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Arabidopsis male sexual lineage exhibits more robust maintenance of CG methylation than somatic tissues

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Cytosine DNA methylation regulates the expression of eukaryotic genes and transposons. Methylation is copied by methyltransferases after DNA replication, which results in faithful transmission of methylation patterns during cell division and, at least in flowering plants, across generations. Transgenerational inheritance is mediated by a small group of cells that includes gametes and their progenitors. However, methylation is usually analyzed in somatic tissues that do not contribute to the next generation, and the mechanisms of transgenerational inheritance are inferred from such studies. To gain a better understanding of how DNA methylation is inherited, we analyzed purified *Arabidopsis thaliana* sperm and vegetative cells—the cell types that comprise pollen—with mutations in the *DRM*, *CMT2*, and *CMT3* methyltransferases. We find that DNA methylation dependency on these enzymes is similar in sperm, vegetative cells, and somatic tissues, although *DRM* activity extends into heterochromatin in vegetative cells, likely reflecting transcription of heterochromatic transposons in this cell type. We also show that lack of histone H1, which elevates heterochromatic DNA methylation in somatic tissues, does not have this effect in pollen. Instead, levels of CG methylation in wild-type sperm and vegetative cells, as well as in wild-type microspores from which both pollen cell types originate, are substantially higher than in wild-type somatic tissues and similar to those of H1-depleted roots. Our results demonstrate that the mechanisms of methylation maintenance are similar between pollen and somatic cells, but the efficiency of CG methylation is higher in pollen, allowing methylation patterns to be accurately inherited across generations.

DNA methylation | epigenetic inheritance | histone H1 | pollen

Cytosine methylation is a covalent DNA modification that regulates transcription in eukaryotes (1). The highest levels of methylation in plant and animal genomes are typically located within symmetric CG dinucleotides (1). Methylation in this sequence context is virtually ubiquitous in plant transposable elements (TEs), which are transcriptionally silenced by methylation, but also occurs within many genes without disrupting their expression (1, 2). CG methylation is catalyzed by the Dnmt1 methyltransferase family, called MET1 in plants (1, 2). MET1 restores full methylation of hemimethylated CG dinucleotides generated by DNA replication, thereby perpetuating methylation patterns after cell division (1, 2). This maintenance activity is thought to allow DNA methylation to carry epigenetic information—and influence gene expression and phenotype—across generations (3, 4). The nature of this mechanism predicts that imperfect maintenance of CG methylation should lead to complete loss as methylation is diluted during each cell division, so that the only stable methylation states for a CG site in a population of cells should be fully methylated or fully unmethylated. However, the methylation levels measured at *Arabidopsis thaliana* CG sites appear to be too low for stable maintenance (5, 6). Therefore, exactly how CG methylation is so robustly inherited in flowering plants is not entirely clear.

In addition to MET1, plants possess the chromomethylase (CMT) and DRM methyltransferase families. In *Arabidopsis*, CMT3 catalyzes methylation of semisymmetric CNG sites, which

is typically analyzed as CHG (H stands for A, T, or G) to avoid overlap with CG (1, 2). A related enzyme, CMT2, catalyzes asymmetric (CHH) methylation, primarily in heterochromatic TEs (7, 8). Both enzymes rely on dimethylation of lysine 9 of histone H3 (H3K9me₂), a histone modification characteristic of plant heterochromatin (8, 9). DRM enzymes (DRM1 and DRM2 in *Arabidopsis*), which are guided by the small RNA-directed DNA methylation (RdDM) pathway (10), catalyze CHH methylation of more euchromatic TEs (7, 11, 12). Methylation mediated by CMT and DRM enzymes, collectively referred to as non-CG methylation, functions to repress TE expression and is almost completely absent from genes (1, 2). Non-CG methylation varies substantially between plant cell types and tissues (13–17) for reasons that remain largely unexplained.

Transgenerational inheritance of DNA methylation patterns is carried out by gametes and the cellular lineages from which they differentiate. The shoot apical meristem, a small group of stem cells that develops early during *Arabidopsis* embryogenesis, gives rise to all above-ground tissues, including the floral meristems that produce the sexual organs (18). In these, certain cells differentiate into meiocytes, which undergo meiosis to produce haploid spores (19, 20). The spores go on to divide by mitosis to create the multicellular male and female gametophytes. The male gametophyte, pollen, consists of two sperm cells and a vegetative cell, which forms the pollen tube that delivers the sperm into the female gametophyte (19, 20). As this

Significance

Cytosine methylation is a mechanism of epigenetic inheritance—the transmission across generations of information that does not reside in DNA sequence. This transmission is mediated by enzymes that copy methylation states following DNA replication. Only a small group of plant cells—gametes and their progenitors—participates in inheritance, yet methylation is usually studied in other cell types, in which cytosine methylation within CG dinucleotides appears to be too low for stable maintenance. Here, we examine methylation in the pollen grains of *Arabidopsis thaliana* plants with methyltransferase mutations and show that although methylation is maintained by similar mechanisms in pollen and somatic cells, maintenance of CG methylation is more efficient in pollen, explaining how methylation can be faithfully inherited across generations.

