Regulation of Seed Size by Hypomethylation of Maternal and Paternal Genomes[1]

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DNA methylation is an epigenetic modification of cytosine that is important for silencing gene transcription and transposons, gene imprinting, development, and seed viability. DNA METHYLTRANSFERASE1 (MET1) is the primary maintenance DNA methyltransferase in Arabidopsis (*Arabidopsis thaliana*). Reciprocal crosses between antisense *MET1* transgenic and wild-type plants show that DNA hypomethylation has a parent-of-origin effect on seed size. However, due to the dominant nature of the antisense *MET1* transgene, the parent with a hypomethylated genome, its gametophyte, and both the maternal and paternal genomes of the F_1 seed become hypomethylated. Thus, the distinct role played by hypomethylation at each generation is not known. To address this issue, we examined F_1 seed from reciprocal crosses using a loss-of-function recessive null allele, *met1-6*. Crosses between wild-type and homozygous *met1-6* parents show that hypomethylated maternal and paternal genomes result in significantly larger and smaller F_1 seeds, respectively. Our analysis of crosses between wild-type and heterozygous *MET1/met1-6* parents revealed that hypomethylation in the female or male gametophytic generation was sufficient to influence F_1 seed size. A recessive mutation in another gene that dramatically reduces DNA methylation, *DECREASE IN DNA METHYLATION1*, also causes parent-of-origin effects on F_1 seed size. By contrast, recessive mutations in genes that regulate a smaller subset of DNA methylation (*CHROMOMETHYLASE3* and *DOMAINS REARRANGED METHYLTRANSFERASES1* and 2) had little effect on seed size. Collectively, these results show that maternal and paternal genomes play distinct roles in the regulation of seed size in Arabidopsis.

DNA methylation usually refers to a covalent addition of a methyl group to cytosine at the 5-position. DNA methylation is a heritable epigenetic process that regulates growth and development in both animals and plants (Martienssen and Colot, 2001; Reik et al., 2001; Li, 2002). In mammals, 5-methylcytosine mainly occurs at CpG dinucleotides, and plays a vital role in genome stabilization, X chromosome inactivation, silencing of transposons and endogenous retrovirus, gene expression, and imprinting (Bird and Wolffe, 1999; Bestor, 2000; Reik et al., 2001). The patterns of DNA methylation are maintained during somatic development by the DNA methyltransferase1 (Dnmt1) DNA methyltransferase (Li et al., 1992). During gametogenesis and embryogenesis, DNA methylation is lost and subsequently reestablished by the Dnmt3a and Dnmt3b DNA methyltransferases (Okano et al., 1999).

In plants, DNA methylation is involved in regulating many epigenetic phenomena (Martienssen and Colot, 2001; Bender, 2004; Gehring et al., 2004; Chan et al., 2005). These include transcriptional silencing of transposons and transgenes, defense against pathogens, regulation of imprinting, as well as the silencing of genes that control flowering time, floral organ identity, fertility, and leaf morphology (Finnegan et al., 1996; Kakutani et al., 1996; Jacobsen et al., 2000; Soppe et al., 2000; Miura et al., 2001). In Arabidopsis (*Arabidopsis thaliana*), CpG DNA methylation is maintained by DNA METHYLTRANSFERASE1 (MET1), a homolog of mammalian DNA methyltransferase Dnmt1 (Finnegan and Dennis, 1993; Finnegan and Kovac, 2000). In addition to CpG methylation, Arabidopsis has CpNpG and asymmetric CpNpN methylation, which are maintained by the CHROMOMETHYLASE3 (CMT3) and the de novo DNA METHYLTRANSFERASES1 and 2 (DRM1 and DRM2; Henikoff and Comai, 1998; Bartee et al., 2001; Lindroth et al., 2001; Cao and Jacobsen, 2002a, 2002b). DNA methylation is intimately associated with histone modifications, chromatin remodeling, and the accessibility of DNA to transcription factors (Martienssen and Colot, 2001; Jackson et al., 2002; Johnson et al., 2002; Soppe et al., 2002; Bender, 2004; Chan et al., 2005).

The seeds of flowering plants are derived from two fertilization events that occur in the female gametophyte. In Arabidopsis, a haploid megaspore undergoes

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three mitotic divisions to form an eight-nucleus, seven-cell female gametophyte containing the egg, central, synergid, and antipodal cells; the fusion of two haploid polar nuclei makes the nucleus of the central cell diploid. Fertilization of the egg cell by a sperm cell gives rise to a diploid embryo that ultimately generates the organs, tissues, and meristems of the plant. Fertilization of the central cell by a second sperm cell produces a triploid, terminally differentiated endosperm that supports embryo or seedling growth and development by producing storage proteins, lipids, and starch, and by mediating the transfer of maternal-derived nutrients to be absorbed by the embryo (Brown et al., 1999).

