

DNA Methylation Dynamics during Sexual Reproduction in *Arabidopsis thaliana*

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Summary

DNA methylation maintains genome stability and regulates gene expression [1]. In mammals, DNA methylation is reprogrammed in the germline from one generation to the next [2]. In plants, it was considered that patterns of DNA methylation are stably maintained through sexual reproduction [3–6]. However, a recent report showed discrete variations of DNA methylation profiles from mother to daughter plants [7]. The mechanisms that explain these variations have remained unknown. Here, we report that maintenance DNA methyltransferases are barely expressed during *Arabidopsis* female gametogenesis. In contrast, after fertilization both maintenance and de novo DNA methyltransferases are expressed strongly in the embryo. Embryogenesis is marked by increased de novo DNA methylation, reaching levels that are further maintained in the adult plant. The accumulation of these epigenetic marks after fertilization silences a methylation-sensitive fluorescent reporter. De novo DNA methylation in the embryo provides a mechanism that could account for the gradual remethylation of experimentally demethylated genomes [8, 9]. In conclusion, we uncover that DNA methylation activity fluctuates during sexual reproduction. This cycle likely explains variations of genome-wide patterns of DNA methylation across generations in *Arabidopsis* [7, 10] and enables a limited degree of reprogramming of the epigenome.

Results and Discussion

De Novo DNA Methyltransferases Are Predominantly Expressed in the Egg Cell

In flowering plants, embryogenesis is initiated by fertilization of the egg cell by one of the two male gametes [11]. Two synergid cells and the central cell surround the egg cell [12]. These cells derive from the same haploid spore and form the mature female gametophyte, where we studied the pattern of expression of all members of the three families of genes encoding DNA methyltransferases in the *Arabidopsis* genome [1] (Figure 1; see also Figure S1 available online).

The DNA METHYLTRANSFERASE 1 (MET1) maintains CG methylation in a semiconservative manner [13]. We expressed

pMET1-MET1:RFP (RFP, red fluorescent protein) (Figure S1A) in the null allele *met1-3* and observed that the fusion protein complemented the loss of function of *MET1* (Figure S1B) and was localized to the nucleus in embryos (Figure S2A) and roots (Figure S2B). These results supported that the reporter line reproduced the wild-type pattern of expression of *MET1* and that the protein fusion MET1:RFP was functional. Expression of MET1:RFP was detected during early stages of female gametogenesis (Figure S1A). Levels of RFP fluorescence appeared always lower in the embryo sac than in surrounding tissues (Figure S1G). At maturity, the embryo sac undergoes cellularization, a specialized form of cell division, which separates the egg cell, the central cell, and the synergid cells [13]. After cellularization, levels of RFP fluorescence decreased both in the central cell and the egg cell until they reached levels below the detection threshold in mature ovules (Figures 1A and S1G) as reported previously using a transcriptional reporter [14]. Low levels of RFP fluorescence could be detected in the egg cell, but not in the central cell. In both female gametes, RFP fluorescence was always much lower than in ovule integuments (Figure S1G). Because *MET1* is the main maintenance DNA methyltransferase, its low expression in female gametes was surprising, and we investigated the expression of three *MET1* homologs present in the *Arabidopsis* genome, *MET2a* (At4g14140), *MET2b* (At4g08990), and *MET3* (AT4G13610), using transcriptional reporter constructs. We detected the expression of pMET2a-H2B:RFP (Figure 1B) and pMET2b-H2B:RFP (Figure 1C) in the central cell of mature ovules. The expression of pMET3-H2B:RFP was not detected in mature female gametes (Figure S1D), leaving little evidence for any sustained activity of DNA methyltransferases acting in CG context in the egg cell.