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developmental sequence illustrates, plants specify dedicated sexual lineages much later than animals, which set aside the germ line during embryogenesis (21). Nonetheless, only a very small fraction of plant cells can give rise to gametes. Despite their importance, these cells are rarely directly examined in studies of DNA methylation, so that most of our knowledge about the mechanisms of epigenetic methylation inheritance is inferred from analyses of differentiated tissues that do not contribute to the next generation.

To help address this deficiency, we analyzed DNA methylation in purified *Arabidopsis* sperm and vegetative cells with mutations in *CMT3*, *CMT2*, and both *DRM1* and *DRM2*, respectively. We also analyzed sperm and vegetative cells with mutation of both genes encoding canonical histone H1, a chromatin protein that globally reduces heterochromatic DNA methylation in all sequence contexts (7). Despite the reported absence of H3K9me2

from the vegetative cell nucleus (22), we find that methyltransferase dependencies of non-CG methylation in the sperm and vegetative cells are similar to those of leaves and other examined tissues, although RdDM partially extends into vegetative cell heterochromatin. Unlike in somatic tissues, mutation of H1 does not substantially increase heterochromatic methylation in either sperm or vegetative cell. Instead, methylation of CG sites is elevated in wild-type (WT) pollen, resembling *h1* mutant somatic tissues. The higher CG methylation levels in pollen are easier to reconcile with stable transgenerational maintenance, indicating that CG methylation efficiency is reduced in somatic cells with limited division potential. This in turn suggests that small DNA methylation differences between somatic cells or tissues may be caused by variance in maintenance efficiency instead of active developmental reprogramming.