Arabidopsis plants with an antisense *MET1* transgene or partial-loss-of-function *met1* mutations caused a reduction of global DNA methylation levels, particularly at CpG dinucleotides (Finnegan et al., 1996; Kakutani et al., 1996; Ronemus et al., 1996; Kankel et al., 2003). It has also been shown that mutations in *CMT3* and *DRM* lead to a reduction in CpNpG and asymmetric CpNpN methylation. Plants with reduced DNA methylation display pleiotropic developmental abnormalities, such as abnormal shoot and leaf development, abnormal flower organ formation, delayed flowering, reduced fertilities, and abnormal embryogenesis (Finnegan et al., 1996; Kakutani et al., 1996, 2005; Ronemus et al., 1996; Cao et al., 2003; Kankel et al., 2003; Kato et al., 2003; Saze et al., 2003; Xiao et al., 2006).

In addition to MET1, DECREASE IN DNA METHYLATION1 (DDM1), an ATP-dependent SWI2/SNF2 chromatin remodeling factor, is also required for normal patterns of genomic DNA methylation in Arabidopsis (Vongs et al., 1993; Jeddeloh et al., 1999). Mutations in the *DDM1* gene result in a rapid loss of cytosine methylation at heterochromatic repetitive sequences and a gradual depletion of methylation at euchromatic low-copy sequences over successive generations (Kakutani et al., 1999). DDM1 has been shown to regulate gene imprinting, transposons, gene silencing, and paramutation (Vielle-Calzada et al., 1999; Hirochika et al., 2000; Singer et al., 2001).

Genetic crosses between wild-type and antisense *MET1* plants revealed that DNA methylation influences F_1 seed size (Adams et al., 2000; Luo et al., 2000). Pollination of antisense *MET1* transgenic pistils with wild-type pollen produced large F_1 seeds, and reciprocal crosses generated small F_1 seeds. Thus, DNA hypomethylation has parent-of-origin effects on seed size. However, due to the dominant nature of the antisense *MET1* transgene, the parent with a hypomethylated genome generates a hypomethylated gametophyte, and both the maternal and paternal genomes of the F_1 embryo and seed become hypomethylated after fertilization. Thus, the distinct role played by hypomethylation at each generation is not known. To address this issue, we carried out reciprocal crosses using a complete loss-of-function allele, *met1-6* (Xiao et al., 2003), to distinguish the roles of maternal versus paternal genomes in parents, gametophytes, and F_1 progeny. We show that larger F_1 seeds were produced when the maternal genome was hypomethylated, whereas smaller F_1 seeds were generated when the male genome was hypomethylated. Reciprocal crosses between wild-type and heterozygous *MET1/met1-6* plants revealed that hypomethylation in either the male or female gamete was sufficient to cause seed size variation. Moreover, reciprocal crosses between wild type and *ddm1-2*, which dramatically reduces genome DNA methylation, produced F_1 seed that displayed parent-of-origin effects on size. By contrast, little effect on size was detected in F_1 seed from reciprocal crosses between wild type and mutations with relatively smaller effects on genome DNA methylation, *cmt3* and *drm1 drm2*. Thus, the DNA methylation status of the maternal and paternal genomes regulates seed size in Arabidopsis.

RESULTS

MET1 Is Expressed in the Developing Seed

To understand whether *MET1* plays a role in regulating seed size, we first examined expression of *MET1* in ovules and developing seeds, including embryo and endosperm. Reverse transcription (RT)-PCR analysis indicates that *MET1* was expressed in ovule-containing flowers at stages 1 to 12 and stage 13, as well as in the early developing seeds at 1, 2, 3, 4, 6, and 10 d after pollination (DAP; Fig. 1A). When seed was separated into embryo and endosperm parts, we found *MET1* was expressed in both embryo and endosperm of Arabidopsis ecotypes Columbia (Col)-0 and Landsberg *erecta* (L*er*; Fig. 1B). This result indicates that *MET1* is expressed in flowers prior to fertilization, as well as the developing embryo and endosperm after fertilization.

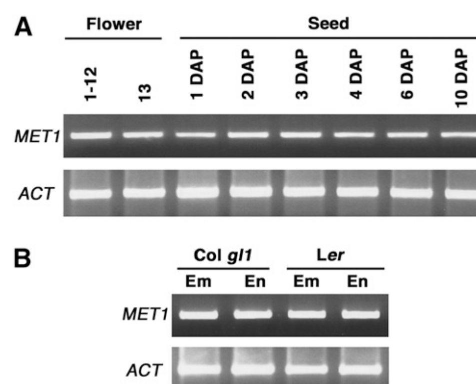

Figure 1. Expression of the *MET1* gene during plant reproduction. *MET1* RNA was amplified by RT-PCR as described in "Materials and Methods." A, RNA was isolated from flowers (stages 1–12 and 13) and developing seeds at the indicated DAP. B, Seeds from wild type (Col *gl1* and L*er* ecotypes) were harvested 8 DAP, dissected, and RNA was isolated from embryos (Em) and endosperm (En).

