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Daniel Bouyer

`daniel.bouyer@ens-lyon.fr`

Centre National de la Recherche Scientifique <https://orcid.org/0000-0002-7438-5551>

Laëtitia De Faria

ENS de Lyon <https://orcid.org/0009-0009-7727-0731>

Fiamma Bunello

University of Udine

Yifang Sui

University of Regensburg

Jeremy Just

Univ Lyon, ENS de Lyon, UCB Lyon 1, CNRS, INRAE <https://orcid.org/0000-0003-0842-9808>

Marie Biharé

Centre National de la Recherche Scientifique

Marie-France Gerentes

Centre National de la Recherche Scientifique

Andrea Bleckmann

University of Regensburg

Philipp Denninger

University of Würzburg

Emanuele De Paoli

Department of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, Udine

Thomas Dresselhaus

University of Regensburg

Article

Keywords: DNA methylation, epigenetics, pollen, plant reproduction, Arabidopsis, DME, ROS1, Oxford Nanopore Technology (ONT)

Posted Date: October 1st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10972510/v1>

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Additional Declarations: There is **NO** Competing Interest.

Antagonistic DNA methylation dynamics at a single locus controls pollen tube guidance in *Arabidopsis*

Laëtitia De Faria¹, Fiamma Bunello^{1,2}, Yifang Sui³, Jérémy Just¹, Marie Biharé¹, Marie-France Gerentes¹, Andrea Bleckmann³, Philipp Denninger⁴, Emanuele de Paoli², Thomas Dresselhaus³ and Daniel Bouyer^{1,*}

¹ Centre National de la Recherche Scientifique (CNRS), ENS de Lyon, Lyon 1 Université, INRAE, INRIA, Laboratoire Reproduction et Développement des Plantes (RDP), UMR5667, F-69342 Lyon, France

² Department of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, 33100 Udine, Italy

³ Plant Cell Biology, Biochemistry, and Biotechnology, Institute of Plant Sciences, University of Regensburg, 93053 Regensburg, Germany

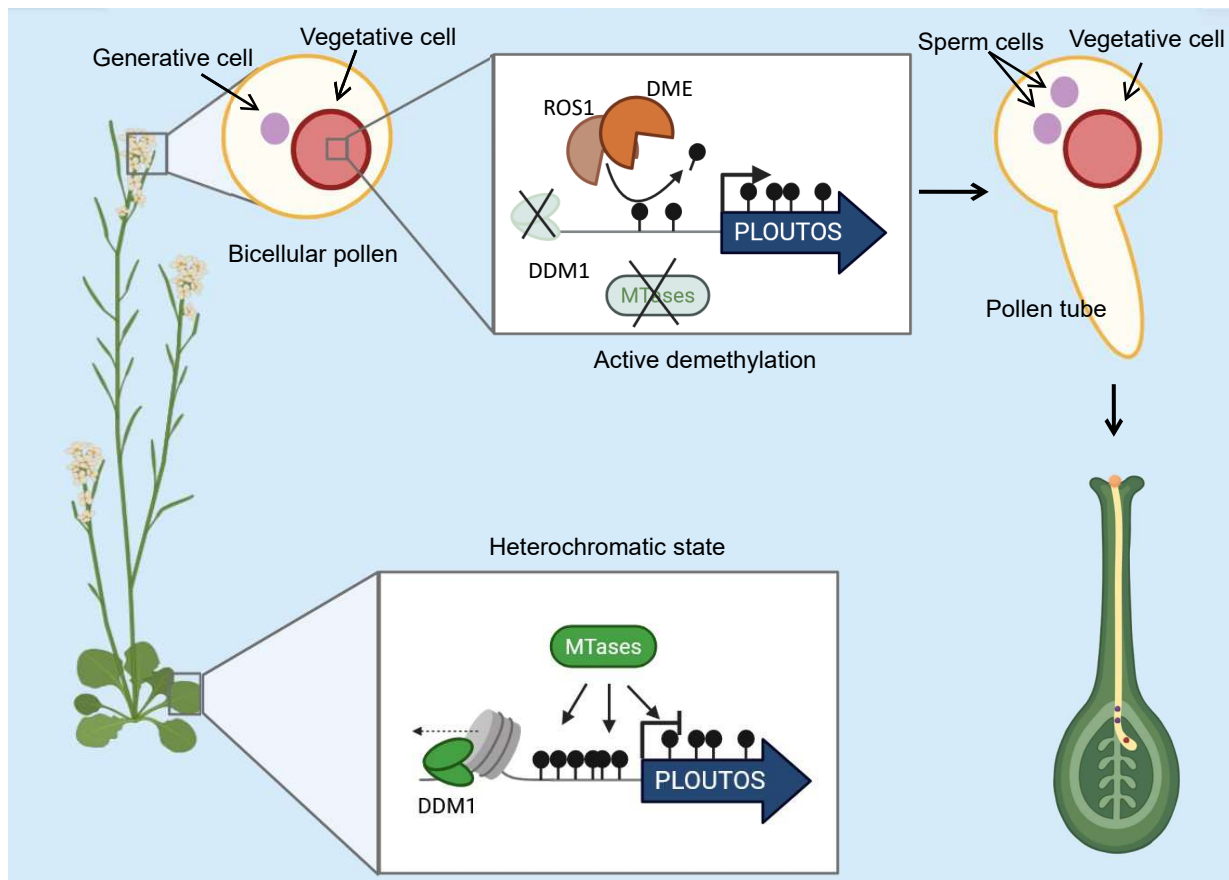
⁴ Lehrstuhl für Botanik I - Molekulare Pflanzenphysiologie und Biophysik, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany

* Correspondence: daniel.bouyer@ens-lyon.fr

Key findings

- Active DNA demethylation in pollen is required for pollen tube guidance by the ovule
- DNA demethylation in the male gametophyte counteracts heterochromatic DNA methylation that is established and maintained during sporophytic vegetative development
- A major causative locus was identified using isogenic, but epigenetically diverse, segregant populations, converging towards a single gene named *PLOUTOS*
- The predicted S-adenosylmethionine-dependent methyltransferase PLOUTOS plays a key role in male fertility, possibly by interacting with signalling pathways required for pollen tube function

28 Graphical abstract



30 Abstract

31 Active DNA demethylation mediated by DEMETER (DME) and REPRESSOR OF SILENCING1
 32 (ROS1) is required for male fertility in *Arabidopsis thaliana*. Here we report that the loss of these
 33 demethylases results in persistent silencing of transposable element-like methylated (teM) genes in
 34 the pollen vegetative cell, leading to pollen tube growth and guidance defects, and reduced fertility.
 35 Consistently, we show that hypomethylation induced by a mutation of the chromatin remodeler
 36 DECREASE IN DNA METHYLATION 1 (DDM1) restores pollen fertility in demethylase mutants and
 37 rescues expression of the majority of down-regulated genes. This hypomethylated state is stably
 38 inherited even after restoration of DDM1 function, resulting in variable demethylase mutant fertility
 39 recovery in epigenetic segregating populations. However, this complementation remains partial due
 40 to RdDM-mediated remethylation and full rescue requires both, *ddm1*-derived hypomethylation and

41 impairment of the RdDM pathway. Using individual whole-genome methylome sequencing of
42 epigenetically segregating lines with contrasting fertility, we identify a key hypomethylated locus
43 associated with fertility restoration, *PLOUTOS*, encoding a predicted SAM-dependent
44 methyltransferase required for pollen tube growth and guidance. Functional validation through
45 CRISPR/Cas9-generated *ploutos* mutants confirms that PLOUTOS is essential for male gametophyte
46 function, with near-complete loss of paternal transmission. Our findings establish that, despite
47 widespread demethylation activity in the pollen vegetative cell, male reproductive success is
48 critically dependent on the epigenetic regulation of a single locus.

49

50 [Keywords](#)

51 DNA methylation, epigenetics, pollen, plant reproduction, Arabidopsis, DME, ROS1, Oxford
52 Nanopore Technology (ONT)

53 Introduction

54 DNA methylation (5mC) is an evolutionarily conserved epigenetic mark involved in
55 transposable element (TEs) silencing, genome integrity and gene expression regulation (Law and
56 Jacobsen 2010). This chromatin modification is maintained by dedicated DNA methyltransferases
57 (MTases), each specific for the respective CG (MET1), CHG (CHROMOMETHYLASE 3/CMT3) and CHH
58 (CMT2) contexts (with H representing all bases except G) in *Arabidopsis thaliana* (Arabidopsis).
59 Plants also contain a RNA-directed DNA methylation (RdDM) pathway responsible for CHH
60 methylation maintenance and *de novo* methylation. In this pathway, small RNAs (sRNAs) are
61 generated by a dedicated RNA polymerase (PolIV), cleaved to 24 nt length by DICER-LIKE 3 (DCL3)
62 and loaded into ARGONAUTE 4 (AGO4), which guides the MTase DOMAINS REARRANGED
63 METHYLTRANSFERASE 2 (DRM2) to genomic sequences complementary to the sRNAs (Xie et al.
64 2025). RdDM acts mainly over repeat sequences in chromosome arms and the borders of
65 pericentromeric TEs, and together with the Swi/Snf2-like chromatin remodeler DECREASE IN DNA
66 METHYLATION 1 (DDM1) establishes TE silencing. DDM1 enables access of MTases to highly
67 compacted heterochromatic regions, thereby maintaining dense DNA methylation for all sequence
68 contexts, a pattern referred to as transposable element-like methylation (teM; Zemach et al. 2013).
69 Remarkably, loss of DNA methylation in *ddm1* mutants can be stably inherited over several
70 generations after re-establishment of DDM1 function and can alter plant development (Johannes et
71 al. 2009; Cortijo et al. 2014). However, not all hypomethylated regions are equally stable: the
72 distinction between stably hypomethylated regions and those that regain DNA methylation depends
73 on the local abundance of sRNAs in the genome, which progressively re-establishes methylation over
74 successive generations (Teixeira et al. 2009; Baduel et al. 2025), indicating that DNA methylation is
75 more dynamic during reproduction compared to vegetative growth (Teixeira and Colot 2010).

76 This epigenetic dynamics during reproduction is marked by active DNA demethylation in
77 gamete accompanying cells, both on the female and male side in flowering plants (Waters et al.
78 2011; Calarco et al. 2012; Ibarra et al. 2012; Rodrigues et al. 2013; Zhang et al. 2014; Moreno-
79 Romero et al. 2016). Demethylation in the central cell (CC) of the embryo sac (female gametophyte)
80 and the vegetative cell (VC) in pollen (male gametophyte) is carried out by DNA glycosylases/lyases
81 in a base excision repair process and is required for successful reproduction (Ibarra et al. 2012; Kim
82 et al. 2019; Khouider et al. 2021; Zhou et al. 2021; Gent et al. 2022; Xu et al. 2022). Moreover,
83 demethylation activity in the pollen VC has been hypothesized to release certain TEs from silencing,
84 which generates 21/22nt sRNAs that are transported into the sperm cells (SCs; Slotkin et al. 2009;

Martínez et al. 2016). Among the four *Arabidopsis* demethylases, DEMETER (DME) plays the major role during reproduction, though partially sharing its function with REPRESSOR OF SILENCING 1 (ROS1) in pollen (Ibarra et al. 2012; Khouider et al. 2021). DME activity is essential for seed development, since *dme* mutations lead to seed abortion when inherited from the female side (Choi et al. 2002), probably due to defects in genomic imprinting (Gehring et al. 2006; Khouider and Gehring 2024). On the male side, we have previously shown that DNA demethylation by both, DME and ROS1, is required for activation of genes implicated in pollen tube function (Khouider et al. 2021). Notably, these genes display teM, a methylation pattern recurrently found over protein-coding genes specifically activated in pollen (Schmitz et al. 2013; Zeng et al. 2024).