To further assess the expression of *MET1* before the onset of embryogenesis, we isolated egg cells and synergid cells and performed RNA deep-sequencing analysis (Figures 1G–1I; Table S1). All maintenance DNA methyltransferases from the *MET1* family showed levels of expression lower in egg cells and synergid cells than in seedlings (Figure 1G). Egg cells showed higher levels of expression of *MET1* than synergid cells. These data were similar to the expression patterns of corresponding fluorescent fusion proteins. We concluded that *MET1* and its homologs are not expressed in the central cell and synergid cells and that *MET1* is expressed in the egg cell at levels lower than in somatic cells.

In plants, DNA methylation in CNG context is maintained by CHROMOMETHYLASE 3 (CMT3) [15]. We detected levels of *CMT3* transcripts higher in egg cells than in synergid cells (Figure 1H). We obtained plants expressing pCMT3-CMT3:CFP (CFP, cyan fluorescent protein). This construct achieved complementation of the *cmt3* loss-of-function allele in one transgenic line (Figure S1E) that was subsequently used for confocal analysis. Despite high levels of transcripts detected by deep sequencing, we could not detect CFP fluorescence in the egg cell (Figures 1D and S1H). This suggests either that *CMT3*:CFP expression pattern or function is not fully comparable with wild-type *CMT3* or that *CMT3* protein is undergoing a specific regulation in the egg cell. In contrast to *MET1*:RFP fluorescence, which was uniformly distributed

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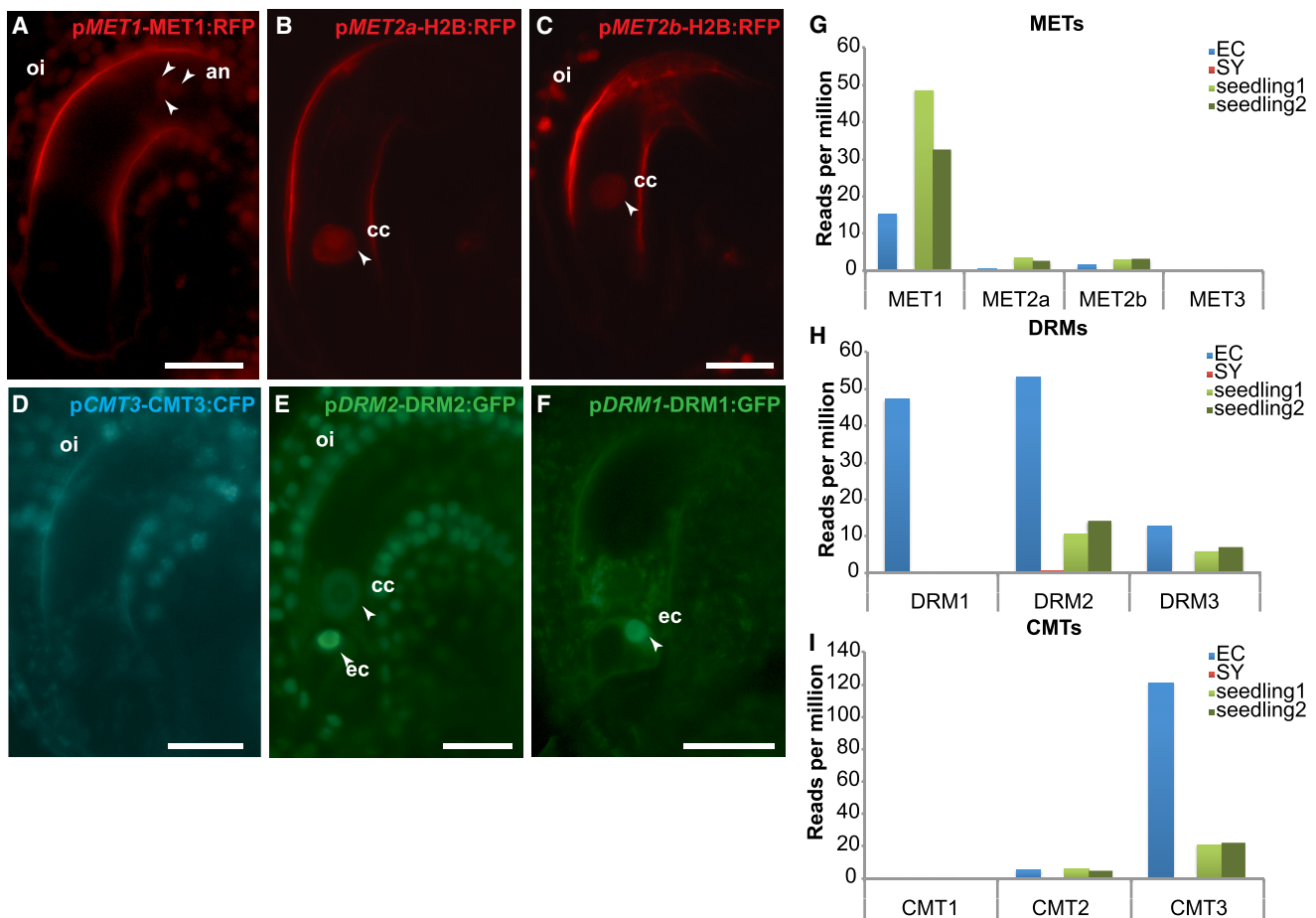