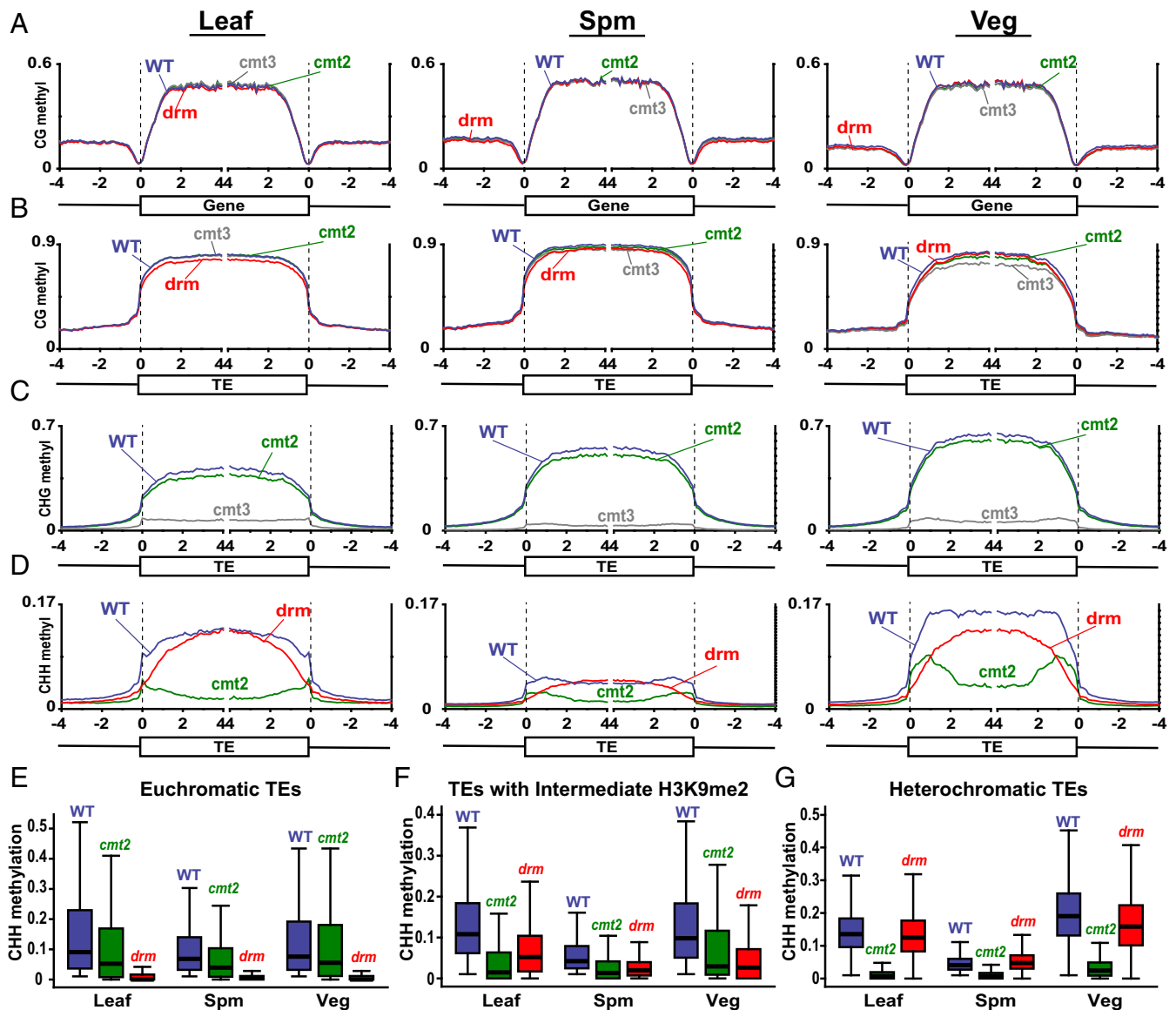


Fig. 1. DNA methylation in *Arabidopsis* leaf, sperm (Spm), and vegetative cell (Veg). Genes (A) and TEs (B–D) were aligned at the 5' and 3' ends. Methylation within each 100-bp interval was averaged and plotted from 4 kb away from the annotated gene or TE (negative numbers) to 4 kb into the annotated region (positive numbers). The dashed lines represent the points of alignment. (E–G) Box plots show CHH methylation levels within 50-bp windows in WT, *cmt2*, and *drm* mutants in TEs with low H3K9me2 in leaves (euchromatic TEs; E), TEs with intermediate H3K9me2 (F), and TEs with high H3K9me2 (heterochromatic TEs; G). Each box encloses the middle 50% of the distribution, with the horizontal line marking the median and vertical lines marking the minimum and maximum values that fall within 1.5 times the height of the box. Only windows with methylation greater than 1% in WT tissues and with at least 20 informative sequenced cytosines across all samples are included.

Results

Mechanisms of DNA Methylation Maintenance Are Similar Between Pollen and Leaves. We isolated sperm and vegetative cell nuclei from *cmt3*, *cmt2*, *drm1drm2* (*drm*), and control WT plants by fluorescence-activated cell sorting (FACS) (15). Genome-wide analysis of DNA methylation by bisulfite sequencing (Table S1) revealed that, as expected, none of the mutations have a major effect on CG DNA methylation in either genes or TEs (Fig. 1*A* and *B*), just as in leaves (Fig. 1*A* and *B*) and other somatic tissues (5–8, 23). The *cmt3* mutation has a similarly strong effect on TE CHG methylation in sperm, vegetative cells, and leaves (Fig. 1*C*), indicating that CHG methylation in both pollen cell types is maintained primarily by CMT3, despite the reported lack of H3K9me2 in the vegetative nucleus (22). Likewise, mutation of either *cmt2* or *drm* affects the patterns of CHH methylation similarly in sperm, vegetative cells, and leaves (Fig. 1*D*), even though CHH methylation is about threefold higher in vegetative cells compared with sperm (Fig. 1*D*) (15, 16). As in leaves and other tissues (7, 8), sperm and vegetative cell CHH methylation in euchromatic TEs—here defined as those with a low level of H3K9me2 in leaves (Fig. S1) (7)—is primarily dependent on *drm* (Fig. 1*E* and Fig. S2*A*). CHH methylation of TEs with intermediate H3K9me2 is dependent on *drm* and *cmt2* (Fig. 1*F*), with *cmt2* causing a stronger reduction in TE bodies and *drm* in TE edges (Fig. S2*B*) (7), and methylation of the most heterochromatic TEs is performed primarily by CMT2 in sperm, vegetative cells, and leaves (Fig. 1*G* and Fig. S2*C*). An interesting feature of the data is that vegetative cells have only modestly higher CHH methylation of euchromatic TEs compared with sperm (Fig. 1*E*), whereas heterochromatic TEs are far more methylated in the vegetative cell (Fig. 1*G*). The elevated CHH

methylation in the vegetative cell is thus largely caused by increased activity of H3K9me2-dependent CMT2 in heterochromatin (Fig. 1*G*). Overall, our results demonstrate that the roles of the major *Arabidopsis* DNA methyltransferases in pollen are remarkably similar to those in somatic tissues.