How these antagonistic DNA methylation and demethylation activities are set up and to what extent they contribute to gene regulation important for male fertility remains unclear. Here we report the genome-wide extent of epigenetically regulated genes implicated in pollen tube function. We show that the synergistic activity of DME and ROS1 is required to activate numerous genes in the male gametophyte, and that loss of this activity mainly affects pollen tube guidance. We provide evidence that this process is indeed regulated by DNA methylation as a large proportion of down-regulated annotations in *dme ros1* mutant pollen show DME/ROS1-dependent demethylation in the VC, in contrast to those being up-regulated. Moreover, demethylase mutant male sterility can be rescued by simultaneous loss of DDM1 function, but not other DNA methylation pathways, in agreement with our observation that many epigenetically regulated male fertility genes are heterochromatic and hence teM. This finding allowed us to exploit the heritable nature of *ddm1*-induced hypomethylation: by using an isogenic epigenetic population in which *ddm1*-derived hypomethylated regions segregate in a wild type DDM1 background, we could separate completely fertile from sterile demethylase mutant lines. Despite thousands of genomic regions that become demethylated in the pollen VC affecting a large number of epigenetically regulated genes, methylome-based comparison of fertile and sterile lines converges on a single locus, *PLOUTOS*, as the primary epigenetic determinant of male fertility.

Results

DNA demethylation is required for pollen tube guidance

Previous analyses suggested that DNA demethylase mutants show aberrant pollen tube trajectories within the female reproductive organ, but are not affected in pollen grain viability or pollen tube growth vigour (Khouider et al. 2021). To better characterize the male fertility defects in *dme* and *dme ros1* mutants, we performed single-pollen pollinations and examined pollen tube trajectories within the transmitting tract 25 hours after pollination, using aniline blue staining to monitor both pollen tube growth and ovule targeting (Fig. 1a). Distorted pollen tube growth was quantified by scoring pollen tubes exhibiting more than three intersection events within the transmitting track, hereafter referred to as “entangled” (see Methods), with representative scoring examples shown in Suppl. Fig. 1. We observed entangled growth in 14.74% wild type plants which increased to 27.14% entanglement in *dme/+* plants and further to 39.34% in *dme/+;ros1* plants. This finding indicates that demethylase mutant pollen tubes are compromised in navigating towards the ovule (Fig. 1b). To test whether demethylase mutant pollen tubes are also defective in ovular guidance, we followed pollen tube trajectories of wild type and mutants by semi-*in vitro* assays using dissected ovules (Zhong et al. 2020). As *dme* mutant lines only exist as heterozygotes, we made use of a fluorescent tag insertion (FTL), located in proximity (around 1 Mb distance) of the *DME* locus and selection of a meiotic recombinant line, physically associating the *dme* mutant allele with the FTL insertion thereafter named *dme^{FTL}* (Francis et al. 2007; Khouider et al. 2021; see Methods for more details). This allowed us to identify mutant pollen by RFP fluorescence. Successful ovule targeting of individual pollen tubes was verified using ovule dragging to discriminate pollen tubes that have entered the embryo sac and those that only locate at ovules without penetration (Liu et al. 2021; Sui et al. 2026) (Fig. 1c). Wild type stigmas were pollinated with pollen from heterozygous *dme^{FTL}/+;ros1* plants under limited pollination conditions, allowing fertile *ros1* single mutants and *dme ros1* double mutant pollen tubes to compete for the same ovules within a single pollination event. Because both pollen types compete on the same stigma, this assay controls for variation in female receptivity between pollinations. Wild type pollen tubes showed an ovule targeting efficiency of 73.4%±7.5%, whereas *dme/+;ros1* mutant pollen tubes showed a reduced targeting efficiency of 26.6%±7.5% (mean±s.d) (Fig. 1d). This indicates that ovule targeting is impaired in *dme/+;ros1* mutant pollen tubes. Taken together, these results suggest that male sterility in DNA demethylase mutants is caused, at least to a large extent, by pollen tube guidance defects.

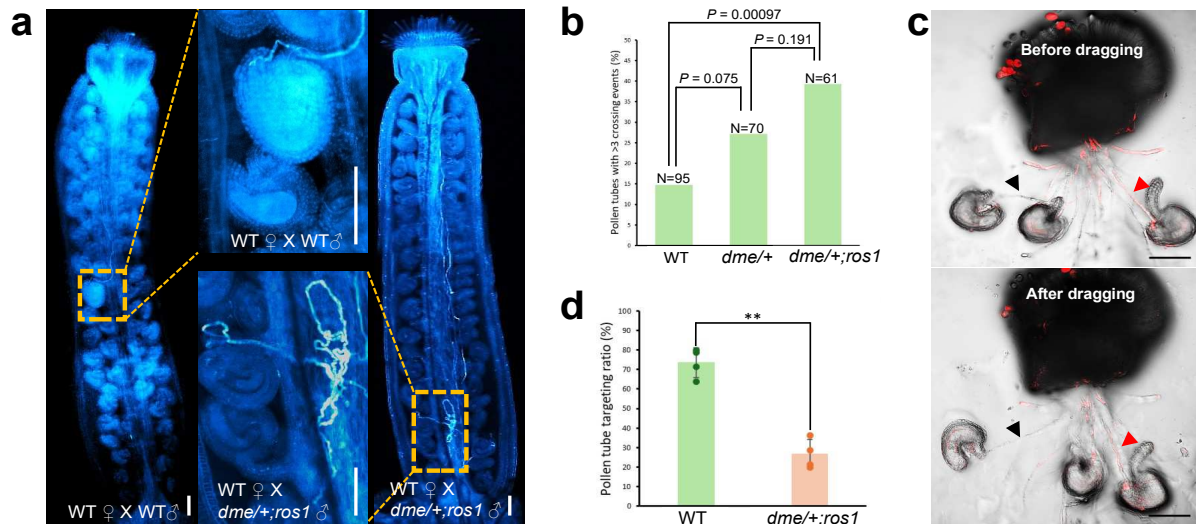


Figure 1: DNA demethylation is required for pollen tube guidance to the ovule. **a** Representative images showing defective pollen tube guidance in DNA demethylation mutants. Pistils were fixed at 25 hours after pollination (HAP) and stained with aniline blue. Regions indicated in orange boxes are enlarged in the middle of the panel. **b** Quantification of the entangled pollen tube phenotype across indicated genotypes at 25 HAP. Pollen tubes with >3 crossing events within the transmitting tract were classified as entangled. Data represent pooled proportions of pollen tubes for each genotype. Statistical significance was determined by a two-sided Fisher's exact test. **c** Semi-in vitro assay for assessing ovule targeting by wild type (WT) and mutant pollen tubes. WT pistils were pollinated with pollen from heterozygous *dme^{FTL}/+* *ros1* plants. *dme* mutant pollen tubes expressing DsRed were distinguished from non-fluorescent *ros1* pollen tubes. Black and red arrowheads indicate *ros1* and *dme ros1* pollen tubes, respectively. **d** Pollen tube targeting ratio of *ros1* and *dme ros1* pollen tubes in the semi-in vitro assay. A total of 37 scorable ovules were analyzed across four replicates. Data are shown as mean $\pm$ s.d. Statistical significance was determined by a two-tailed paired Student's *t*-test. Scale bars in **a** and **c**, 100 μ m.

DME and ROS1 act synergistically to allow gene activation in pollen

We had previously identified four DME/ROS1 target genes, the absence of activation of which could partially explain the demethylase mutant sterility. These genes had been selected based on their established or presumed implication in pollen tube function (Khouider et al. 2021), though the whole extent of gene regulatory activity of pollen-expressed demethylases has remained unclear. This is partially due to the heterozygosity of *dme* mutants, causing half of the pollen population to be wild type, although RNA-seq experiments has revealed differential expression between *dme-7* and wild type pollen (He et al. 2019). To isolate specifically *dme* mutant pollen we made use of the *dme^{FTL}* line described above. We have determined the recombination rate between the *DME* locus and the *FTL* insertion to be 1.5%, indicating that fluorescent pollen should be *dme* mutant with 98.5% purity. This line was used in Fluorescent Activated Cell Sorting (FACS) experiments to isolate *dme* and *dme ros1* mutant pollen (Fig. 2a). This method was also applied to obtain wild type and *ros1* pollen in biological triplicates for each sample (16-25M reads/sample; 93-

96% uniquely mapped reads, 2-4% multiple mapped reads; 2-3% unmapped; Table S1). Supporting the synergistic activity of DME and ROS1 in pollen, we identified a large increase in differentially expressed genes (DEGs) in the *dme ros1* double mutant relative to the single mutants with 236 genes down-regulated and 183 up-regulated in *dme ros1*, compared to 49 down- and 6 up-regulated annotations in *dme*, and 27 down- and 30 up-regulated genes in *ros1* (all versus wild type; Fig. 2b and 2c; Table S2). In agreement with an important role of active DNA demethylation for pollen tube function, Gene Ontology (GO)-term analyses of *dme ros1* down regulated genes revealed a strong enrichment in GO terms such as “Regulation of pollen tube growth” (18.31-fold, p -value: $5.3 \cdot 10^{-4}$) and “Pollen tube growth” (8.8-fold; p -value $6.9 \cdot 10^{-6}$) categories (Fig. 2d). In contrast, *ros1* and *dme* single mutant DEG as well as up-regulated genes in *dme ros1* double mutant did not display statistically significant GO-terms enrichment ($p > 0.05$; Suppl. Fig. 2a). In addition, transcriptome analysis revealed differential expression of several TEs, predominantly downregulated, suggesting that DNA demethylation promotes their activation (Suppl. Fig. 2b; Table S2).