Hypomethylation of Maternal and Paternal Genomes Causes Parent-of-Origin Effects on Seed Size

To assess the effect of maternal versus paternal genome hypomethylation on seed size, we reciprocally crossed wild type with the first generation of the *met1-6* homozygous plants. As shown in Figure 2, larger F_1 seed was generated when the maternal parent was homozygous *met1-6*. By contrast, smaller F_1 seed was produced when the paternal parent was homozygous for the *met1-6* mutation.

We quantitatively measured F_1 seed size from reciprocal crosses by sifting them through sieves with different size openings, weighing seeds, and measuring seed length and width. As shown in Table I, when wild-type pistils were pollinated with pollen from a homozygous *met1-6* plant, F_1 seed size was significantly reduced with 68% and 28% of the seed retained on sieve numbers 60 (250-μm opening) and 70 (212-μm opening), respectively. For the wild-type control cross, 64% and 35% F_1 seed was retained on sieve numbers 50 (300-μm opening) and 60, respectively. Pollination of *met1-6* pistils with wild-type pollen produced the largest F_1 seed, in which 10% seed was retained on sieve number 45 (355-μm opening), 84% was retained on sieve number 50, and only 6% was retained on sieve number 60 (Table I).

The F_1 seed size of the different crosses was reflected in their seed mass. Average weight of 100 F_1 seeds of the wild-type control cross was 2.8 mg. Pollination of wild-type pistils with *met1-6* pollen produced F_1 seeds with an average weight of 1.6 mg per 100 seeds, whereas the reciprocal cross generated 3.9 mg per 100 F_1 seeds.

We also measured F_1 seed length and width of the above three crosses. As shown in Table II, the wild-type F_1 seed from control crosses were on average 497-μm long and 302-μm wide. Pollination of wild-type pistils with *met1-6* pollen produced F_1 seeds that were on average 404-μm long and 245-μm wide, approximately 19% smaller than F_1 wild-type seeds. By contrast, F_1 seed from the reciprocal cross were approximately 27% longer and 23% wider. Taken together, the above results indicate that hypomethylation of the maternal genome results in larger and heavier seeds, whereas hypomethylation of the paternal genome results in smaller, lighter seed.

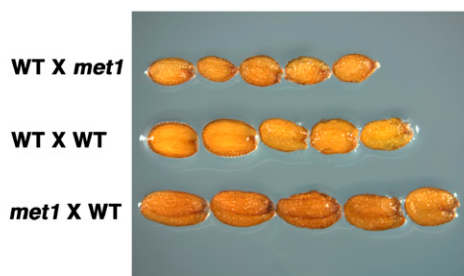

Figure 2. Parent-of-origin effects of the *met1-6* mutation on seed size in Arabidopsis. For each cross, the genotype of the maternal parent is indicated first, followed by the paternal parent. WT, Wild-type parent; *met1*, homozygous *met1-6* parent.

Hypomethylation of Maternal and Paternal Genomes Causes Parent-of-Origin Effects on Endosperm Structure and Development

We next examined F_1 seed to see if parental hypomethylation affects structure and the timing of seed development. We sectioned F_1 seed at 3, 4, 5, and 7 DAP (Fig. 3). We found that development of small F_1 seed with a hypomethylated paternal genome was accelerated compared to wild-type controls. For example, in F_1 seeds with a paternal hypomethylated genome, endosperm started to cellularize at 4 DAP and almost finished cellularization at 5 DAP, whereas wild-type endosperm had not started to cellularize at 5 DAP. Smaller F_1 seed also displayed a smaller endosperm volume than wild-type seeds. By contrast, larger F_1 seed with a hypomethylated maternal genome displayed delayed development and had a larger endosperm volume when compared to wild-type controls. In particular, these F_1 seed had enlarged chalazal endosperm and nodule at the chalazal end of the seed (Fig. 3). These results show that maternal and paternal genome hypomethylation results in distinct patterns of endosperm development within F_1 seed.