New Heterochromatic RdDM Targets in the Vegetative Cell. Although pollen and somatic heterochromatic CHH methylation is largely dependent on CMT2, the effect of the *cmt2* mutation is weaker in the vegetative cell compared with leaf and sperm (Fig. 1*G* and Fig. S2*C*). The *cmt2* mutation also does not reduce CHH methylation in TEs with intermediate H3K9me2 as much in the vegetative cell as it does in leaf (Fig. 1*F* and Fig. S2*B*). This is easily noticeable in the methylation data as heterochromatic TEs that retain substantial CHH methylation in *cmt2* vegetative cells (Fig. 2*A*) and as intermediate TEs that retain essentially WT methylation levels in *cmt2* vegetative cells (Fig. S2*D*). To systematically identify heterochromatic loci with CMT2-independent CHH methylation, we compared methylation levels between all 50-bp windows in heterochromatic TEs that retain CHH methylation in either *cmt2* vegetative cell or *cmt2* leaf (Fig. 2*B*). The vast majority of windows have more methylation in the vegetative cell (Fig. 2*B*). As expected, CHH methylation of these windows is dependent on CMT2 in leaf and sperm (Fig. 2*C*). WT vegetative cells have higher CHH methylation of these loci than leaf or sperm, which is dependent on both CMT2 and DRM (Fig. 2*C*), resembling the pattern observed in TEs with intermediate H3K9me2 (Fig. 1*F*). TEs containing such loci also exhibit higher CHH and CHG methylation than other heterochromatic TEs in the vegetative cell (Fig. 2*D*). Thus, in addition to the usual maintenance of heterochromatic CHH methylation by