Figure 1. Expression of Reporters for DNA Methyltransferases in *Arabidopsis* Mature Ovules

(A) pMET1-MET1:RFP expression is not detected in the gametes but is detected in the nucleus of antipodal cells (an) and the ovule integuments (oi). Arrowheads in (A)–(F) point to nuclei. Other fluorescence results from background. Scale bars in (A)–(F) represent 20 μ m.

(B) pMET2a-H2B:RFP expression in the central cell nucleus (cc).

(C) pMET2b-H2B:RFP expression in the central cell nucleus (cc) and the outer layers of the integuments (oi).

(D) pCMT3-CMT3:CFP expression is not detected in the gametes but is detected in the nucleus of the ovule integuments (oi).

(E) pDRM2-DRM2:GFP expression is detected in the central cell (cc), the egg cell (ec), and in the nucleus of the ovule integuments (oi).

(F) pDRM1-DRM1:GFP expression is detected in the egg cell (ec).

(G–I) RNA deep-sequencing analysis of transcripts from isolated egg cells (EC), synergid cells (SY), and two seedling samples, used as a sporophytic tissue reference. Transcript abundance is indicated in reads per million for transcripts from genes encoding members of MET1 class (G), CMT3 class (H), and DRM class (I) of DNA methyltransferases. Raw data are available in Table S1.

See also Figure S1.

in the nucleoplasm, CMT3:CFP was localized to intensely fluorescent dots likely corresponding to chromocenters (Figures S2A and S2B). Levels of transcripts and fluorescent protein fusions of CFP with the two homologs of CMT3, CMT1 (At1g80740) and CMT2 (At4g19020), were below our detection threshold during female gametogenesis and in mature gametes (Figures 1H and S1D). We conclude that in both female gametes, the levels of the two main maintenance DNA methyltransferases MET1 and CMT3 are lower in the egg cell than in somatic cells.

The DOMAINS REARRANGED METHYLTRANSFERASES (DRMs) cause de novo methylation of cytosine residues in CHH context [16]. DRM2 was fused to green fluorescent protein (GFP) and expressed under the control of its own promoter. The construct pDRM2-DRM2:GFP complemented the *drm2-2* null allele (Figure S1C), supporting that the DRM2:GFP fusion protein was functional and expressed according to the endogenous pattern of expression of DRM2.

DRM2:GFP was localized to the nucleus of root cells (Figure S2B) in agreement with its function.