DNA Methylation Status of Gametes Affects Seed Size

To investigate whether hypomethylation in male or female gametes is sufficient to affect seed size, we reciprocally crossed wild type with the heterozygous *MET1/ met1-6* plants. Wild-type pistils pollinated with pollen from a heterozygous *MET1/ met1-6* plant produced a subset of smaller F_1 seeds compared to wild-type controls (Table I). That is, nearly half of the F_1 seed (47%) was retained on sieve number 60 (250 μm), and there was also 12% and 4% seed retained on sieve numbers 70 (212 μm) and 80 (180 μm), respectively. By contrast, when *MET1/ met1-6* pistils were pollinated with wild-type pollen, we detected a subtle increase in seed size. Most F_1 seeds (72%) were retained on sieve number 50 (300 μm), and no seed was retained on sieve number 70 or number 80.

To examine the correlation between changes in seed size and gamete hypomethylation, we determined the genotype of F_1 seeds produced by reciprocal crosses between wild-type and heterozygous *MET1/ met1-6* plants. Wild-type pistils were pollinated with pollen from a *MET1/ met1-6* plant, F_1 seed were visually divided into two size categories (medium-sized and small-sized seeds), and the genotype of seeds was determined as described in ''Materials and Methods.'' We found that 92% of the medium-sized seeds inherited the wild-type *MET1* allele from the paternal parent. The probability that this deviation from 1:1 segregation of paternal-derived *MET1* and *met1-6* alleles within the medium-sized population occurred by

Table I. *Parent-of-origin effects of the met1-6 mutation on seed size*

Genetic Cross		Percentage of Seeds Retained on Indicated Sieve[a]					Weight
Maternal Parent	Paternal Parent	No. 45 355 μm	No. 50 300 μm	No. 60 250 μm	No. 70 212 μm	No. 80 180 μm	
							mg[b]
Wild type	*met1-6*	0	0	68 ± 4	28 ± 6	4 ± 2	1.6 ± 0.1
Wild type	Wild type	0	64 ± 8	35 ± 8	0	0	2.8 ± 0.1
met1-6	Wild type	10 ± 2	84 ± 5	6 ± 4	0	0	3.9 ± 0.2
Wild type	*MET1/met1-6*	0	37 ± 6	47 ± 5	12 ± 7	4 ± 2	2.2 ± 0.3
MET1/met1-6	Wild type	0	72 ± 7	27 ± 7	0	0	3.0 ± 0.3

[a]Two hundred seeds were passed through sieves. Standard deviation for three replicates is shown. [b]Two hundred seeds were weighed. Weight per 100 seeds is shown. Standard deviation for three replicates is shown.

chance is extremely low (paternal *MET1*:*met1-6*, 106:9, $\chi^2 = 82$, $P \ll 0.005$). Moreover, 84% of the small-sized F_1 seeds inherited the mutant *met1-6* allele from the paternal parent (paternal *MET1*:*met1-6*, 14:73, $\chi^2 = 40$, $P \ll 0.005$). These results suggest that hypomethylation in the male gametophyte is sufficient to reduce F_1 seed size. We also determined the genotype of F_1 seed from the reciprocal cross, *MET1*/ *met1-6* pistils pollinated with wild-type pollen. We found that 78% of the medium-sized F_1 seed inherited the paternal wild-type *MET1* allele. The probability that this deviation from 1:1 segregation of maternal-derived *MET1* and *met1-6* alleles within the medium-sized population occurred by chance is again extremely low (maternal *MET1*:*met1-6*, 78:22, $\chi^2 = 36$, $P \ll 0.005$). Moreover, 75% of the large F_1 seed inherited the mutant *met1-6* allele (maternal *MET1*:*met1-6*, 25:75, $\chi^2 = 21$, $P \ll 0.005$). Thus, hypomethylation in the gametophyte generation is sufficient to affect F_1 seed size.

Hypomethylation of the Maternal or Paternal Genome by the *ddm1-2* Mutation Affects Seed Size

Does *met1-6* mutant hypomethylation uniquely influence seed size? To address this question, we looked at the effect of mutations in the *DDM1* gene on seed size. DDM1, an ATP-dependent SWI2/SNF2 chromatin remodeling protein, also is required for genomic DNA methylation. Mutations in *DDM1* result in a rapid loss of cytosine methylation at repetitive sequences and a gradual depletion of methylation at low-copy sequences (Jeddeloh et al., 1999). We reciprocally crossed the recessive *ddm1-2* mutant with wild-type plants and analyzed F_1 seed size by measuring their width and length. As shown in Figure 4 and Table III, when pistils were pollinated with pollen from a homozygous *ddm1-2* mutant male plant, a modest decrease in F_1 seed size was detected. By contrast, F_1 seeds from the reciprocal cross were significantly larger than the wild-type controls (Fig. 4; Table III). This result is consistent with the increased size of F_1 seeds that bear mutations in *DDM1* as well as in the *MEDEA* (*MEA*) Polycomb group gene (Vielle-Calzada et al., 1999). Thus, a hypomethylated maternal or paternal genome caused by either *met1-6* or *ddm1-2* mutation is sufficient to influence F_1 seed size.