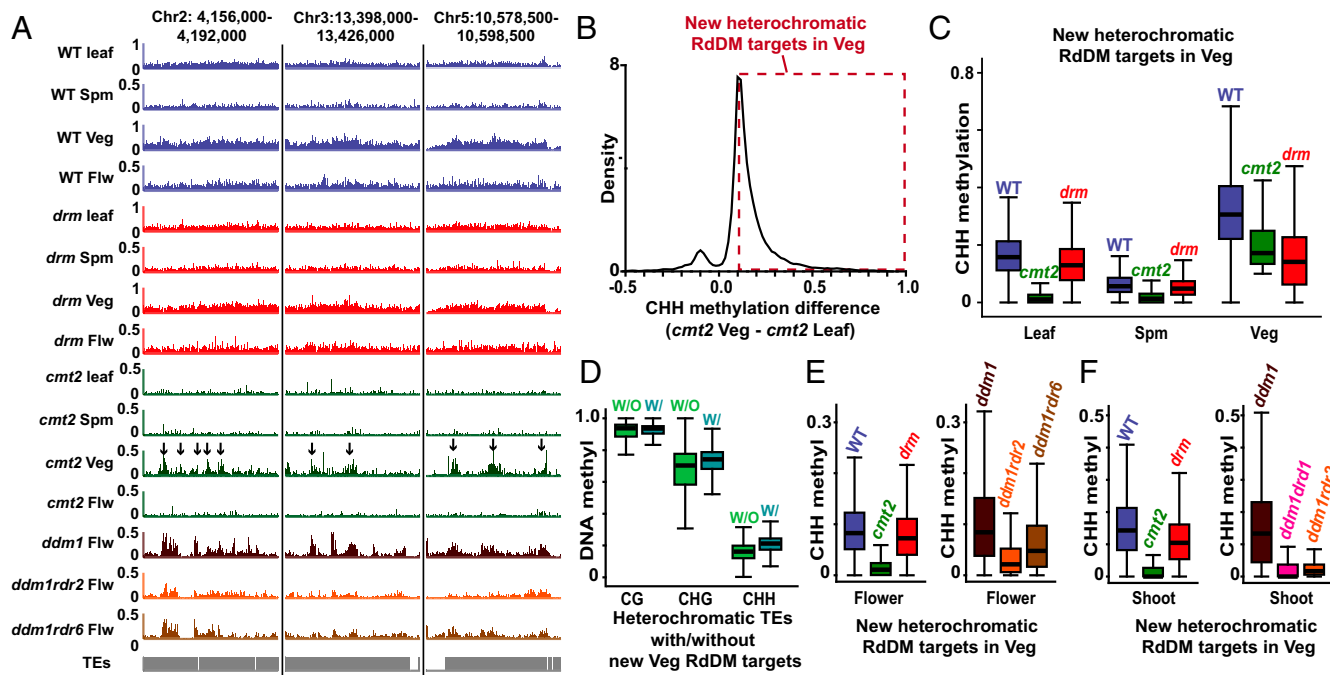


Fig. 2. RdDM extends into heterochromatin in the vegetative cell. (A) Snapshots of CHH methylation in leaf, sperm (Spm), vegetative cell (Veg), and flower (Flw) of WT, *cmt2*, and *drm* mutants (the arrows point to loci that retain CHH methylation in *cmt2* mutant vegetative cell, but not in leaf, sperm, and flower). (B) A density plot showing the frequency distribution of CHH DNA methylation differences between 50-bp windows in heterochromatic TEs that retain at least 10% CHH methylation in either *cmt2* vegetative cell or *cmt2* leaf. Only windows with at least 20 informative sequenced cytosines in both samples are included. The dashed box indicates windows in which CHH methylation is at least 10% greater in *cmt2* vegetative cell than in *cmt2* leaf. These windows are defined as new heterochromatic RdDM targets in the vegetative cell. (C) Box plots show CHH methylation levels of 50-bp windows marked by the dashed box in *B* (heterochromatic RdDM targets in the vegetative cell) in WT, *cmt2*, and *drm* mutants. Only windows with at least 20 informative sequenced cytosines in each sample are included. (D) Box plots show vegetative cell methylation levels of heterochromatic TEs with RdDM targets in the vegetative cell (W) and other heterochromatic TEs (WO). (E and F) Box plots show CHH methylation levels of 50-bp windows marked by the dashed box in *B* in the indicated genotypes for flowers (E) and shoots (F).

CMT2, some parts of vegetative cell heterochromatin are also targeted by DRM via RdDM, suggesting that these loci are less heterochromatic in this cell type. However, the 466,450 bp covered by such loci in our analysis (Dataset S1) represent only 7.9% of CMT2-dependent heterochromatic loci in the vegetative cell (5,905,450 bp), so that the vast majority of vegetative cell heterochromatic CHH methylation requires CMT2 (Fig. 1G).

RdDM-Targeted Heterochromatic TEs Are Likely Transcribed in the Vegetative Cell. *Arabidopsis* TEs are generally transcriptionally silenced, but activation of heterochromatic TEs has been reported in the vegetative cell (24). Furthermore, transcriptional activation of such TEs in the *ddm1* mutant background has been associated with RdDM targeting (25), suggesting that DRM-targeted heterochromatic TEs (Fig. 2A–C) may be transcribed in the vegetative cell. To test this hypothesis, we examined how TEs targeted by RdDM in the vegetative cell are methylated in *ddm1* mutant flowers (25) and shoots (7). As expected, CHH methylation of these loci is maintained by CMT2 in WT flowers and shoots (Fig. 2A, E, and F). However, CHH methylation becomes RdDM-dependent in both tissues in *ddm1* plants, as it is greatly reduced by additional mutation of either *RDR2* or *DRD1* (Fig. 2E and F), genes that are required for RdDM (10). CHH methylation in *ddm1* flowers is also reduced by mutation of *RDR6*, which contributes to RdDM of transcriptionally active loci (Fig. 2E) (25). This can be seen at individual TEs, where the *RDR2*- and *RDR6*-dependent methylation patterns of *ddm1* flowers resemble those of *cmt2* vegetative cells (Fig. 2A). Heterochromatic TEs targeted by RdDM in the vegetative cell are also more likely to be transcribed in *ddm1* mutants than other heterochromatic TEs (Fig. S3A and Dataset S2), and they tend to be much longer (Fig. S3B), suggesting they are mostly full-length, transcriptionally competent elements (25). Overall, our results support the interpretation that RdDM targets transcriptionally activated heterochromatic TEs in the vegetative cell.

Histone H1 Is Present in Sperm but Absent from the Vegetative Cell. The targeting of heterochromatic loci by RdDM in the vegetative

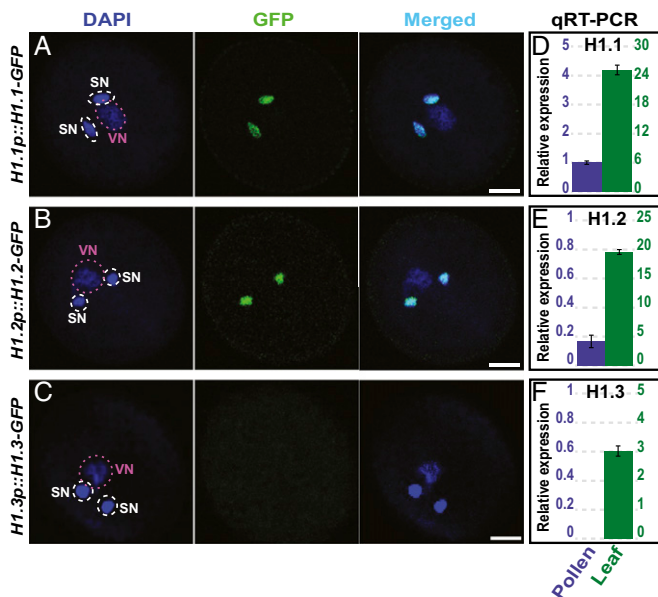


Fig. 3. H1 expression in pollen. (A–C) H1.1-GFP (A) and H1.2-GFP (B) reporter constructs (27) are expressed in the sperm cells but not in the vegetative cell of pollen, whereas H1.3-GFP is not expressed in pollen (C). DAPI stains DNA in the pollen nuclei. SN, sperm nucleus; VN, vegetative nucleus. (Scale bar, 5 μ m.) (D–F) Quantitative RT-PCR of *H1.1* (D), *H1.2* (E), and *H1.3* (F) in pollen and leaf. The y axis scales show relative expression in pollen (left) and leaf (right).