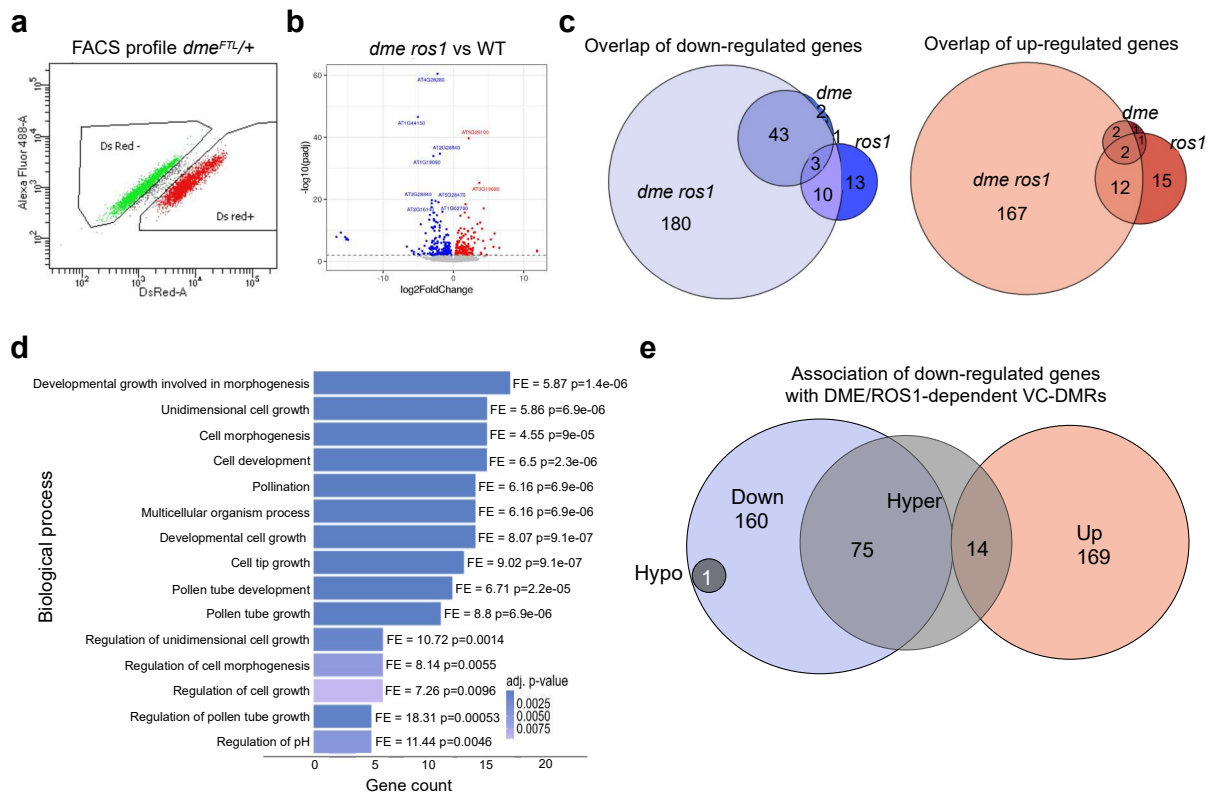
184

Genes down-regulated in demethylase mutants overlap with DNA demethylation in the VC and are associated with heterochromatic signatures

Next, we assessed if DEGs in demethylase mutants are direct DNA demethylation targets by making use of our previously identified pollen VC vs. SC methylome analyses (Khouider et al. 2021). Whereas up-regulated genes were only marginally associated with these DNA methylation differences (14 out of 183), a substantial fraction of down-regulated genes (75 out of 236) were associated with CG hypomethylation in wild type VC vs. SC and/or *dme/+ ros1* vs. wild type hypermethylation within 500bp upstream of the transcription start site (TSS; Fig. 2e; Table S3). This is even more pronounced for down-regulated genes in the *dme* single mutant for which the majority is also hypomethylated in the wild type VC (Suppl. Fig. 2c; Table S3). This suggests that DNA demethylation is indeed required for transcriptional activation in the pollen VC. Among the 75 down-regulated annotations associated with hypomethylation, 5 corresponded to TEs; the other 70 were gene annotations, comprising non-coding RNAs (9 annotations), uncharacterised “novel transcripts” (17), pseudogenes (4), and protein-coding genes. Of these 70 gene annotations, 43 were teM (Table S3), showing DNA methylation in all sequence contexts and thus resembling the pattern typical of heterochromatic TEs, a feature also observed for down-regulated genes in demethylase mutant maize pollen (Zeng et al. 2024). This is a strong enrichment of genes showing teM (genome-wide: 2,415 teM out of 29,051 classified gene annotations [Table S4]; for *dme ros1*: Representation factor

203 (RF): 11.0, $p < 4.1 \cdot 10^{-21}$ for 43/70 genes; for *dme*: RF: 10.8, $p < 6.0 \cdot 10^{-17}$ for 17/19 genes;
 204 hypergeometric test). In agreement with this notion of a heterochromatic signature of epigenetically
 205 regulated pollen genes, the majority (80%) of them are DDM1-dependently methylated in the CG
 206 and CHG context (60 out of 75 down-regulated in *dme ros1* vs. wild type and associated with
 207 demethylase-dependent CG-hypomethylation; Table S5), which suggests an antagonistic role of
 208 DDM1 and demethylase activity in their transcriptional regulation.

209



210 **Figure 2: DME and ROS1 act synergistically to regulate pollen expressed genes implicated in**
 211 **pollen tube growth processes.** **a** FACS profile of *dme*^{FTL} pollen separating red fluorescent pollen
 212 associated with the *dme* mutation from non-fluorescent wild type (WT) pollen. **b** Volcano plot of
 213 differentially expressed genes ($p_{adj} \leq 0.01$) in *dme ros1* mutant compared to WT pollen. The x-axis
 214 represents log2 fold change, and the y-axis represents $-\log_{10}$ adjusted p-value (p_{adj}). Significantly
 215 upregulated genes are shown in red, significantly downregulated genes in blue, and non-significant
 216 genes in gray. Labels indicate the 10 most significant DEGs based on their adjusted p-values. **c** Venn
 217 diagram with the number and overlap of up and down regulated genes in *dme*, *ros1* and *dme ros1*
 218 compared to WT pollen. **d** GO-term analyses for down-regulated genes in the *dme ros1* double
 219 mutant, showing enrichment in pollen tube-related biological processes (BP). Gene count indicates
 220 the number of genes associated with each GO term. Bar colours represent adjusted p -values. FE: fold
 221 enrichment. Statistical significance was determined using Fisher's exact test with Bonferroni
 222 correction. **e** Venn diagram giving the number and overlap of up- and down-regulated genes in *dme*
 223 *ros1* mutant with differentially methylated regions (DMRs) in the sperm cell vs. vegetative cell (VC) in
 224 CG context.

Demethylation in pollen counteracts DDM1 activity during the vegetative phase

Loss of DDM1 restores male fertility in demethylase mutants

To test if active DNA demethylation in pollen indeed counteracts DDM1-mediated teM, we generated *dme* and *dme ros1* mutants in combination with *ddm1* by crossing with heterozygous *ddm1/+* lines to obtain first-generation homozygous *ddm1* mutants in the F2 segregating population to minimize TE transposition and epigenetic effects associated with prolonged *ddm1* homozygosity. Unlike other DNA methylation pathway mutants, *ddm1* was able to completely restore male fertility in the demethylase mutants (Fig. 3a and 3b; Suppl. Fig. 3). In line with an antagonistic activity of DDM1 and DME, previous reports have demonstrated that DDM1 is depleted in the pollen VC (Slotkin et al. 2009). This indicates that DNA methylation is established during the vegetative phase and that the absence of DDM1 in the pollen VC is part of the epigenetic reprogramming taking place in the gamete accompanying cell. Supporting this hypothesis, single-cell transcriptomic data indicate a global reduction in the expression of maintenance DNA MTases in the pollen VC (Ichino et al. 2022). To exclude a role for DDM1 in the VC, we ectopically expressed a DDM1–GFP fusion protein under the control of the *LAT52* promoter that confers strong expression in the VC (Suppl. Fig. 4a). To assess pollen fertility, we selected four independent single-insertion lines and measured the transmission rate of the pLAT52::DDM1–GFP transgene. No deviation from Mendelian inheritance was observed, with the transgene transmitted at approximately 50%, indicating absence of detectable effects on pollen fertility by ectopic expression of *DDM1* in the VC (Suppl. Fig. 4b). In addition, and confirming the absence of DDM1 activity in pollen, no effect on demethylase mutant male sterility was observed in combination with heterozygous *ddm1/+* lines (Fig. 3a and 3b). This corroborates the antagonistic relationship between active DNA demethylation during reproduction and DNA methylation maintenance in the vegetative phase for epigenetically regulated male fertility genes. To further test whether the *dme/+ ddm1* fertility rescue results from global hypomethylation affecting preferentially heterochromatic non-CG methylation, we generated a *dme/+* mutant combination with *cmt2 cmt3*, which affect CHH and CHG methylation, respectively, over regions targeted by DDM1 (Zemach et al. 2013). Indeed, the triple mutant showed a stronger fertility restoration than either double mutant (Suppl. Fig. 3a), supporting a cumulative effect of reduced non-CG methylation. Our attempts to establish a *dme/+ met1 cmt2 cmt3* quadruple mutant were not successful, although we managed to generate CRISPR/Cas9-mediated *met1* in the *dme/+* background, using the same sgRNAs and mutagenesis strategy as He et al. (2022). Notably, active DNA demethylation targets on the male and female side are methylated by different pathways as

female sterility of *dme* mutants is rescued by simultaneous loss of *met1* corroborating previous reports (Xiao et al. 2003), but not *ddm1* and vice versa (Fig. 3a-b; Suppl. Fig. 3a; Suppl. Fig. 5a).