Plants have CpNpG and asymmetric CpNpN DNA methylation that are mainly maintained by CMT3 and DRM2. Does non-CpG DNA methylation play a role in determining seed size? To answer this, we reciprocally crossed recessive mutants *cmt3-7* and *drm1 drm2* with their corresponding wild-type plants, and then compared F_1 seed size with wild-type control F_1 seed. We did not observe a significant change in F_1 seed size from these crosses (data not shown). This suggests loss of CpNpG and CpNpN methylation has little effect on seed size.

DISCUSSION

DNA methylation is essential for regulating development in plants (Martienssen and Colot, 2001; Bender, 2004; Gehring et al., 2004; Chan et al., 2005). DNA methylation silences transposons, regulates gene imprinting, and influences flowering time, floral organ identity, fertility, embryogenesis, and leaf morphology (Finnegan et al., 1996; Kakutani et al., 1996; Jacobsen

Table II. *Parent-of-origin effects of the met1-6 mutation on F_1 seed length and width*

Genetic Cross		Length[a]	Change[b]	Width[a]	Change[b]
Maternal Parent	Paternal Parent				
		μm		μm	
Wild type	*met1-6*	404 ± 20	−19%	245 ± 22	−19%
Wild type	Wild type	497 ± 27		302 ± 19	
met1-6	Wild type	631 ± 35	+27%	372 ± 22	+23%

[a]Average length and width of 50 F_1 seeds is shown. [b]Change is relative to wild-type F_1 seed.

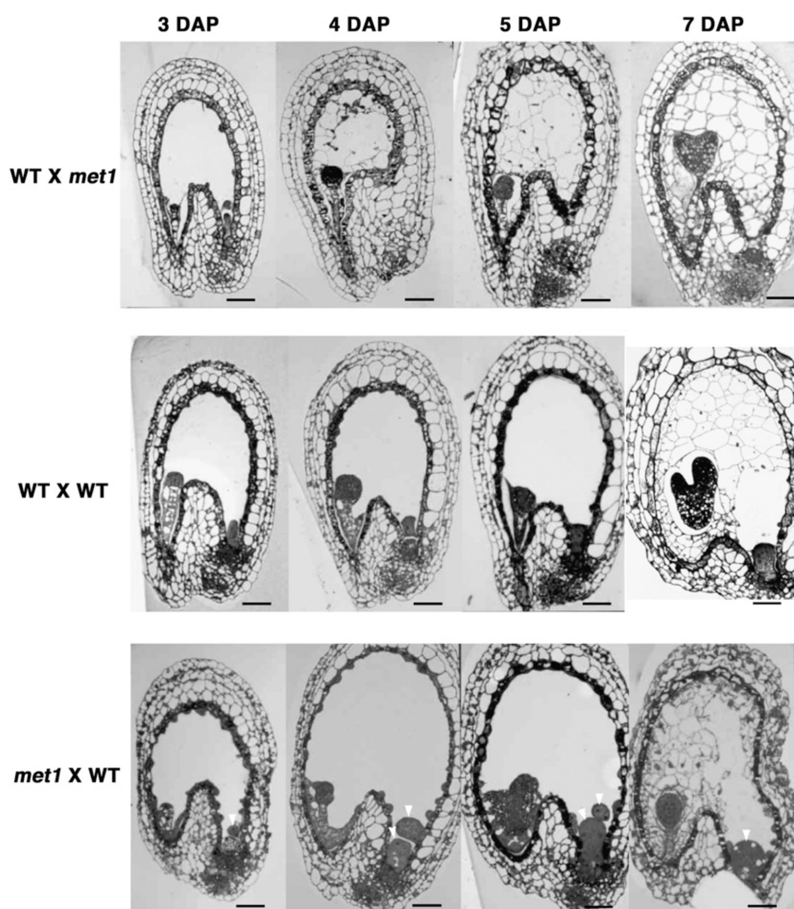

Figure 3. Structure differences of F_1 seeds from reciprocal crosses between wild-type and *met1-6* mutant parents. Thin sections of seeds were isolated, fixed, and stained as described in "Materials and Methods." For each cross, the genotype of the maternal parent is indicated first, followed by the paternal parent. WT, Wild-type parent; *met1*, homozygous *met1-6* parent. Arrowheads indicate large chalazal endosperm and nodule at the chalazal end. Scale bars represent 50 μm.

et al., 2000; Soppe et al., 2000; Miura et al., 2001). Many of these phenomena resulted from gene silencing by DNA methylation and gene activation by DNA hypomethylation. Here, we show that maternal or paternal genome hypomethylation has a parent-of-origin effect on seed size in Arabidopsis (Table I; Fig. 2). Maternal genome hypomethylation causes seeds to be larger, whereas paternal genome hypomethylation generates smaller seeds.