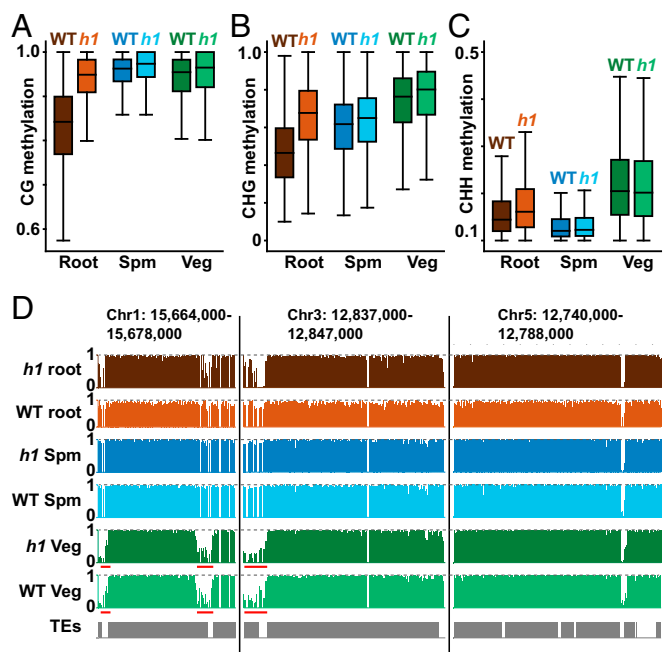


Fig. 4. Lack of H1 does not increase heterochromatic methylation in pollen. (A–C) Box plots show DNA methylation levels in 50-bp windows within heterochromatic TEs in WT and *h1* mutant root, sperm (Spm), and vegetative cell (Veg). Only windows with at least 10 informative sequenced cytosines and methylation of at least 30% for CG and 10% for CHG and CHH are included. (D) Snapshots of CG methylation in root, sperm, and vegetative cell of WT and *h1* mutants. Note how DNA methylation levels compare with the horizontal dashed line that denotes 100% methylation. Underlined regions bordering heterochromatic TEs are subject to active DNA demethylation in the vegetative cell (15).

cell (Fig. 2) as well as much higher levels of heterochromatic CHH methylation compared with sperm (Fig. 1G) suggest that heterochromatin may be more accessible to DNA methyltransferases in the vegetative nucleus. This is consistent with the decondensation of chromatin and lack of heterochromatic foci reported in the vegetative cell (22). Histone H1 is a chromatin protein that reduces the efficiency of heterochromatic DNA methylation (7). We therefore wondered if increased heterochromatic methylation may be caused by reduced abundance of H1 in the vegetative cell. The *Arabidopsis* genome encodes two canonical, widely expressed *H1* genes, *H1.1* and *H1.2*, as well as a truncated, stress-induced *H1.3* gene generally expressed at a much lower level (26). We find that *H1.1-GFP* and *H1.2-GFP* reporter proteins (27) are present in sperm but undetectable in vegetative nuclei (Fig. 3A and B). *H1.3-GFP* is not detectable in pollen (Fig. 3C). Quantitative RT-PCR experiments show that *H1.1* and *H1.2* transcript levels are much lower in pollen than in leaf (Fig. 3D and E), whereas *H1.3* is undetectable in pollen (Fig. 3F). Vegetative cell chromatin thus appears to be depleted of histone H1, and sperm chromatin may contain less H1 than leaves and other tissues.

Lack of H1 Does Not Increase Heterochromatic Methylation in Pollen.

To determine how histone H1 influences DNA methylation in pollen, we analyzed methylation of FACS-purified sperm and vegetative cells with mutations in *H1.1* and *H1.2* (*h1* mutants) (Table S1). Unlike somatic tissues (7), vegetative cells do not show substantially increased heterochromatic methylation in any sequence context (Fig. 4A–C), consistent with undetectable expression of H1 in the vegetative cell (Fig. 3). Heterochromatic methylation is also largely unaffected in sperm cells (Fig. 4A–C), perhaps due to lower levels of H1 in this cell type (Fig. 3). Overall, heterochromatic CG methylation in WT sperm and vegetative cells is similar to *h1* roots

and substantially higher than in WT roots (Fig. 4A), which is readily apparent even at individual loci (Fig. 4D). Pollen heterochromatic CG methylation is therefore substantially higher than in somatic tissues, potentially due to reduced levels of H1.