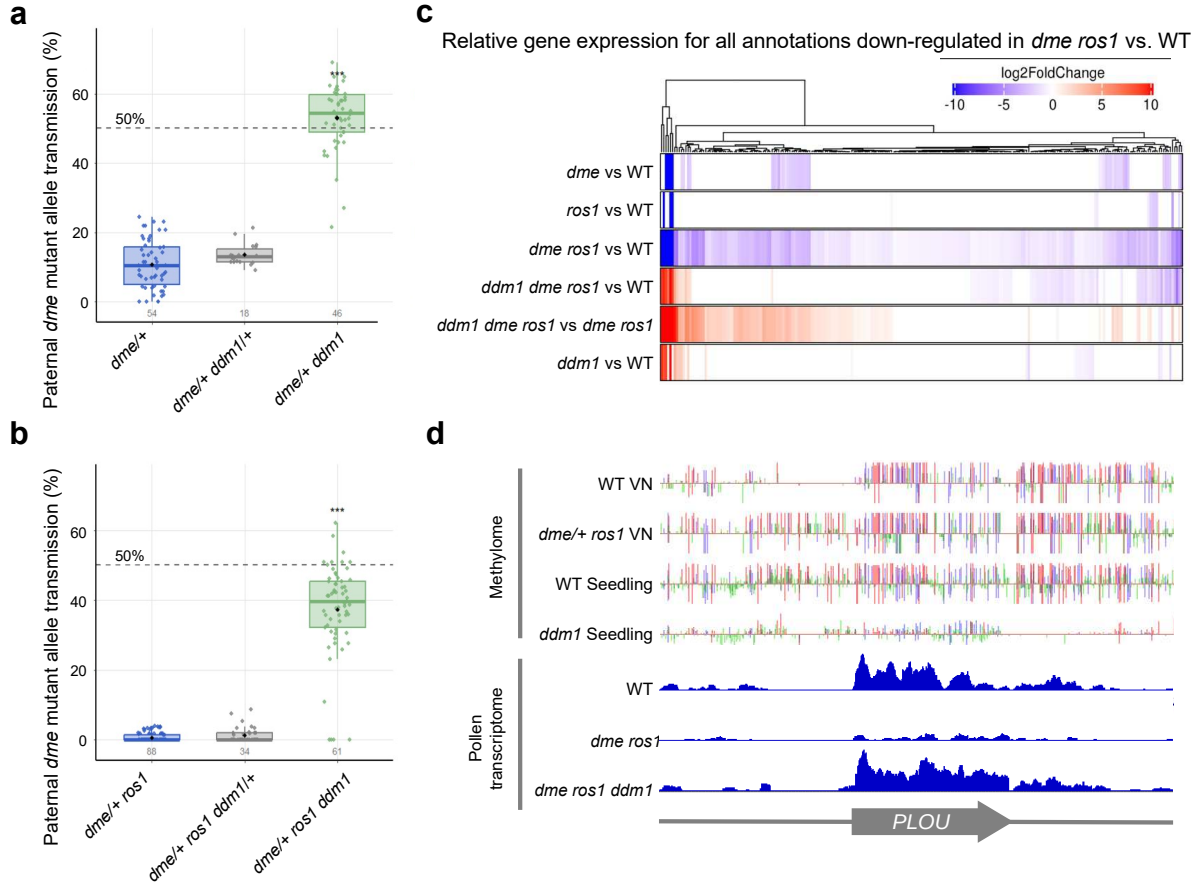


Figure 3: Loss of *DDM1* restores *dme/+* and *dme/+ ros1* male fertility and gene expression. **a & b** Paternal transmission frequency of the *dme* mutant allele in crosses between maternal wild type plants and mutant pollen donors for **a** *dme/+* and **b** *dme/+ ros1* in combination with heterozygous (*ddm1/+*) and homozygous *ddm1* (*ddm1*) mutants. Boxplots show the median (center line) and the interquartile range (box limits), with whiskers representing the data distribution. Black diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of biological replicates is indicated for each genotype. Pairwise comparisons of *dme* transmission rates relative to the *dme/+* control were performed using a Dunnett's test. Statistical significance *** = $p \leq 0.001$. The dashed horizontal line indicates the expected Mendelian transmission rate (50%). **c** K-means clustered heatmap of relative gene expression with log₂-fold change for comparison between respective genotypes as indicated. **d** IGV screenshot of the methylome and transcriptome landscape over the *PLOU* locus for the genotypes and tissues labelled on the left side. mC is colour-labelled according to its context (mCG = red; mCHG = blue; mCHH = green).

Loss of DDM1 restores gene expression of down-regulated genes in *dme* and *dme ros1*

To test the role of DDM1 to maintain DNA methylation for pollen demethylation targets, RNA-seq of demethylase mutant combinations with *ddm1* was carried out, using the same FACS protocol that we applied to isolate *dme* mutant pollen (Suppl. Fig. 6). In agreement with the assumption that demethylase targets are maintained silenced by DDM1, *ddm1 dme* vs. *dme* and *ddm1 dme ros1* vs. *dme ros1* show re-establishment of expression for the majority of transcripts (42 out of 49 down-regulated genes in *dme* and 163 out of 236 down-regulated annotations in *dme ros1*; Fig. 3c, Suppl. Fig. S7a-b; Table S5). Contrary to the minor effect of expression restoration for up-regulated genes in the *dme* background (2 out of 11; Table S5), many up-regulated genes in *dme ros1* were also restored upon *DDM1* depletion, indicating indirect feedback mechanisms (Suppl. Fig. 7a, Table S5). Underlining the absence of demethylase mutant male fertility restoration of lines heterozygous for *ddm1/+*, we detected only marginal effects on transcription in these mutant combinations (Suppl. Fig. 8; Table S2). It should be noted that the loss of DDM1 has a strong effect on gene activity in pollen, which affect both, TEs and non-TE transcripts (Suppl. Fig. 8; Table S2). This is reflected by the principle component analysis (PCA) of the transcriptome samples that clearly separate *ddm1* and its combinations from the other samples in the PC1 axis (Suppl. Fig. 9).

In the following we will focus on the effects concerning the annotations requiring DNA demethylation in the pollen VC for their transcriptional activation (75 annotations, Fig. 2e). From these DME/ROS1 targets, 64 (36 protein coding genes) were transcriptionally rescued by *ddm1* in the *dme ros1* mutant background of which 54 (21 protein coding genes) require DDM1 for their teM pattern (mCG and mCHG within and/or 500bp 5' of the transcription unit, Table S5, see Fig. 3d for an example). These are further-on defined as candidates. To identify the causative loci required for pollen tube function within this group, we made use of the fact that *ddm1*-induced DNA hypomethylation can be transmitted independently of the *ddm1* mutation (Johannes et al. 2009; Teixeira et al. 2009; Zhang et al. 2021; Baduel et al. 2025), which allowed us to disentangle the genetic cause (*ddm1* mutation) from its epigenetic consequence (DNA hypomethylation), by creating an isogenic population with segregating variable epigenomes.

Maintenance of *ddm1*-induced male fertility in demethylase mutants after restoration of DDM1 function

Previous work has shown that the loss of DNA methylation in the *ddm1* mutant can persist over several generations after the restoration of DDM1 by backcrossing the mutant to the wild type,

thereby creating an epigenetic recombinant inbred line population (EpiRILs; Johannes et al. 2009; Teixeira et al. 2009; Zhang et al. 2021; Baduel et al. 2025). We made use of this inheritance of DNA hypomethylation by establishing a genetically identical, but epigenetically diverse population using the F2 segregants after the initial cross of *ddm1* mother plants with *dme/+* mutant pollen. To limit progressive remethylation effects of these regions (Teixeira et al. 2009; Baduel et al. 2025) and the potential accumulation of TE insertions, analyses were restricted to the F3 generation or earlier. Thus, after DDM1 restoration, hypomethylated regions are expected to segregate within the population and should therefore result in lines inheriting such regions affecting the candidate genes.

To this end, we isolated 90 *DDM1* wild type lines from this F2 population (epiLines) carrying the *dme/+* mutation and established their male fertility by measuring the transmission rate of the mutant allele *dme* in self-fertilized siliques. We confirmed that selfing and crossing of *dme/+* plants results in the same mutant allele transmission rates, corroborating that the *dme* mutation can only be passed on from the male side (Suppl. Fig. 5b). Male fertility was restored to varying degrees across these epiLines following a continuous distribution of *dme* allele transmission rates, consistent with a quantitative effect of DNA methylation levels on fertility (Fig. 4a). Importantly, as these lines are by definition isogenic, but different in their DNA methylation profiles, the observed variation in sterility phenotypes likely reflects epigenetic variation. Within these epiLines, 21 significantly restored fertility of *dme* mutant pollen ($p\text{-val} < 0.01$, Dunnett test), suggesting that candidate genes have been inherited in a hypomethylated state for at least two generations in the presence of DDM1. Thus, in order to identify the hypomethylated loci responsible for the fertility restoration, we established the methylomes of the vegetative tissues of ten individual lines from the fertility restoration group and compared them with ten individual lines that did not restore male fertility (fertile vs. sterile epiLines; F1-10 and S1-10 as indicated in Fig. 4a). We established cytosine methylation directly from Oxford Nanopore Technology (ONT) sequencing data, using the novel 5mC-calling capabilities of ONT's base-calling pipeline, Dorado (see Methods for details; Table S6). To validate this approach, Dorado-called methylation levels were compared with those from DeepSignal3, successor of DeepSignal-plant (Ni et al. 2021). Despite differing training data, both tools produced highly concordant CG, CHG, and CHH methylation calls (Suppl. Fig. 15, 16). Both were further benchmarked against independent, Bismark-reprocessed whole-genome bisulfite sequencing (WGBS) data from matching genetic backgrounds; Dorado showed closer concordance and was used for subsequent DMR analyses.

