCR amplification was with Invitrogen Abridged Anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) and *MEA* sense-R2 (5′-CTCGTCTTCTCTGATGTT-3′). After 95°C for 5 min, 35 PCR cycles were carried out (94°C 30 s, 55°C 30 s, 72°C 4 min). PCR products were diluted 100-fold and amplified again with Invitrogen abridged universal amplification primer (5′-GGCCACGCGTCGAC TAGTAC) and *MEA* sense-R3 (5′-GGTGAAAAGGATAATGCAAAG

GGT-3′). *FIE* sense primers, R1 (TGGAGTCAAAGACCCAACTATT GACTCGT), R2 (TCTCTCTCTGTCTGACTCTCGCACAC), R3 (TCG ATTAGACACAGATTCACAGGT); *AG* sense primers, R1 (AGGTA AGGTTGTGCTGGTG), R2 (CATCCATATAGTGTCTTGTC), R3 (CTG GTGTTTCTTTTCAGTAC); and *AP1* sense primers, R1 (CCAAG AATCAGTGGAGTATTCG), R2 (GACCAGCTCTTCTTTTCG), R3 (GAA GAGCTCAGACTTTGGT). For *AG*, the PCR annealing temperature was 52°C.

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Accession Numbers

The *DME* cDNA sequence has been deposited in GenBank as accession number AF521596.