CG Methylation Is More Robustly Maintained in Pollen than in Leaf or Root. We were intrigued by the higher levels of heterochromatic CG methylation in sperm and vegetative cells (Fig. 4A and D) because they suggested an explanation for a longstanding mystery. The semiconservative model of CG methylation maintenance (1) implies that even a modest reduction in maintenance efficiency below 100% should lead to dilution of methylation with each cell division that eventually causes complete loss (28). However, CG methylation levels measured in somatic cells (Fig. 4A) (5, 6) appear to be below what would be required for stable maintenance (28). Somatic tissues do not contribute to the next generation, so higher methylation efficiency in cells that do, including gametes, would solve the problem. Indeed, methylation of individual CG sites in sperm and vegetative cells, as well as in the microspores from which they arise, is much higher than in leaves and roots, both in heterochromatic TEs and genes (Fig. 5A and B). CG methylation of euchromatic TEs is also higher in sperm and microspores than in somatic tissues (Fig. 5C). The low methylation of euchromatic TEs in vegetative cells (Fig. 5C) is

attributable to extensive active DNA demethylation of such sequences in this cell type (Fig. 4D) (15, 16), which may also explain the somewhat lower CG methylation of genes compared with sperm and microspores (Fig. 5A).

A potential concern regarding our analysis is that we are comparing methylation between pure male reproductive cells and complex somatic tissues. Methylation heterogeneity between cell types within leaves and roots could, when averaged, create the impression of an overall lower methylation efficiency even though methylation within each cell type is as robust as in pollen. To circumvent this issue, we analyzed published methylation data from multiple purified root cell types (17). These cells show CG methylation levels comparable to those of whole roots and well below those of male reproductive cells (Fig. 5D–F). Therefore, the lower CG methylation levels we observe in somatic cells and tissues are caused by reduced methylation efficiency rather than tissue heterogeneity.

To understand the mechanism of increased CG methylation efficiency in pollen, we tested several key known pathways. First, we asked if active DNA demethylation contributes to reduced efficiency in somatic tissues by analyzing methylation of leaves lacking ROS1, DML2, and DML3, the *Arabidopsis* DNA demethylases expressed in somatic tissues (29). The *ros1dml2dml3* (*rdd*) triple mutant did not substantially affect CG methylation in leaves compared with sperm and vegetative cells (Fig. 5A–C), consistent with the limited genomic hypermethylation observed in this mutant (6). We also found that CG methylation of *drm*, *cmt2*, and *cmt3* mutant sperm remained higher than in leaves in TEs and genes (Fig. S4). Our data indicate that—with the exception of MET1—no single known pathway can explain the increased CG methylation efficiency in pollen.

Discussion

The nuclei of sperm and vegetative cells are drastically different. The sperm nucleus is small, with densely packed chromatin (Fig. 3) and obvious H3K9me2-containing heterochromatic foci (22). The vegetative nucleus is larger (Fig. 3), lacks heterochromatic foci and cytologically detectable H3K9me2 (22), and has much higher levels of CHH methylation (Fig. 1D) (15, 16). CMTs are dependent on H3K9me2, so it is reasonable to hypothesize that CHH and even CHG methylation in the vegetative nucleus may be largely dependent on RdDM. The columella cells in the *Arabidopsis* root cap also have much higher CHH methylation than neighboring cells, with elevated RdDM proposed as the cause (17). Our data do show that RdDM extends somewhat into heterochromatic TEs in vegetative cells (Fig. 2), which is consistent with published results (22) and may reflect TE activation in the vegetative nucleus (24). However, CHG methylation is still dependent on CMT3 (Fig. 1C) and heterochromatic CHH methylation primarily on CMT2 (Fig. 1G). In general, maintenance of non-CG methylation is remarkably similar between vegetative, sperm, and leaf cells (Fig. 1C–G). It is likely that the active removal of H3K9me2 in the vegetative nucleus (30) occurs after DNA methylation is deposited, allowing CMTs to work. Overall, our results demonstrate that large, global CHH methylation changes can occur with only minor alterations of pathway specificity.

