Regulation of Seed Size by Hypomethylation of Maternal and Paternal Genomes

Wenyan Xiao, Roy C. Brown, Betty E. Lemmon, John J. Harada, Robert B. Goldberg, and Robert L. Fischer*

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720 (W.X., R.L.F.); Department of Biology, University of Louisiana, Lafayette, Louisiana 70504 (R.C.B., B.E.L.); Section of Plant Biology, Division of Biological Sciences, University of California, Davis, California 95616 (J.J.H.); and Department of Molecular, Cell, and Developmental Biology, University of California, Los Angeles, California 90095 (R.B.G.)

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 F1 seed become hypomethylated. Thus, the distinct role played by hypomethylation at each generation is not known. To address this issue, we examined F1 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 F1 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 F1 seed size. A recessive mutation in another gene that dramatically reduces DNA methylation, DECREASE IN DNA METHYLATION1, also causes parent-of-origin effects on F1 seed size. By contrast, recessive mutations in genes that regulate a smaller subset of DNA methylation (CHROMOMETHYLASE3 and DOMAINS REARRANGED METHYLTRANSFERASE1 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 genomic stabilization, X chromosome inactivation, silencing of transposons and endogenous retroviruses, 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 (Dmnt1) DNA methyltransferase(Li et al., 1992). During gametogenesis and embryogenesis, DNA methylation is lost and subsequently reestablished by the Dmnt3a and Dmnt3b 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 Dmnt1 (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 METHYLTRANSFERASE1 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...
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; Kankan 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 embryo-ogenesis (Finnegan et al., 1996; Kakutani et al., 1996, 2005; Ronemus et al., 1996; Cao et al., 2003; Kankan et al., 2003; Kato et al., 2003; Saize 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 F1 seed size (Adams et al., 2000; Luo et al., 2000). Pollination of antisense MET1 transgenic pistils with wild-type pollen produced large F1 seeds, and reciprocal crosses generated small F1 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 F1 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 F1 progeny. We show that larger F1 seeds were produced when the maternal genome was hypomethylated, whereas smaller F1 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 F1 seed that displayed parent-of-origin effects on size. By contrast, little effect on size was detected in F1 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 (Ler; 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.](image-url)
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 F₁ seed was generated when the maternal parent was homozygous met1-6. By contrast, smaller F₁ seed was produced when the paternal parent was homozygous for the met1-6 mutation.

We quantitatively measured F₁ 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, F₁ 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% F₁ 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 F₁ 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 F₁ seed size of the different crosses was reflected in their seed mass. Average weight of 100 F₁ seeds of the wild-type control cross was 2.8 mg. Pollination of wild-type pistils with met1-6 pollen produced F₁ seeds with an average weight of 1.6 mg per 100 seeds, whereas the reciprocal cross generated 3.9 mg per 100 F₁ seeds.

We also measured F₁ seed length and width of the above three crosses. As shown in Table II, the wild-type F₁ seed from control crosses were on average 497-μm long and 302-μm wide. Pollination of wild-type pistils with met1-6 pollen produced F₁ seeds that were on average 404-μm long and 245-μm wide, approximately 19% smaller than F₁ wild-type seeds. By contrast, F₁ 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.

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

We next examined F₁ seed to see if parental hypomethylation affects structure and the timing of seed development. We sectioned F₁ seed at 3, 4, 5, and 7 DAP (Fig. 3). We found that development of small F₁ seed with a hypomethylated paternal genome was accelerated compared to wild-type controls. For example, in F₁ 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 F₁ seed also displayed a smaller endosperm volume than wild-type seeds. By contrast, larger F₁ seed with a hypomethylated maternal genome displayed delayed development and had a larger endosperm volume when compared to wild-type controls. In particular, these F₁ 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 F₁ 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 F₁ seeds compared to wild-type controls (Table I). That is, nearly half of the F₁ 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 F₁ 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 F₁ 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, F₁ 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

![Figure 2](image-url)

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

<table>
<thead>
<tr>
<th>Genetic Cross</th>
<th>Percentage of Seeds Retained on Indicated Sieve*</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 45 355 μm</td>
<td>No. 50 300 μm</td>
</tr>
<tr>
<td>Wild type</td>
<td>68 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>35 ± 8</td>
<td>0</td>
</tr>
<tr>
<td>met1-6</td>
<td>6 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>Wild type</td>
<td>12 ± 7</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>MET1/met1-6</td>
<td>72 ± 7</td>
<td>27 ± 7</td>
</tr>
</tbody>
</table>

*Two hundred seeds were passed through sieves. Standard deviation for three replicates is shown. ^kTwo hundred seeds were weighed. Weight per 100 seeds is shown. Standard deviation for three replicates is shown.