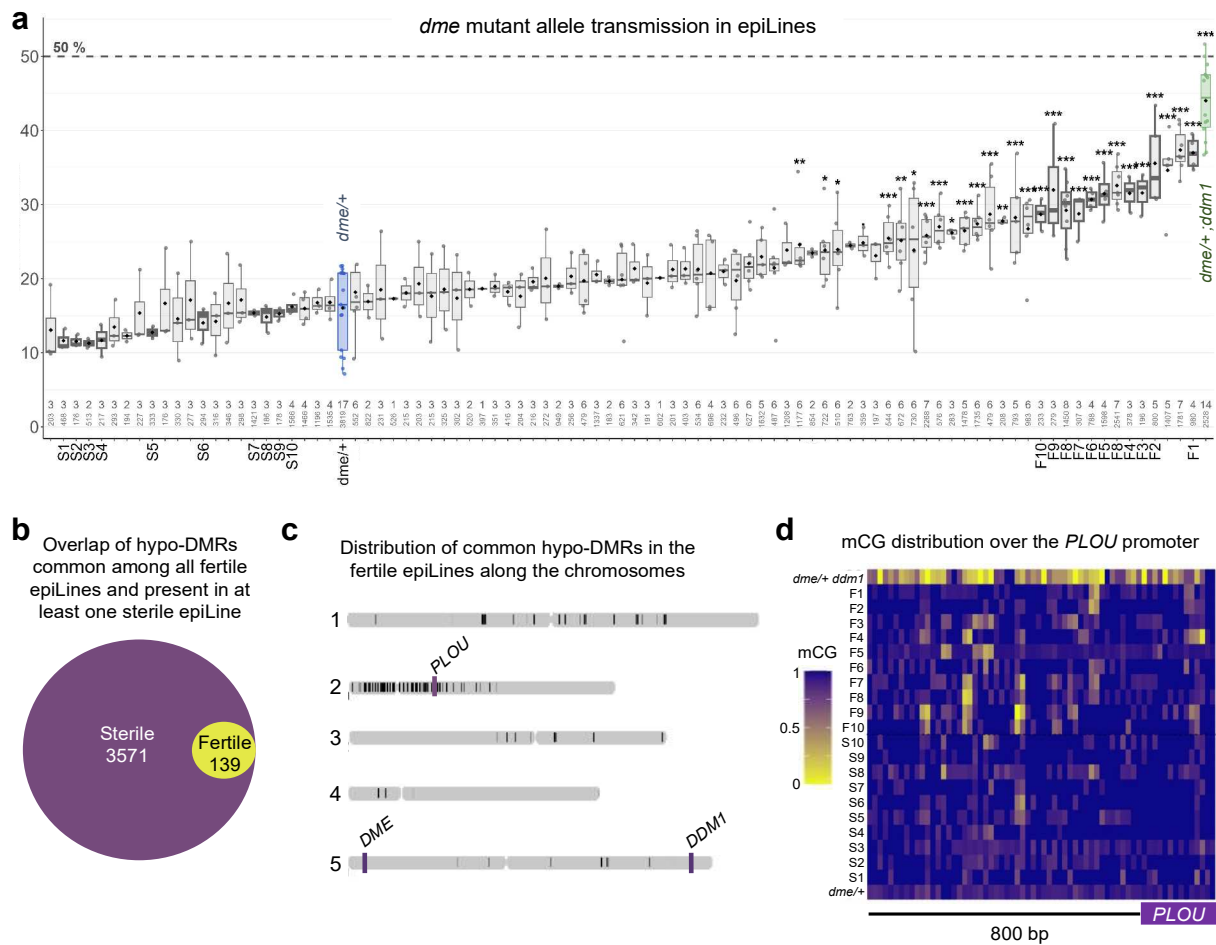


Figure 4: *dme* male fertility can be epigenetically restored and segregates with DNA hypomethylation on the upper half of chromosome 2. Transmission frequency of the *dme* mutant allele in a F2 segregating population derived from a cross of *ddm1* and *dme/+*. These lines have been selected for the WT allele of *DDM1* and are genetically identical. Bold-labelled and named lines correspond to those selected for whole-genome methylome analyses. Boxplots show the median (central line) and the interquartile range (box limits), with whiskers representing the data distribution. Diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of biological replicates is indicated below each line. Pairwise comparisons of *dme* transmission rates relative to the *dme/+* control were performed using Dunnett's test. Statistical significance is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. The dashed horizontal line indicates the expected Mendelian transmission rate (50%). **b** Venn diagram showing the overlap between genes containing promoter hypomethylated DMRs in all fertile lines and those identified in at least one sterile line. **c** Genome-wide distribution of promoter hypomethylated DMRs (black lines) in fertile lines. Localisation of *PLOU*, *DME* and *DDM1* is labelled and indicated by violet line. **d** Absolute CG methylation percentage of each cytosine in this sequence context located within the demethylation target region of *PLOU*, spanning 800 bp in the promoter and 150 bp in the coding sequence.

Isolation of a hypomethylated locus associated with fertility restoration in the demethylase mutant

Global DNA methylation differences were not detected between fertile and sterile lines across genes and TEs in CG, CHG and CHH contexts. Instead, all lines exhibited methylation levels similar to the *dme/+* control and distinct from the hypomethylated *ddm1 dme/+* mutant reference (Suppl. Fig. 10). As differences in fertility could not be explained by global DNA methylation variation, we next investigated local differentially methylated regions (DMRs, see Methods for DMR thresholds and definition). To this end, we established DMRs in fertile and sterile epiLines using the same criteria as in Khouider *et al.* (2021). First, among the 139 genes carrying promoter hypo-DMRs shared by the 10 fertile lines, all were also found to be hypomethylated in at least one sterile line (Fig. 4b; Table S7). Although this did not allow a clear separation of DMRs exclusively found in the fertile epiLines, this approach revealed a significant enrichment of hypo-DMRs around the centromere of chromosome 2 (Fig. 4c; Suppl. Fig. 11a). This enrichment may reflect a selection of one or several hypomethylated loci in this region, as it does not represent irregularities in its meiotic recombination behaviour (Singer *et al.* 2006; Salomé *et al.* 2012; Choi *et al.* 2013), DNA methylation (Fields *et al.* 2022), or distribution of *ddm1*-induced DMRs in a previously established epigenetic recombinant inbred line (epiRIL) population (Johannes *et al.* 2009; Zhang *et al.* 2021; Baduel *et al.* 2025). Whereas only few common hyper-DMRs were detected in the fertile and sterile epiLines, respectively (Suppl. Fig. 12, Table S7), the distribution of hypo-DMRs present in all sterile lines revealed an enrichment around the centromere of chromosome 3, indicating that it contains a locus or several loci that might contribute negatively to male fertility in the *dme* mutant when hypomethylated (Suppl. Fig. 11a-b, Table S7). Yet, differences in *dme* mutant allele transmission rates in the sterile epiLines are only subtle and not statistically significant compared to the *dme/+* mutant plant (Fig. 4a), suggesting that a potential epigenetically regulated locus in this region would have a rather limited impact.

To single out candidates that associate with epigenetic changes, we relaxed our selection criteria to identify hypo-DMRs present in at least 9 out of 10 of fertile epiLines (292 genes) while excluding genes hypomethylated in more than one sterile epiLine. This analysis identified three annotations, all located on chromosome 2: *AT2G16620*, *AT2G23220* and *AT2G16030*. Among these, only one gene overlapped with our previously defined candidates: *AT2G16030*, also known as *PLOUTOS* (*PLOU*; Khouider *et al.* 2021). Further relaxing the criteria to hypo-DMRs present in at least

80% of fertile lines and in no more than one sterile line resulted in 8 annotations, including two from the candidate gene list: *PLOU* and *AT2G00590*, annotated as novel transcribed region.

Hence, among the candidates, only two fulfilled all the selection criteria and displayed consistent hypo-DMRs (Suppl. Fig. 11c). To exclude the possibility that our DMR criteria exclude more subtle DNA hypo-methylation, we investigated the DNA methylation profile using the coordinates of the DMRs that have been detected between VC and SC in pollen for each candidate loci individually. Several candidates, including *AT5G04875*, *AT3G42075/AT3G42130*, *AT1G35540*, *AT1G06907*, *AT1G19086*, and *AT1G19090 (RKF2)*, either showed similar methylation levels in both fertile and sterile epiLines or methylation levels comparable to the *dme/+* control (Suppl. Fig. 11d, Table S7). Other candidates, such as *AT3G00620/AT3G06095*, *AT3G30720* and *AT3G44070*, all located around the centromere on chromosome 3, were on average more methylated in fertile than in sterile epiLines (Suppl. Fig. 11d, Table S7). Therefore, these might represent genes negatively affecting demethylase-dependent male fertility when hypomethylated, although their impact might be marginal.

In contrast, *PLOU* again emerged as the only convincing candidate locus showing consistent hypomethylation in fertile lines. Notably, only one fertile line (F10) did not show a clear hypo-DMR at this locus, but also displayed the lowest fertility within this group (28.6% vs. 30.9% average *dme* transmission rate in the fertile epiLines), while only a single 50 bp CHH hypo-DMR, with a methylation loss of -0.19, was detected in one sterile line (Table S7). Based on these observations, we hypothesized that specific hypomethylated regions or even individual cytosines within the *PLOU* promoter might contribute to fertility restoration of the *dme* mutant. Indeed, a higher number of hypomethylated CG sites are present in the fertile compared with sterile epiLines at this locus, though the majority of CG sites show re-establishment of normal methylation levels compared to *ddm1* (Fig. 4d). This partial hypomethylation observed for *PLOU* would be consistent with the limited re-establishment of pollen function in the fertile epiLines with a maximal *dme* allele transmission rate of 34% as compared to the 50% expected by Mendelian segregation upon complete fertility restoration.

This suggests DNA remethylation at this site, which is indeed the case for about half of the *ddm1*-dependent hypomethylated DMRs in the canonical epiRIL population due to the presence of sRNAs, thus successively gaining methylation by RdDM in further generations (Teixeira et al. 2009, Baduel et al. 2025). Consistently, out of the 25 DME/DDM1-regulated pollen genes (*dme* DOWN+Pollen-DMR+*ddm1*-DMR), *PLOU* and 10 further candidates are targeted by 24nt sRNAs in

pollen and/or leaves (Borges et al. 2018) and are systematically (re)methylated in the epiRILs (Table S5; Suppl Fig. 13a; Baduel et al. 2025). In line with this, fertile epiLines have reduced fertility in the F3 compared to its previous F2 generation indicating further remethylation (Suppl Fig. 13b). Taken together, we have identified a single locus, *PLOU*, among the candidates that associates consistently with DNA hypomethylation in the fertile epiLines, yet substantial remethylation compared to their *ddm1* grandparent suggests RdDM activity targeting this gene.

Identification of *PLOUTOS* as the main epigenetically regulated locus required for male fertility

To avoid remethylation by RdDM and its effect on male fertility in the demethylase mutant, we generated a second *ddm1*-derived epigenetic population using the double mutant *dme/+ ros1* in a RdDM defective background through the introduction of a Pol IV (*nprpd1a*) mutation from both parents. Thus, we established a F2 population after an initial cross of *ros1 nprpd1a* with *dme ros1 ddm1 nprpd1a* and isolated 99 *DDM1*-WT individuals (epi-pIV-lines).

Although the loss of RdDM itself does neither increase male fertility in *dme ros1* nor impact its restoration in the *dme ros1 ddm1* triple mutant, it dramatically changes the effect on fertility in the epi-pIV-lines (Fig. 5a). In contrast to the initial epiLines, *dme* mutant allele transmission rates were significantly elevated for the majority of lines compared to the control crosses when pollinated to wild type plants (Fig. 5a). Thus, 29 epi-pIV-lines fully rescued male fertility in the *dme/+* background (~50% *dme* transmission, χ^2 test, $p>0.1$), and 62 lines restore *dme* transmission rates between 25 and 40% (Fig. 5a). The remaining eight lines are comprised of seven sterile plants and only one line showing an intermediate, although significant level of rescue (~15% *dme* transmission; Fig. 5a). This distribution would be in line with a bi-allelic segregation of a single locus where one allele leads to complete restoration of fertility in the absence of demethylase activity. In this scenario the number of lines existing in a heterozygous state for this allele is expected to be twice the number of lines carrying a homozygous “fertile” allele, which fits our observation (62 vs. 29 lines). The reduction in the number of sterile lines (7 instead of ~30 lines), and therefore plants with a homozygous “sterile” allele in the epi pIV lines can be explained by the selection applied to the paternal transmission of *dme* that favours the presence of the “fertile” allele in the initial F1 cross. In epigenetic terms, this would translate into two epi-allelic states determining the reproductive success in the demethylase mutant background, one “sterile” methylated and one “fertile” unmethylated allele.