DRM2:GFP was expressed during female gametogenesis (Figure S1A). In mature ovules, DRM2:GFP was expressed in the ovule integuments and in both female gametes and showed in the egg cell a signal of higher intensity than in the central cell (Figure 1E). DRM1 expression was not detected in somatic tissues, and DRM1:GFP functionality could not be evaluated due to the absence of an established phenotype for *drm1-2* null mutants. We confirmed the lack of expression of DRM1:GFP in roots, ovule integuments, and seed somatic tissues (Figure S1F). Interestingly, we observed that DRM1:GFP was expressed only in the mature egg cell (Figure 1F). The patterns of expression of DRM1 and DRM2 proteins were mirrored by the levels of corresponding transcripts detected in the egg cell, at least twice higher than in somatic cells (Figure 1I; Table S1). DRM3 transcript was also preferentially expressed in the egg cell (Figure 1I), but we could

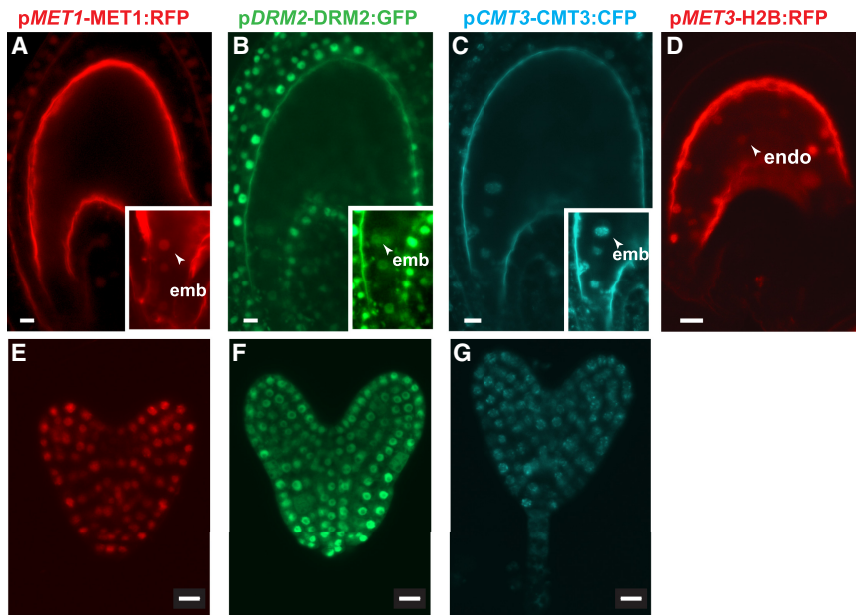


Figure 2. Expression of DNA Methyltransferases Reporters in Endosperm and the Embryo

(A and E) *pMET1-MET1:RFP* expression is not detected in the endosperm but is detected in the nuclei of the young one cell embryo (A, inset, emb) and the seed coat, and in the heart stage embryo (E).

(B and F) *pDRM2-DRM2:GFP* expression is not detected in the endosperm but is detected in the nuclei of the young two cell embryo (B, inset, emb), and in the heart stage embryo (F).

(C and G) *pCMT3-CMT3:CFP* expression is not detected in the endosperm but is detected in the nuclei of the young two cell embryo (C, inset, emb), and in the heart stage embryo (G).

(D) *pMET3-H2B:RFP* expression is detected in the endosperm (endo) (a slightly earlier developmental stage than shown in A–C).

Arrowheads point to nuclei. Other fluorescence results from background. Scale bars represent 20 μm . See also Figure S2.

not detect the expression of *DRM3:GFP* in the egg cell (Figure S1D). We concluded that among all DNA methyltransferases, the egg cell expresses predominantly the de novo DNA methyltransferases, *DRM1* and *DRM2*. We propose that this enrichment might compensate the deprivation of maintenance DNA methyltransferases *MET1* and *CMT3* during female gametogenesis. In contrast with the egg cell, the central cell and the synergid cells expressed low levels of de novo DNA methyltransferases and undetectable levels of maintenance DNA methyltransferases, which likely explains why these cells express genes that are silenced by DNA methylation in vegetative tissues [14, 17–19].