Role of Continuing Genome Hypomethylation in F_1 Seed

F_1 seed from crosses between wild type and plants bearing an antisense *MET1* transgene displayed parent-of-origin effects on seed size (Adams et al., 2000; Luo et al., 2000). In these experiments, due to the dominant nature of the antisense *MET1* transgene, the parent with a hypomethylated genome generates a hypomethylated gametophyte, and both the maternal and paternal genomes of the F_1 embryo and seed become hypomethylated after fertilization. This is because reduction of *MET1* expression in the seed by the antisense *MET1* transgene would be expected to reduce the level of methylation of both maternal and paternal genomes. Thus, the distinct role played by hypomethylation at each generation is not known. We have carried out reciprocal crosses between wild-type plants and plants homozygous for a recessive *met1-6* allele. In these experiments, the F_1 zygote has a hypomethylated set of maternal-derived or paternal-derived chromosomes. This state is preserved because the F_1 embryo and endosperm have a wild-type *MET1* allele. Wild-type MET1 protein maintains the DNA methylation on chromosomes inherited from the wild-type parent. However, methylation of chromosomes from the *met1-6* parent, in particular, CpG methylation, is not restored

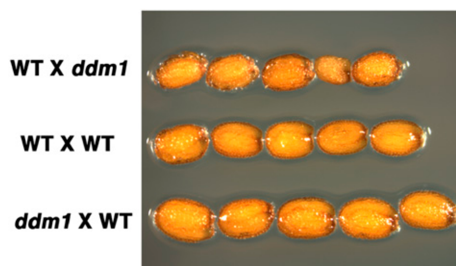

Figure 4. Parent-of-origin effects of the *ddm1-2* mutation on seed size in Arabidopsis. For each cross, the genotype of the maternal parent is indicated first, followed by the paternal parent. WT, Wild-type parent; *ddm1*, homozygous *ddm1-2* parent.

Table III. *Parent-of-origin effects of the ddm1-2 mutation on F_1 seed length and width*

Genetic Cross		Length[a]	Change[b]	Width[a]	Change[b]
Maternal Parent	Paternal Parent				
		μm		*μm*	
Wild type	*ddm1-2*	462 ± 32	−6%	271 ± 24	−9%
Wild type	Wild type	491 ± 27		299 ± 16	
ddm1-2	Wild type	607 ± 38	+24%	346 ± 21	+16%

[a]Average length and width of 50 F_1 seeds is shown. [b]Change is relative to wild-type F_1 seed.

by de novo DNA methyltransferases (Cao and Jacobsen, 2002b; Chan et al., 2006). Thus, the continuous hypomethylation of both maternal and paternal chromosomes in the F_1 seed is not a prerequisite for parent-of-origin effects on seed size.

Role of Genome Hypomethylation in the Preceding Sporophytic Generation and Its Gametophyte

During embryogenesis and gametogenesis in mammals, patterns of DNA methylation at imprinted loci are erased and then reestablished (Reik et al., 2001; Li, 2002). Dnmt1 and de novo methyltransferases Dnmt3a and Dnmt3b are important for maintenance of DNA methylation in oocytes and spermatogenesis (Chen and Li, 2006). By contrast, in flowering plants there is no evidence for erasure and reestablishment of DNA methylation during gametogenesis or embryogenesis (Cao and Jacobsen, 2002b). Rather, DNA methylation is maintained in the gametophyte and sporophyte generations by DNA methyltransferases, primarily by MET1, and also by CMT3 and DRM2 (Kankel et al., 2003; Saze et al., 2003; Xiao et al., 2003; Kinoshita et al., 2004).

In Arabidopsis, there are two or three mitotic cell divisions during male or female gametogenesis, respectively. In *met1-6* mutant gametophytes, each cell division results in an approximate halving of the genome DNA methylation (Saze et al., 2003). Due to the small number of cell divisions, genome hypomethylation in the *met1-6* male and female gametophytes is incomplete and varies from gamete to gamete (Saze et al., 2003). After fertilization, the wild-type *MET1* allele in the heterozygous F_1 seed produces MET1 protein that maintains DNA methylation inherited from gametes. Reciprocal crosses with wild-type and heterozygous *MET1*/ *met1-6* plants indicated that this hypomethylation within the gametophyte is sufficient to cause changes in F_1 seed size. Thus, genome hypomethylation during the parental sporophyte generation is not required to generate parent-of-origin effects on seed size. Genome hypomethylation in the female or male gametophyte, which is preserved in the F_1 seed, is sufficient.