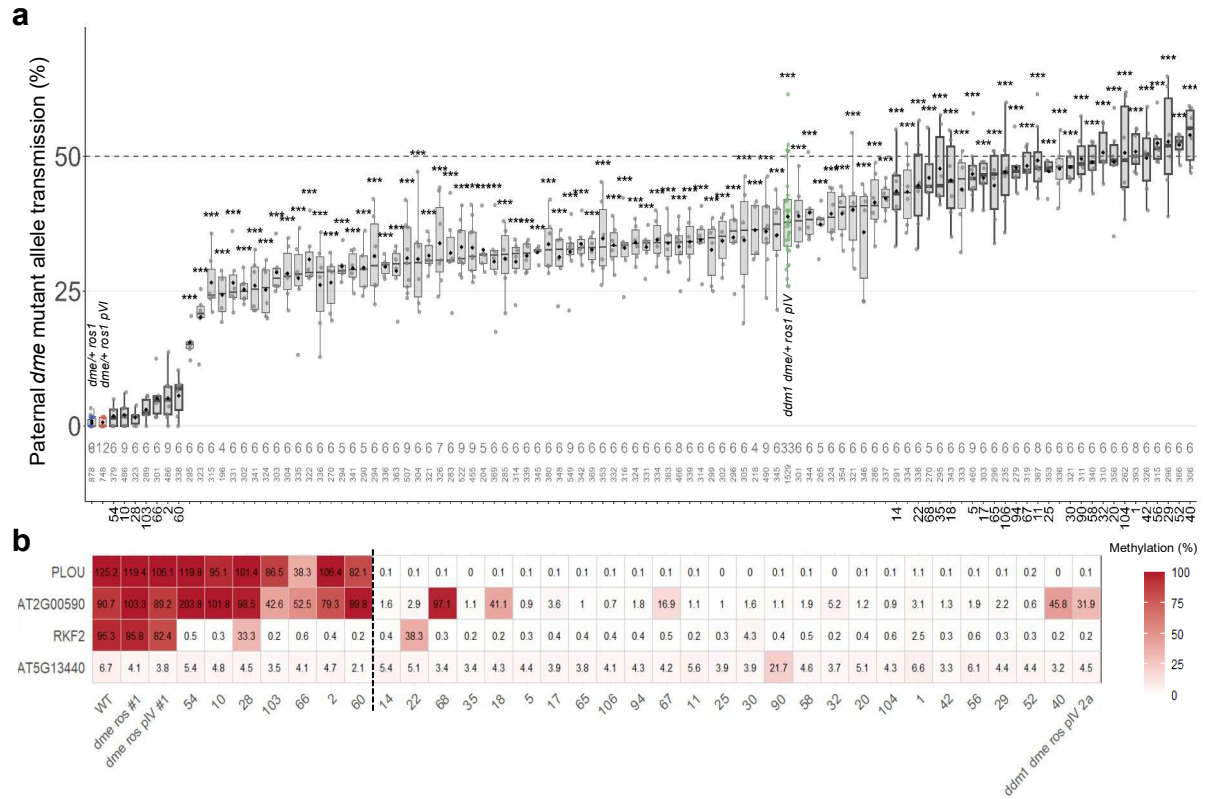


Figure 5: DNA hypomethylation of *PLOU* strictly correlates with fertility restoration in the demethylase mutant. **a** *dme* mutant transmission frequency of pollinations of wild type (WT) mother plants with *dme*^{+/+} *ros1*, *dme*^{+/+} *ros1* *nrpd1a* (pIV) and *ddm1* *dme*^{+/+} *ros1* *nrpd1a* (pIV) as well as 99 individual pollen donors from a F2 epigenetic segregating population derived from *ros1* *nrpd1a* × *ddm1* *nrpd1a* *ros1* *dme*^{+/+}. These lines are genetically identical and WT for *DDM1* but presumably differ epigenetically, inheriting *ddm1*-induced DNA hypomethylated states segregating within the population. Boxplots show the median (central line) and the interquartile range (box limits), with whiskers representing the data distribution. Diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of individual plants and biological replicates is indicated below each boxplot. Pairwise comparisons of *dme* transmission rates relative to the *dme*^{+/+} *ros1* *nrpd1a* control were performed using Dunnett's test. Statistical significance is indicated as follows: *p* *** ≤ 0.001. Lines used in the following DNA methylation assay are labeled in bold. **b** Heatmap of methylation levels assessed by chop-qPCR of the two candidate genes: *PLOU* and *AT2G00590*, as well as *RKF2* as a neutral control and *AT5G13440* as an unmethylated control. WT, *dme*^{+/+} *ros1* and *dme*^{+/+} *ros1* *nrpd1a* (pIV) are used as methylated as well as *ddm1* *dme*^{+/+} *ros1* *nrpd1a* (pIV) as unmethylated controls. Sterile epi-pIV-Lines are grouped on the left side and are separated from the fertile epi-pIV-Lines on the right by a dashed line in the heat-map table. Calculated DNA methylated percentages are indicated in the respective boxes.

To test this hypothesis, we performed chop-qPCR on DNA digested with cytosine methylation-sensitive restriction enzymes on the 7 sterile and 25 fully rescued lines from the epi pIV population on *PLOU* and *AT2G00590*, both candidates that associate with DNA hypomethylation in fertile epiLines (Suppl. Fig. 11c). *RKF2* (*AT1G19090*) served as a control, as it exhibits similar methylation levels in both fertile and sterile epiLines, as well as *AT5G13440* as a consistently

unmethylated reference sequence (Fig. 5b; Baduel et al. 2025). As expected from our previous methylome analyses (Table S6), *RKF2* is hypomethylated in the absence of *DDM1* (Fig. 5b), but as in the epiLines, this locus remained hypomethylated regardless of the fertility status, excluding it as a potential causative locus. Although *AT2G00590*, about 0.5Mbp away from *PLOU*, was systematically methylated in sterile lines, it was methylated in four fertile epi pIV lines, one showing complete (wild type-like) and three intermediate DNA methylation levels, indicating dissociation of the epigenetic state of both annotations by meiotic recombination. As a consequence, DNA hypomethylation at this locus unlikely associates with pollen fertility. In contrast, *PLOU* shows a clear distinction between fertile and sterile epi pIV lines, where the latter are persistently methylated and on the other hand, fertile lines consistently unmethylated. Taken together, numerous criteria support the notion of *PLOU* being the main epigenetically regulated locus required for pollen tube guidance: its DNA methylation pattern outside reproductive growth depends on DDM1 function, but requires demethylase activity in pollen for its activity. Loss of DDM1-mediated DNA methylation restores proper gene expression and male fertility, which can be obtained in two epigenetically segregating populations with and without remethylation, both showing consistent and specific association of *PLOU* hypomethylation. Moreover, the bi-(epi-)allelic segregation pattern in the epi pIV lines and the uncoupling from the closely located *AT2G00590* annotation strongly suggest *PLOU* as the central epigenetically regulated locus required for pollen tube guidance.

496

497 *PLOUTOS* is a main actor of male fertility

Indeed, *PLOU* had previously been identified by us as an epigenetically regulated male fertility gene as it was able to partially complement male sterility in the demethylase mutant backgrounds when ectopically expressed in pollen (Khouider et al. 2021). This gene codes for an uncharacterised putative S-adenosyl-L-methionine-dependant methyl-transferase. To further test the function of *PLOU* in pollen, we generated a CRISPR/Cas9 mutant by deleting the enzymatic methyltransferase domain (Suppl. Fig. 14). Phenotypic analysis showed that homozygous mutants crossed as pollen donors onto wild type plants exhibited a reduced number of seeds per silique (– 45%), whereas reciprocal crosses of wild type pollen over *plou* mothers had normal seed set (Fig. 6b). This indicates that *PLOU* is indeed required for male fertility. To test its male gametophytic function, we assessed the mutant allele transmission rates using heterozygous lines as either maternal or paternal crossing partner with wild type plants. Confirming *PLOU* as a major player in pollen function only 4% of the offspring carry the mutant allele when derived from a cross with

plou/+ fathers against the expected Mendelian segregation when used as a heterozygous mother (Fig. 6c). To exclude the possibility of a CRISPR/Cas9-generated off target mutation we complemented the mutant with a PLOU-mCitrine reporter line under its own promoter, leading to full restoration of male fertility (Fig. 6d) and exhibiting non-nuclear cytoplasmic localisation in pollen (Fig. 6a). Colocalization of PLOU-mCitrine together with a mScarlet (mSct) fusion of POLLEN RECEPTOR KINASE 4 (PRK4), a member of the pollen-specific LRR-receptor kinase family which are plasma membrane localized (Duckney, New Phytologist 2017) and enriched in the tip of growing pollen tubes, showed non-overlapping localisation patterns (Fig. 6A, below). This shows that PLOU-mCit exhibits a cytoplasmic localization, consistent with a function in post-translational modification of target proteins (Fig. 6a). In summary, we demonstrate that the main epigenetically regulated locus implicated in pollen tube growth and guidance, PLOU, is an important actor in male fertility.

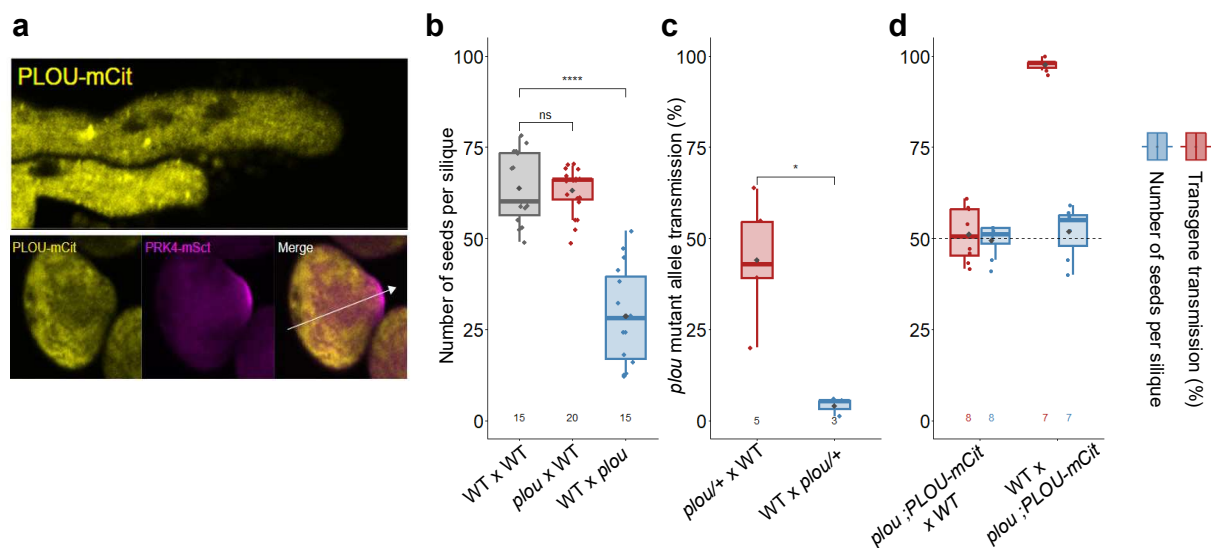


Figure 6: PLOU is required for male fertility. **a** Confocal microscopy of pPLOU:PLOU-mCitrine (PLOU-mCit) localisation in growing tubes (above) and germinating pollen grains (below); co-imaging with pPRK4:PRK4-mScarlet (PRK4-mSct) labelling the pollen tube germination site. **b** Seed set per silique in WT crosses compared with crosses involving *plou* mutant plants used either as female (maternal) or male (pollen donor). **c** Transmission frequency of the *plou* mutant allele in crosses with heterozygous *plou/+* lines used either as female (maternal) or male (pollen donor). **d** Transmission frequency of a heterozygote, single-locus PLOU-mCit insertion in a homozygous *plou* background used as male or female crossing partner with WT plants. Box-plots show the median (central line) and the interquartile range (box limits), with whiskers representing the data distribution. Diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of biological replicates is indicated below each box-plot. Pairwise comparisons between groups were performed using a non-parametric Wilcoxon rank-sum test (two-sided). Statistical significance is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Discussion