DNA Methyltransferases Are Strongly Expressed during Embryo Development

We analyzed the expression of the DNA methyltransferases after fertilization. Fertilization of the central cell gives rise to the endosperm [11]. We were unable to detect the expression of *MET1:RFP*, *DRM2:GFP*, and *CMT3:CFP* in the endosperm in contrast with the high levels observed in the seed coat, suggesting that the endosperm expresses low levels of DNA methyltransferases when compared with somatic tissues (Figures 2A–2C). From all DNA methyltransferases studied, we could only detect the expression of *MET3* in the endosperm (Figure 2D). Those results are consistent with endosperm transcriptome analyses (available online at <http://seedgenenetwork.net/> [20]; Figure S2C). Although only low levels of *MET1* transcripts are detected in endosperm, they appear sufficient to support *MET1* activity in the maintenance of imprinted expression in this tissue [17]. Together with the expression of the DNA demethylase *DEMETER* in the central cell [21], the reduced expression of DNA methyltransferases in the central cell and endosperm likely accounts for the low levels of DNA methylation in endosperm [22, 23].

The fertilized egg cell elongates and after the first asymmetric zygotic division gives rise to a long suspensor cell and a small apical cell, from which derives most of the embryo [24]. In contrast with the endosperm, all three major DNA methyltransferases, *MET1*, *DRM2*, and *CMT3*, were strongly expressed in the embryo proper and the suspensor (Figures

2A–2C and S2C). These results were supported by detection of the respective transcripts at a similar stage [25] (Figure S2D). These enzymes were also expressed at later stages of embryo development from the globular to the torpedo stage (Figures 2E–2G and S2A) at levels comparable with that observed in the integuments. We conclude that fertilization triggers the expression of all major DNA methyltransferases in the embryo, reaching expression levels comparable to or higher than that in somatic tissues and that this intense transcriptional activity is maintained during embryo development.

Increase of CHH Methylation during Embryo Development

To evaluate the impact of high levels of DNA methyltransferases during embryogenesis, we quantified directly DNA methylation using bisulfite sequencing on leaves and dissected embryos at successive stages of development. We analyzed five loci that are silenced by DNA methylation in adult plants: *AtSN1*, *FERTILIZATION-INDEPENDENT SEED 2 (FIS2)* [17], *FLOWERING WAGENINGEN A (FWA)* [18], *MEDEA (MEA)* [26], and *SUPPRESSOR OF DRM CMT3 (SDC)* [27]. We did not observe any significant change in methylation in CG (Figures 3A and S3) and CHG (Figures 3B and S3) contexts throughout embryo development. However, CHH methylation increased during embryo development (Figures 3C and S3).

We studied the effect of de novo methyltransferases *DRM1* and *DRM2* in embryos at the heart and torpedo stages (Figures 3D, 3E, and S3). In comparison to wild-type, CHH methylation was lost in the double mutant *drm1-2 drm2-2* during embryo development and in rosette leaves. The RNA-directed DNA methylation (RdDM) pathway targets CHH methylation by *DRM1* and *DRM2* and requires short interfering RNAs and the RNA polymerases IV and V (*PolIV* and *PolV*, respectively) [28–31]. A similar loss of CHH methylation was observed in mutants for RNA *PolIV* and RNA *PolV* (Figure 3D), which further supported that the RdDM pathway is involved in gradual de novo methylation in the embryo. During early embryogenesis, CHH methylation was reduced in the *drm1 drm2* double mutant further than in single mutants *drm1* and *drm2* (Figure 3D). Later, during embryo development and in the adult plant, we no longer observed an impact of *drm1* on CHH