Although DNA methylation pathways function similarly in pollen and somatic tissues, the efficiency of CG methylation is substantially higher in pollen (Fig. 5). The semiconservative model of CG methylation inheritance (1), and the considerable genetic evidence in support of this model (31–33), is more compatible with the higher efficiency of CG methylation in the male germ line (microspores and sperm), and with the similarly high efficiency observed in female plant gametes (34), than with that observed in somatic tissues (28). In heterochromatic TEs, much or all of the increased efficiency might be accounted by reduced levels of histone H1 (Fig. 3), but the mechanism must be different in genes and euchromatic TEs, where loss of H1 does not facilitate CG methylation (Fig. 5B and C). Instead of a unifying

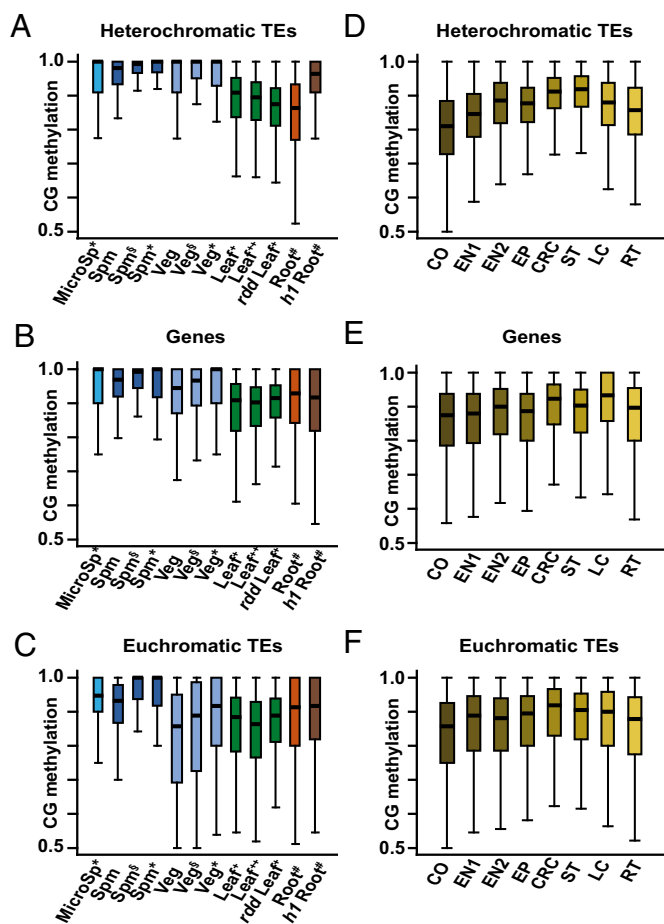


Fig. 5. More robust maintenance of CG methylation in pollen. (A–F) Box plots show CG methylation for individual CG sites with methylation greater than 50% and at least 10 informative sequenced cytosines. Published data in A–C are from the following: *, ref. 16; §, ref. 15; +, ref. 23; ++, ref. 8; #, ref. 7. Published data in D–F are from ref. 17. CO, cortex; CRC, columella root cap; EN, endodermis; EP, epidermis; LC, lower columella; MicroSp, microspore; RT, root tip; Spm, sperm; ST, stele; Veg, vegetative cell.

mechanism, the similar pollen–soma methylation efficiency differences in genes and TEs (Fig. 5) may be an unavoidable consequence of distinct selection pressures. Gametes—and cells that might give rise to gametes—have the potential to undergo an essentially unlimited number of divisions that span generations and should therefore be under strong selection for very efficient methylation maintenance. In comparison, somatic cells will divide very few times and need a methylation maintenance activity that is just sufficient to keep TE silencing and other methylation functions from degenerating. Therefore, mutations that reduce methylation efficiency in somatic cells but keep it above this threshold would not be counterselected. Under these conditions, somatic methylation efficiency would be expected to settle at this equilibrium threshold. An important consequence of less efficient somatic methylation is that small methylation differences between somatic tissues or cell types (17) may be caused by maintenance fluctuations rather than developmental reprogramming.

Materials and Methods

Isolation of *A. thaliana* Sperm and Vegetative Cell Nuclei. *A. thaliana* plants were grown under 16 h light/8 h dark in a growth chamber (20 °C, 80% humidity). Sperm and vegetative cell nuclei were isolated by FACS based on SYBR Green staining, as previously described (15).

Whole-Genome Bisulfite Sequencing. Bisulfite sequencing libraries for sperm and vegetative cells were constructed using the Ovation Ultralow Methyl-Seq Library Systems (Nugen, 0336) and EpiTect Fast Bisulfite Conversion (Qiagen, 59802) kits according to the kit protocols, except the incorporation of two rounds of bisulfite conversion. Illumina sequencing was performed at the UC Berkeley Vincent J. Coates Genomics Sequencing Laboratory, the DNA sequencing facility of the University of Cambridge Department of Biochemistry, Novogene Ltd., and the Bauer Core Facility at Harvard University. Sequenced reads (75 or 100 base single end) were mapped to the TAIR10 reference genome, and DNA methylation of each cytosine was ascertained as previously described (15).

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