Here we showed that DNA methylation dynamics at pollen reproductive genes is regulated by an antagonistic activity of DDM1 that tightly maintains DNA methylation during the vegetative phase and pollen-specific function of DME and ROS1 to remove DNA methylation during the reproductive phase. Accordingly, the depletion of *DDM1* fully rescues the fertility defects observed in demethylase mutants by restoring the expression of reproductive genes that are downregulated in the absence of active DNA demethylation in pollen. Consistently, these genes are teM and rapidly remethylated in *ddm1*-derived hypomethylated populations where epialleles segregate after the restoration of DDM1 function as described in epiRILs (Johannes et al. 2009). This remethylation therefore results in only partial fertility rescue in an epigenetic population with segregating *ddm1*-derived hypomethylated genomic regions, which coincides with the absence of stably inherited DNA hypomethylation over the key locus *PLOU* pinpointed in this work. In line with this, *PLOU* is heavily targeted by sRNAs and its remethylation indeed relies on the thereby mediated RdDM activity. Preventing RdDM from occurring within a *ddm1*-derived epigenetic population indeed identifies *PLOU* as the main locus the hypomethylation of which associates with a complete restoration of fertility in the otherwise severely sterile *dme ros1* double mutant. As we demonstrate in this work, demethylase mutants have defects in ovular pollen tube growth and guidance, which in turn would result from the absence of *PLOU*. In agreement with this notion, we provide functional genetic validation as a *plou* mutant shows male sterility corroborating an important role in pollen tube functions. Although we cannot exclude some contribution of other similarly epigenetically regulated loci to establish male fertility, here we provide ample evidence that this process converges primarily on *PLOU*. In light of this, it is puzzling that thousands of regions become demethylated with hundreds of annotations requiring this interference with epigenetic silencing to establish proper transcriptional activation. Notably, a *PLOU* ortholog in maize with preferential expression in male reproductive tissues, Zm00001eb097260, also shows teM, although it was not detected as a demethylase target in pollen, possibly due to lower detection levels associated with single pollen RNA-seq used in this publication (Zeng et al. 2023, 2024). Nonetheless, altogether this suggests that the heterochromatic state and the pollen-specific expression of this locus are evolutionarily conserved.

Earlier reports have hypothesized a role of sRNAs generated by transcriptional up-regulation of TEs in the pollen VC, which in turn would lead to the non-cell-autonomous transcriptional silencing of corresponding TE sequences in the SCs (Slotkin et al. 2009; Martínez et al. 2016). Despite

a broad demethylation activity targeting repeat sequences in the VC (Calarco et al. 2012; Ibarra et al. 2012; Khouider et al. 2021), we observed only a few TEs being transcriptionally dependent on this activity, although we probably miss many transcripts in our RNA-seq due to 3' polyA-based sequencing.

There are further indications that methyltransferase activity is involved in polar growth, such as root and root hair growth, which requires sequential methylation of membrane lipid precursors (Cruz-Ramirez et al. 2004). Similarly, methylation of pectins, important components of polar pollen tube growth, might contribute to fine-tune the ratio of methylated- and unmethylated pectins, the cross-methyl esterification of which determine mechanical properties of the cell wall (Bosch and Hepler 2005). Alternatively, and based on predicted membrane-localised protein interaction assays (Jones et al. 2014; Muschietti and Wengier 2018), PLOU was shown to bind to multiple receptor-like kinases, including PRK2 and MALE DISCOVERER 2 (MDIS2). MDIS2 was shown to mediate ovule-guided pollen tube growth and guidance (Wang et al. 2016), which offers the possibility that PLOU is involved in pollen tube guidance signalling pathway by modifying receptor kinase activity through methylation. We had previously identified three additional candidate genes encoding pollen receptor-like kinases (*RKF2*, *PRK2* and *PRK4*), which led to partial rescue of demethylase mutant sterility when ectopically expressed in pollen (Khouider et al. 2021). Thus, the ectopic expression of the aforementioned pollen receptor kinases in the demethylase mutant background might partially overcome the loss of PLOU activity in this background. Thus, PLOU might act as a hub integrating receptor kinase signalling pathways involved in pollen tube guidance, as previously proposed (Khouider et al. 2021). In line with this, pollen receptor kinases involved in ovular pollen tube guidance seem to act redundantly (Muschietti and Wengier 2018; Zhong et al. 2019; Zhu et al. 2024), and an integrating function within a thereby mediated signalling process by the S-adenosyl-dependent methylation pathway is further supported by previous findings demonstrating that other components in this pathway also show male sterility (Chen et al. 2016). Further biochemical and cell biological analyses will be required to elucidate its precise molecular targets and mechanism of action.

Altogether, our study identifies *PLOUTOS* as a key epigenetically regulated determinant of male fertility in an evolutionary conserved antagonistic interplay of heterochromatic DNA methylation maintenance during somatic growth and active demethylation during reproduction.

Methods

Plant material and growth conditions.

All mutants and lines were in the *Arabidopsis thaliana* Col-0 background. The fluorescent-tagged line (FTL) used was obtained from the FTL insertion collection created by Francis et al. (2007) and provided by G. P. Copenhaver. This line carries a single insertion fluorescent marker driven by the *LAT52* promoter. The FTL insertion located closest to the *DME* locus (Chr5: 1,309,099–1,318,520) is FTL1311 (Chr5: 2,293,101), which contains a DsRed marker conferring red fluorescence. The *dme*^{FTL} line was generated by crossing FTL1311 with *dme-6* mutant allele and selecting a recombinant line, which provides a concomitant localisation of the fluorescent marker on the same chromosome as the T-DNA insertion of *dme-6*. The recombination rate allowed us to determine a genetic distance of 1.5cM, indicating that both loci co-segregate to 98.5%.

The *dme-6* (GK-252E03014577) and *ros1-5* (SAIL_1249_A01) mutant alleles have been characterised previously (Penterman et al. 2007; He et al. 2019; Khouider et al. 2021). As well as *cmt2-7* (Wisc7E02), *cmt3-11* (SALK14831), *ago4-3* (WiscDSL0x338A06), *drm1* (SALK031705) and *drm2* (SALK150863), *nrpd1a-4* (SALK_083051), *met1-3* (SAIL_809_E03) (Saze et al. 2003; Herr et al. 2005; Chan et al. 2006; Jullien et al. 2006; Havecker et al. 2010; Stroud et al. 2014), all in the Col-0 background.

CRISPR/Cas9 generated mutant lines

The *ddm1-67* mutant used here was a CRISPR/Cas9-generated deletion straddling exon 6 and intron 7, established in the laboratory of Pierre Baduel/IBENS/Paris (*ddm1-67*; Baduel et al., unpublished) and characterised by whole genome nanopore sequencing for global DNA methylation loss, and compared to *ddm1-2* mutant BSseq data (Suppl. Fig. 16 and see below for methods). The *ploutos* mutant (*plou*; *AT2G16030*) and the *met1* mutants used to test the rescue of the *dme* seed abortion phenotype were also generated using CRISPR/Cas9 driven by the *RPS5A* promoter. For each construct, two sgRNAs (see sequences in primers list) were designed under *U6* promoter. The *plou* construct was transformed into a wild type background, whereas the *met1* construct was introduced into a *dme/+* background. The T1 transformants were FAST-selected (Shimada et al. 2010) analysed by sequencing the sgRNAs target regions of the genes, which were amplified by PCR using the primers listed in Supplementary Data (Table S9). In *met1* T1 lines with a ~425 bp deletion was identified, and methylation loss was further validated by Chop-PCR using the methylation-sensitive restriction enzymes HpaII and AclI at the *DCL1* (*AT1G01040*) locus, confirming CG methylation loss.

630 Fluorescent marker lines

631 The pPLOU::PLOU-mCitrine and pPRK4::PRK4-mScarlet2 constructs were generated using the
632 Greengate cloning system (Lampropoulos et al. 2013) by separate amplification of a 1129bp (*PLOU*)/
633 1156bp (*PRK4*) promoter- and the entire coding region without the Stop codon (gDNA), subcloned
634 into the intermediary pGGA000 and pGGC000 vectors, respectively. Fluorescent proteins in the
635 pGGB000 vector system were used to generate fusions of the three parts and finally establish binary
636 vector constructs in the pGGX000 backbone (Bouatta et al. 2025) conferring BASTA (glyphosate)
637 resistance. All constructs were Sanger-sequenced to confirm accurate sequence and vector integrity
638 prior to transformation. Plants were transformed using the floral dip method (Clough and Bent 1998)
639 and seeds selected on 50 μ M glyphosate-containing $\frac{1}{2}$ MS plates. Seeds were surface-sterilized and
640 sowed on $\frac{1}{2}$ MS plates under sterile conditions. After few days of 4°C stratification, plates were
641 transferred to 21°C/16 h light–18 °C/8 h dark growth conditions for 10–15 days before transfer to
642 soil under the same growth conditions with 60% humidity control.

643 Localization of Plou-mCit and PRK4-mSct was done using *in vitro* germinated growing pollen tubes.
644 Pollen were germinated as described (Bouatta et al. 2025) and imaged on an Olympus FV-1000 with
645 a 40 $\times$ water immersion (1.15 NA) objective. Confocal signals were detected with a 1 AU pinhole and
646 high-sensitivity detectors, exciting mCit at 515 nm and mSct at 559 nm, and detecting between 520–
647 550 nm (mCit) and 580-653 nm (mSct), respectively.

648 Quantifications of mutant alleles transmission rates

649 Pollen fertility in *dme/+* and *dme/+ ros1* plants was assessed by measuring the transmission rate of
650 the *dme* mutant allele to the next generation. Transmission rates were calculated as the percentage
651 of mutant progeny relative to the total number of progenies. Because *dme* is transmitted exclusively
652 through the male gametophyte, plants were either self-fertilized (see Suppl. Fig. 5b) or used as
653 pollen donors in crosses with emasculated wild type flowers. Thus, Col-0 wild type plants were
654 emasculated using fine forceps (Swiss Forceps size 5) and pollinated two days later by applying
655 saturating amounts of pollen. Successful fertilization was verified by harvesting individual siliques
656 and counting the number of seeds. Siliques containing less than 25 seeds were not considered. As
657 the T-DNA insertion conferring the *dme* mutation provides sulfadiazine resistance, seeds were
658 germinated on $\frac{1}{2}$ MS medium containing 37.5mg/mL of sulfadiazine to select for *dme*-carrying
659 progeny. Pollen fertility of the *plou* mutants was assessed by the number of seeds per silique
660 obtained from emasculated Col-0 wild type flowers pollinated with pollen from homozygous

661 mutants, and by PCR genotyping for the transmission rate of the mutant allele for heterozygous
662 plants.