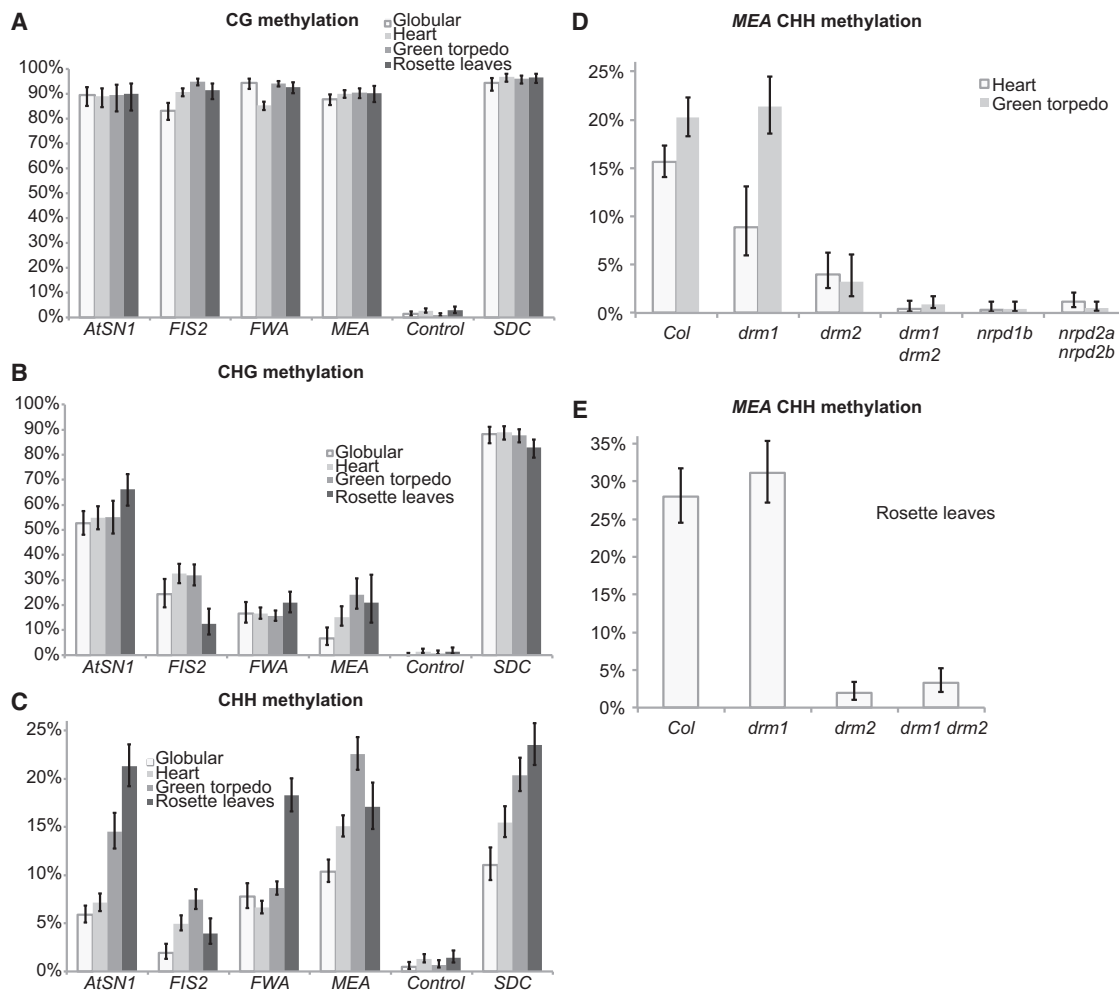


Figure 3. DNA Methylation during Embryogenesis

(A–C) Levels of DNA methylation at five loci during embryogenesis. Levels of DNA methylation in CG context (A), CHG context (B), and CHH context (C) at the methylation-sensitive loci *AtSN1*, *FIS2*, *FWA*, *MEA*, and *SDC* are compared with the unmethylated *Control* locus *At2g20610*. Rosette leaves provide a control for levels of DNA methylation in adult plants.

(D and E) Impact of the RdDM pathway on the methylation levels of the locus *MEA* during embryogenesis.

(D) DNA methylation in CHH context in heart-stage and green torpedo-stage embryos at the *MEA* locus. Wild-type levels in Columbia (*Col*) background are compared with mutants for the RdDM pathway.

(E) Same measurement in rosette leaves.