Table II. Parent-of-origin effects of the met1-6 mutation on F1 seed length and width

<table>
<thead>
<tr>
<th>Genetic Cross</th>
<th>Length*</th>
<th>Change^b</th>
<th>Width*</th>
<th>Change^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μm</td>
<td></td>
<td>μm</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>404 ± 20</td>
<td>-19%</td>
<td>245 ± 22</td>
<td>-19%</td>
</tr>
<tr>
<td>Wild type</td>
<td>497 ± 27</td>
<td></td>
<td>302 ± 19</td>
<td></td>
</tr>
<tr>
<td>met1-6</td>
<td>631 ± 35</td>
<td>+23%</td>
<td>372 ± 22</td>
<td>+23%</td>
</tr>
</tbody>
</table>

*aAverage length and width of 50 F1 seeds is shown. ^bChange is relative to wild-type F1 seed.
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 F1 Seed

F1 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 F1 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 F1 zygote has a hypomethylated set of maternal-derived or paternal-derived chromosomes. This state is preserved because the F1 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 3. Structure differences of F1 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.

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.
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 F₁ 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; Saize 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 (Saize 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 (Saize et al., 2003). After fertilization, the wild-type MET1 allele in the heterozygous F₁ 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 in the gametophyte is sufficient to cause changes in F₁ 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 F₁ 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

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**Table III. Parent-of-origin effects of the ddm1-2 mutation on F₁ seed length and width**

<table>
<thead>
<tr>
<th>Genetic Cross</th>
<th>Length* μm</th>
<th>Change%</th>
<th>Width* μm</th>
<th>Change%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Parent</td>
<td>Paternal Parent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>ddm1-2</td>
<td>462 ± 32</td>
<td>-6%</td>
<td>271 ± 24</td>
</tr>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>491 ± 27</td>
<td></td>
<td>299 ± 16</td>
</tr>
<tr>
<td>ddm1-2</td>
<td>Wild type</td>
<td>607 ± 38</td>
<td>+24%</td>
<td>346 ± 21</td>
</tr>
</tbody>
</table>

*aAverage length and width of 50 F₁ seeds is shown. bChange is relative to wild-type F₁ seed.*
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 F1 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; Dilkies 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 (Dilkies and Comai, 2004). The cross between a diploid female parent and tetraploid male parent produces larger F1 seeds, whereas the reciprocal cross generates smaller F1 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 g11 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 g11. 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 g11) were used in crosses with met1-6 (Col g11) plants. Wild-type Col-0 plants were used in crosses with atm1-2 (Col g11; 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 histarange 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 (Kinosita 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 g11. For MET1 expression in embryo and endosperm, seeds of Col-0 and Ler at 8 DAP were used. Seed was dissected into embryo and endosperm parts as described (Kinosita et al., 1999). Primers used in the experiment were as follows: MET1: DD-F4, 5’-TTAACCAGCTCGACACGGG-3’ and DD-R1, 5’-GGATTCCAAGTTCAGTCTCCT-3’. ACT: ACT-con.F, 5’-GATTTGGGACATACACCTTCTACAATG-3’ and ACT-con.R, 5’-GATTTGGGACATACACCTTCTCAGATG-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 F1 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 and 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 crossess. For measuring seed length and width, photos of seed population of each cross were taken under a microscope (model SZX-ILL.B100; 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 F1 Seed

After pollinating wild-type pistils with pollen from MET1/met1-6 plants, we separated F1 seeds into two categories: medium- and small-sized seeds. F1 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 F1 seeds into medium- and large-sized categories.
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