Modulation of Seed Size Requires Genome Hypomethylation at CpG Sites

Both MET1 and DDM1 are required for maintaining genome methylation patterns in Arabidopsis (Kakutani et al., 1999; Lindroth et al., 2001; Xiao et al., 2003). MET1 is a DNA methyltransferase, whereas DDM1 is an ATP-dependent SWI2/SNF2 chromatin remodeling protein and the mechanism by which DDM1 regulates DNA methylation is likely indirect (Verbsky and Richards, 2001). Here, we show that mutations in either *MET1* or *DDM1* affect seed size. By contrast, mutations in *CMT3* and *DRM1/DRM2*, which primarily reduce CpNpG and CpNpN methylation and have a modest effect on overall genome methylation (Cao and Jacobsen, 2002a), have little effect on seed size. One possibility is that significant hypomethylation of all three classes of plant DNA methylation is a prerequisite for alterations in seed size. This may reflect the redundant suppression of gene expression by the three classes of DNA methylation. Alternatively, CpG DNA methylation may be particularly important for regulation of genes that influence seed size. Indeed, CpG DNA methylation is important in the regulation of genes imprinted in the endosperm, *MEA*, *FWA*, and *FIS2* (Kinoshita et al., 2004; Gehring et al., 2006; Jullien et al., 2006b).

Mechanisms for the Control of Seed Size by DNA Methylation

How does hypomethylation of maternal and paternal genomes influence seed size? Genomic imprinting in the endosperm has been shown to regulate seed size and development in plants (Haig and Westoby, 1991; Kohler et al., 2005; Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006a, 2006b). One theory to explain the evolution of genomic imprinting is the parental conflict theory (Haig and Westoby, 1991). If a mother has offspring by more than one father, the theory predicts that alleles of some genes active in the offspring in allocating resources from maternal tissue to offspring will have a different pattern of expression depending on the parent of origin of those alleles. For endosperm activators, the maternal allele is likely silenced and paternal allele expressed so that mother has resources for each individual of her offspring. By contrast, for the endosperm repressors, the maternal allele is expressed and the paternal allele is silenced. DNA methylation usually represses gene transcription and loss of DNA methylation might activate silenced genes or alleles. In the cross of the *met1-6* female with the wild-type male plant, considering the nature of

the loss-of-function recessive *met1-6* allele, only the maternal genome derived from the *met1-6* mutant plant becomes hypomethylated, and the paternal genome derived from wild-type plant is not epigenetically changed by hypomethylated maternal genome during or after fertilization. Thus, the maternal alleles of endosperm activators, which are normally silenced, are expressed and lead to larger F_1 seeds (Fig. 2; Table II). In the cross of a wild-type female with the *met1-6* male parent, the maternal-derived, wild-type genome is not modified and the paternal-derived genome is hypomethylated. Thus, normally silenced paternal alleles of endosperm repressors are expressed, which leads to repressed endosperm development and smaller seeds (Fig. 2; Table II).

Another hypothesis to explain endosperm imprinting and seed development is dosage effect (Haig and Westoby, 1991; Scott et al., 1998; Dilkes and Comai, 2004). In this case, seed development is hypothesized to depend on a ratio of maternal and paternal genomes in endosperm. The ratio is normally two maternal genomes to one paternal genome. If the 2:1 ratio is disturbed, expression from maternal and paternal genomes may be imbalanced, and a breakdown of endosperm or even failure of seed development might occur (Dilkes and Comai, 2004). The cross between a diploid female parent and tetraploid male parent produces larger F_1 seeds, whereas the reciprocal cross generates smaller F_1 seeds (Scott et al., 1998). Paternal excess promotes growth of seed, whereas maternal excess inhibits seed development (Haig and Westoby, 1991; Adams et al., 2000). In reciprocal crosses of the *met1-6* mutant with wild-type plants, inheritance of the hypomethylated paternal *met1-6* allele resembles maternal genome excess, resulting in smaller seed (Figs. 2 and 3). By contrast, inheritance of the hypomethylated maternal *met1-6* allele resembles paternal genome excess, thus resulting in larger seed.