Raw data are available in Figure S3. Error bars represent a confidence interval with 95% confidence limits (Wilson score interval; see details in Figure S3).

methylation, and DRM2 appeared sufficient to account for DNA methylation during these stages (Figure 3E). We conclude that DRM1 functions as a de novo methyltransferase. We could not detect levels of DRM1 expression during early embryogenesis and propose that DRM1 activity largely derives from its expression in the egg cell. In the late embryo and in the adult plant, DRM1 is no longer expressed and DRM2 becomes the main de novo methyltransferase. Altogether, our results suggest that the RdDM pathway contributes actively de novo DNA methylation during embryo development.

Silencing of a DNA Methylation-Sensitive Reporter during Embryo Development

In order to investigate the impact of increased DNA methylation at embryo stages younger than could be studied using bisulfite sequencing, we used the *FWA-GFP* fluorescent reporter, whose expression is directly silenced by both CG

and CHH DNA methylation [32]. As reported earlier [17], *FWA-GFP* is expressed ectopically in 50% of the pollen grain in a *met1-3/+* mutant (Figure 4A). We crossed *met1-3/+*; *FWA-GFP/FWA-GFP* plant as pollen donor with wild-type ovules and imaged GFP fluorescence in developing seeds. We observed that the paternal *FWA-GFP* allele was ectopically expressed in 50% developing endosperm. The absence of expression of DRMs in the endosperm (Figures 2B and S2C) is sufficient to explain why low MET1 activity in endosperm (Figure S2C; [14]) is sufficient to maintain the *FWA-GFP* paternal allele silenced as shown previously [17].

All sperm cells inheriting the *met1-3* mutation provided a demethylated transcriptionally active paternal *FWA-GFP* allele. Because sperm cells are genetically identical and have equal capacity to fertilize the egg cell and the central cell [33], 50% of developing embryos also inherited a demethylated active paternal *FWA-GFP* allele (Figures 4B–4D). However, in opposition to the endosperm lineage, only 13.7% (n = 167) of

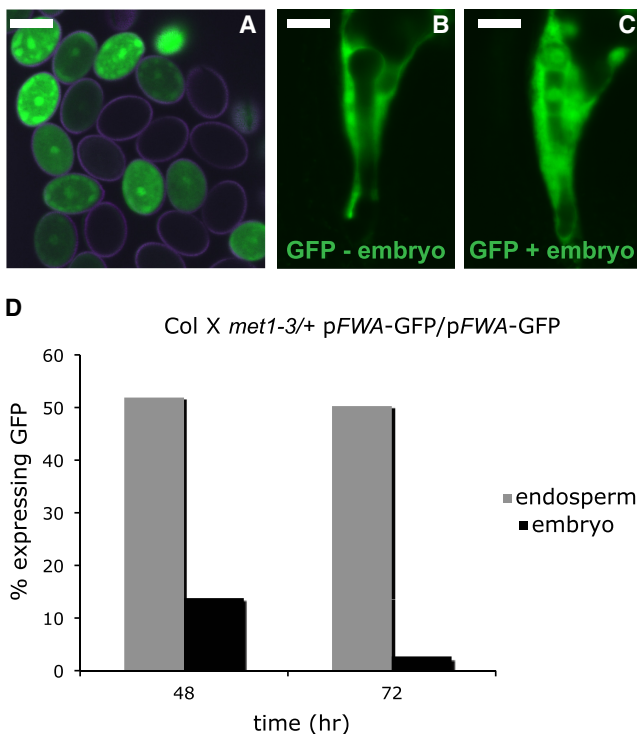


Figure 4. Active Silencing of a DNA Methylation-Sensitive Reporter in the Early Embryo

(A) GFP signal in segregating pollen from *met1-3/+; FWA-GFP/FWA-GFP* plants. The pollen wall is outlined in purple.

(B) Detail from a seed that inherited a *met1-3; FWA-GFP* paternal allele, showing GFP signal in the endosperm but not in the embryo where *FWA-GFP* is silenced (48 hr after fertilization).