DNA methylation likely regulates genes and pathways affecting seed size. The target genes and pathways are not known. However, the study of Polycomb group proteins may provide clues. Polycomb group proteins are epigenetic regulators that form complexes that alter chromatin structure and silence gene expression (Lund and van Lohuizen, 2004). A direct connection between gene silencing by DNA methyltransferases and Polycomb group proteins has recently been shown (Vire et al., 2006). In Arabidopsis, mutations in the *FIE*, *FIS2*, and *MEA* Polycomb Group genes cause parent-of-origin effects on seed viability (Hsieh et al., 2003), as well as endosperm cell proliferation (Kiyosue et al., 1999), a key component in the regulation of seed size (Fig. 3). Also, genetic interactions have been detected between *MET1* and *FIE* (Vinkenoog et al., 2000), *FIS2* (Jullien et al., 2006b), and *DME*, a regulator of *MEA* gene imprinting (Xiao et al., 2003). Thus, it is possible that there is a relationship between DNA methylation and Polycomb group proteins in the parent-of-origin regulation of seed size.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Arabidopsis (*Arabidopsis thaliana*) plants were grown in greenhouses under continuous light at 23°C. The heterozygous *MET1/met1-6* plants (Col *gl1* ecotype) were obtained in the original genetic screen for suppressors of *dme*-mediated seed abortion (Lindroth et al., 2001; Xiao et al., 2003) and maintained in the heterozygous state by backcrossing with wild-type Col *gl1*. The homozygous *met1-6/met1-6* plants we used were selected from the first generation progeny produced by self-pollinated heterozygous *MET1/met1-6* plants. Wild-type plants (Col *gl1*) were used in crosses with *met1-6* (Col *gl1*) plants. Wild-type Col-0 plants were used in crosses with *ddm1-2* (Col *gl1*; Jeddeloh et al., 1999). Genotyping plants for the *met1-6* was performed as described (Lindroth et al., 2001; Xiao et al., 2003).

Sectioning and Microscopy

Thin-section studies of seeds were carried out using methods as described (Brown et al., 1999). Briefly, seeds were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 6.9, postfixed in osmium ferricyanide, dehydrated through a graded acetone series, and infiltrated with Spurr's resin (EMS). Seeds were sectioned sagittally in the plane of the micropyle and stalk with a LKB historange microtome equipped with glass knives. Then 2.0- to 5.0-μm sections were mounted on glass slides (Brown and Lemmon, 1995) and stained with Polychrome stain (Fox, 1997). Thin sections were observed and photographed under light microscopy. The approximate volume of endosperm was estimated by observations of the area of endosperm in photographs of sectioned seeds.

MET1 Expression Analysis

RT-PCR analysis was performed as described (Kinoshita et al., 1999). Total RNA was isolated from flowers at flower stages 1 to 12 and stage 13 and seeds at 1, 2, 3, 4, 6, and 10 DAP, and the ecotype used was Col *gl1*. For *MET1* expression in embryo and endosperm, seeds of Col-0 and L*er* at 8 DAP were used. Seed was dissected into embryo and endosperm parts as described (Kinoshita et al., 1999). Primers used in the experiment were as follows: *MET1*: *DD-F4*, 5′-TTAAACGATCCTGACAGCGG-3′ and *DD-R1*, 5′-GGATTCAGATTCAGTTCCTTC-3′; *ACT*: *ACT.con.F*, 5′-GATTTGGCATCACACTTTCTACAATG-3′ and *ACT.con.R*, 5′-GATTTGGCATCACACTTTCTACAATG-3′. Primer pairs spanned intron sequences so that amplification of RNA could be distinguished from amplification of any contaminating DNA.

Sieving, Weighing, and Measuring Seed Size

Mature F_1 dry seeds in batches of 200 were weighed using an analytical balance and sieved through a series of fine wire sieves (nos. 40, 45, 50, 60, 70, and 80 with openings of 425, 355, 300, 250, 212, and 180 μm, respectively; USA Standard Testing Sieve; Fisher Scientific). Seeds retained by each sieve were counted. Three replicates were done for all crosses. For measuring seed length and width, photos of seed population of each cross were taken under a microscope (model SZX-ILLB100; Olympus), then seed length and width were measured using NIH Image 1.63. Fifty seeds from each cross were measured for seed size.

Determining the Genotype of F_1 Seed

After pollinating wild-type pistils with pollen from *MET1/ met1-6* plants, we separated F_1 seeds into two categories: medium- and small-sized seeds. F_1 seeds were placed on Murashige and Skoog medium plates, treated for 3 d at 4°C, and then incubated in a growth chamber for 10 d at 22°C. Young seedlings were transferred to soil in pots and grown in the greenhouse at 22°C. A rosette leaf from each 4-week-old plant was harvested. Genomic DNA was isolated, and determining the genotype of *MET1* and *met1-6* alleles was performed as described previously (Lindroth et al., 2001; Xiao et al., 2003). For the cross between the heterozygous *MET1/met1-6* female and wild-type male parent, the same procedure was used except that we separated F_1 seeds into medium- and large-sized categories.

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