(C) Detail from a seed that inherited a *met1-3; FWA-GFP* paternal allele, showing GFP signal in the endosperm and in the embryo (48 hr after fertilization).

(D) Percentage of embryos and endosperm showing GFP signal in the endosperm (as shown in B, endosperm) and in the embryo and endosperm (as shown in C, embryo) at 48 and 72 hr after fertilization. Scale bars represent 10 μm for (A) and 25 μm for (B) and (C).

embryos expressed *FWA-GFP* at 48 hr after pollination (Figures 4B–4D). This percentage gradually decreased to reach 2.6% ($n = 151$) at 72 hr after pollination (Figures 4B–4D). Hence, active silencing of DNA methylation-sensitive targets, reflected by gradual silencing of *FWA-GFP*, indicates that DNA methylation takes place in the embryo, but not in the endosperm. Silencing of *FWA-GFP* requires methylation in CG and CHH contexts [34], and 25% of sperm cells provide a copy of *FWA-GFP* demethylated on both strands. MET1 and CMT3 require a methylated template and are unable to methylate completely demethylated paternal alleles of *FWA-GFP*. Hence, the complete silencing of *FWA-GFP* observed in more than 25% embryos implies that RdDM would methylate CG, CHG, and CHH de novo in the embryo. This result suggests that DNA methylation levels may be lower in the zygote and early embryo than those detected by bisulfite sequencing at the globular stage (Figure 3A).

Conclusions

Our data show that in the egg cell, DNA methylation relies predominantly on de novo DNA methyltransferases DRM1 and DRM2. We show that developing embryos experience active de novo DNA methylation by the RdDM pathway. The

egg cell specifically expresses DRM1, which together with DRM2 initiates de novo DNA methylation after fertilization and silences loci sensitive to DNA methylation. De novo DNA methylation in *Arabidopsis* requires 24 nt small interfering RNAs (siRNAs) [28–31]. siRNAs might originate from the male gametes [35], maternal ovule tissues [36], or the endosperm [37]. However, the high activity of RdDM in the embryo lineage suggests that the embryo might produce the 24 nt siRNAs required for its own de novo DNA methylation.

DNA methylation during *Arabidopsis* sexual reproduction is more dynamic than previously expected. Active de novo DNA methylation in the developing embryo provides a mechanism that accounts for the gradual remethylation observed in successive generations of plants that descend from an ancestor carrying a demethylated genome [8, 9, 38]. De novo methylation might also reset silencing of the few imprinted genes expressed in the embryo of maize and rice [39–41]. In succession of wild-type generations, de novo methylation likely reinforces the pattern of DNA methylation on target loci like transposons to maintain genome stability, and this mechanism is compatible with the stable maintenance of epialleles and silenced transgenes across generations [3–6]. In addition, the wave of de novo DNA methylation during early embryogenesis implies a limited loss of DNA methylation during female gametogenesis, which would not be comparable to the large-scale reprogramming of DNA methylation observed in mammals [42]. Nevertheless, our results predict that DNA methylation fluctuates from one generation to the next and may provide a mechanism that explains the transgenerational epigenetic instability observed in wild-type [7, 43].

Supplemental Information

Supplemental Information includes three figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2012.07.061>.

Acknowledgments

P.E.J., F.B., and R.Y. were funded by Temasek Life Sciences Laboratory. D.S. was supported by grant number 7811 from the Japan Society for the Promotion of Science Fellowships. T.H. was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 18075004) and the Japan Science and Technology Agency ERATO project. We thank Toshifumi Nagata, Nori Kurata, Atsushi Toyoda, and Asao Fujiyama at the National Institute of Genetics (Mishima, Japan) for their contribution to the RNAseq data from isolated egg cells and synergid cells.

Received: April 26, 2012

Revised: June 26, 2012

Accepted: July 27, 2012

Published online: August 30, 2012

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