663

664 *In vivo* and *in-vitro* pollen tube visualisation

665 To assess pollen tube growth and guidance defects in demethylase mutants, single-pollen pollination
666 was performed (Sui et al. 2026). Approximately 25 h after pollination, pistils were collected and fixed
667 in ethanol:acetic acid (3:1). Samples were rehydrated through an ethanol series (70%, 50% and 30%),
668 softened in 8M NaOH solution and stained with 0.1% aniline blue for pollen tube visualization.
669 Stained pistils were initially screened using a Zeiss ApoTome.2 fluorescence microscope, and
670 representative images were acquired using a Zeiss LSM980 confocal microscope.

671 Semi-*in vitro* pollen tube guidance assays were performed as previously described with minor
672 modifications (Desnoyer and Grossniklaus 2023; Zhong et al. 2025; Sui et al. 2026). Flowers at stage
673 12 (Smyth et al. 1990) were emasculated 1 day before pollination. Following hand pollination, pistils
674 and mature ovules were dissected in a custom humid chamber under high-humidity conditions to
675 maintain ovule viability and prevent tissue desiccation. Excised ovules with attached funiculi were
676 arranged on pollen germination medium with micropyles oriented upward. Stigmas were excised
677 30–60 min after pollination and positioned such that the cut end of the style faced the ovules at a
678 distance of approximately two ovule diameters. Dishes with cut styles and ovules were incubated in
679 a growth chamber (21/18 °C day/night, 16 h light/8 h dark) for 5–6 h to allow pollen tube emergence
680 and ovule targeting. Pollen tube guidance behavior was observed using a Zeiss LSM980 confocal
681 microscope. Successful targeting was confirmed using the ovule-dragging assay (Liu et al. 2021) in
682 which ovules remained attached to the pollen tube after gentle displacement with a glass needle.

683 For statistical analysis of the *in vivo* pollen tube assay, pollen tubes exhibiting more than three
684 crossing events within the transmitting tract were classified as entangled. The proportions of
685 entangled pollen tubes between genotypes were compared using a two-sided Fisher's exact test. For
686 the semi-*in vitro* ovule-targeting assay, ovule-targeting efficiency was calculated as the percentage
687 of successfully targeted ovules relative to the total number of scorable ovules in each biological
688 replicate. Data are presented as mean $\pm$ s.d. from four independent biological replicates. Statistical
689 significance between wild type and mutant pollen tubes was determined using a two-tailed paired
690 Student's *t*-test. Microscopy images were processed and analyzed using ZEN software (Zeiss Zen
691 3.10) and ImageJ. Graphs and statistical analyses were generated using Microsoft Excel.

692 Pollen isolation and *dme*^{FTL} sorting and RNAseq

693 Mature flowers of about 40 plants were collected for one replicate and put into 30 mL of cold
694 mannitol (0.3M). After 3 min of thorough shaking, the solution was filtered using a 100 µm nylon
695 mesh. Isolated pollen was concentrated by 5 min centrifugation (4000 × *g* at 4 °C) and resuspended
696 in 2 mL PBS buffer. Fluorescence Activated Cell Sorting (FACS) of the pollen grains was performed
697 using a FACS Aria II cell sorter (BD Biosciences) and the integrated FACSDiva software. Sorting was
698 performed with a 100 µm nozzle at 20 psi and 4°C. A 488 nm laser was used for DsRed excitation and
699 emission was detected after passing through a 585/42 nm band pass filter. Between 60,000 and
700 100,000 pollen were collected in one or two FACS sessions to obtain one replicate used for RNA-seq.

701 Sorted pollen was centrifuged again at 10,000 × *g* for 5 min. Remaining liquid was removed as much
702 as possible and the pellet stored at -80°C. The material was homogenized with metallic beads using a
703 Qiagen tissue-lyser avoiding thawing (2 × 1' 30 Hz) prior to perform total RNA extraction with the
704 Qiagen RNeasy Mini Kit, following the manufacturer's instructions. RNA integrity was verified by
705 TapeStation (Agilent). All RNA samples (around 0.5 – 1 µg of total RNA/replicate) were sequenced in
706 triplicates and in the same run to avoid batch effects. Library preparation was carried out using
707 Lexogen CORALL mRNA-seq Library Seq Kit following the manufacturer's instructions. Each sample
708 was bar-coded using Unique Molecular Identifier coupled to the amplification adapters provided by
709 the kit and sequencing was carried out on an Illumina NextSeq500 platform producing directional
710 75nt single-end reads.

711 Transcriptome analysis

712 Raw sequencing data was quality-controlled using FastQC (v0.11.4). UMI extraction and quality
713 trimming were performed according to the CORALL Data Analysis Pipeline using awk (v1.3.3) and
714 Cutadapt (v4.5) (Martin 2011). Structural annotations for the Araport11 genome assembly (Cheng et
715 al. 2017) were processed in order to allow TEs to be taken into account prior to alignment to allow
716 the quantification of all features including protein coding genes, pseudogenes, TEs and TE genes.
717 Indexing for the TAIR10 genome assembly and read alignment were carried out using STAR (v2.7.9a)
718 (Dobin et al. 2013), performing transcript quantification during alignment with the quantMode
719 GeneCounts parameter. Raw transcript counts derived from the 3rd column of the STAR output
720 were used for exploratory and differential gene expression analysis using the DESeq2 (v1.26.0) R
721 package (Love et al. 2014). The median of ratios method was applied to normalise the raw counts for
722 sequencing depth and RNA composition, and genes with no counts or with a single count across
723 samples were filtered out. The r-log transformation was applied to ensure data homoskedasticity

during exploratory analysis, and used to perform Principal Component Analysis using the plotPCA() function implemented in DESeq2. Pearson correlation coefficients were computed from the r-log normalised data with the cor() R function and sample clustering was performed with the R package ComplexHeatmap (v2.2.0) (Gu et al. 2016). Differential expression analysis was performed on the DESeq2-normalised counts, and significant differentially expressed genes (DEGs) were selected based on an adjusted *p*-value threshold of 0.01.

Gene Ontology (GO) enrichment analysis was performed using the clusterProfiler package (v4.14.6) with the org.At.tair.db annotation database (v3.20.0) in R (v4.4.3). Statistical significance was assessed using Fisher's exact test with Bonferroni correction. Differentially Methylated Regions (DMRs) for *dme*, *ros1* and *dme ros1* mutant vegetative cells (Khouider et al. 2021) were mapped against gene promoters of the Araport11 annotations using bedtools intersect (v2.25.0) (Quinlan and Hall 2010). Promoters were defined with bedtools flank (v2.25.0) as the 500bp region upstream of the TSS. DMRs for *ddm1-67* mutant seedlings (Baduel et al., unpublished) were analogously mapped against promoters and exons.

Whole-genome long-read sequencing and cytosine methylation detection.

DNA was extracted from flower buds of individual epiLines and epi pIV lines, as well as control plants, before testing their fertility. Vegetative tissues were frozen in liquid nitrogen and ground with metallic beads using a Qiagen TissueLyser (2 × 1 min at 30 Hz). CTAB (cetyltrimethylammonium bromide) DNA extraction was used to obtain purified genomic DNA, according to Clarke (2009). Individual samples were barcoded with the kit SQK-NBD114-24 prior to pooling and sequenced using an Oxford Nanopore Technologies (ONT) R10.4.1 flow cell with E8.2 sequencing motor, to obtain a sequencing depth of 39x on average. Demultiplexing was done independently using ONT Dorado v0.9.6 (in both single-end and both-ends barcode modes) and Porechop v0.2.4 (Ryan Wick, <https://github.com/rrwick/Porechop>); a read's barcode assignment required agreement between Dorado (single-end mode) and Porechop, falling back to Dorado's both-ends call when Porechop found no barcode. Raw reads were preprocessed using the more recent Dorado v1.1.1 for adapter trimming and basecalling, and subsequently aligned to the *A. thaliana* TAIR10 reference genome and Araport11 annotation using Minimap2 v2.28 with option "--ax map-ont -y --secondary=no", preserving methylation (MM/ML) tags through the BAM-to-FASTQ conversion required for realignment. Detection of modified cytosines in the CG, CHG, and CHH sequence contexts was performed using Dorado's all-context modified-base model (5mC_5hmC).

The resulting methylation data were compared with a parallel analysis performed using a second deep learning tool, DeepSignal3 (P. Ni; <https://github.com/PengNi/deepsignal3>), an updated, Dorado-compatible successor of DeepSignal-plant (Ni et al., 2021), using the plant-specific model trained on rice R10.4.1 (5kHz) data for CG/CHG/CHH detection. Unlike DeepSignal3's plant-specific model, Dorado's modified-base models are trained on non-plant reference data (human, *C. elegans*, and microbial community standards), providing a methodologically independent basis for comparison. Methylation levels called independently by the two ONT-based in the CG, CHG, and CHH contexts of the *dme/+* and *dme/+ ddm1-67* controls were highly concordant (Suppl. Fig. 15 and 16). As this agreement alone could not indicate which tool was more accurate, both were further benchmarked against publicly available whole-genome bisulfite sequencing (WGBS) data, a fully independent standard, obtained from Col-0 four-day-old seedlings (GEO GSE85975, GSM2290018 sample; Bouyer et al. 2017; used as a proxy for *dme/+*, whose methylation levels are comparable to wild type levels in vegetative tissues) and *ddm1-2* mutant plants (GEO GSE62206, sample GSM1522202), both reprocessed with Bismark (v0.22.3) from raw reads. Across all three pairwise comparisons, most cytosines displayed similar methylation levels and comparable methylation density distributions in each sequence context (Suppl. Fig. 15 and 16), confirming overall consistency of the results, particularly in *ddm1* mutants, where extensive hypomethylation may introduce biases in deep learning models, predominantly trained on canonical methylation patterns. Notably, ONT sequencing, owing to its broader genomic coverage, enabled the detection of a higher proportion of cytosines in the CHH context (+7%) compared to WGBS, resulting in increased apparent CHH methylation levels, as already described by Ni (2023). Methylation profiles generated with Dorado showed slightly better concordance with WGBS than those obtained with DeepSignal3, the only criterion available to arbitrate between the two ONT-based tools. Global methylation levels across the three contexts were closer to WGBS estimates with Dorado, and methylation signals detected in mitochondrial and chloroplast genomes—used here as indicators of false-positive methylation calls—were lower. This improved accuracy supports the use of Dorado-derived data for subsequent DMR calling.

Metaplot DNA methylation analysis

Global DNA methylation levels across genes and TEs were compared among individual lines using metaplots. Cytosines were assigned to genomic features based on Araport11 annotations using Bedtools (v2.31.1) closest and were categorized as promoter regions (1kb upstream of the transcription start site [TSS] to the TSS), 5' ends (from the TSS to the first 500 bp), 3' ends (from the

last 500 nt to the transcription termination site [TTS]), or terminator regions (from the TTS to 1kb downstream). Positions were further filtered to include only cytosines covered by at least one methylated read in the *dme/+* control, thereby defining a common reference set for all comparisons. Cytosines were then grouped into 50-bp bins, and the median methylation percentage was calculated for each bin. File processing and intermediate calculations were performed using custom Bash and AWK scripts. Plots were generated in R (v4.4.3) using ggplot2 package (v4.0.1).

DMR calling

Differentially methylated regions (DMRs) were identified using a DMAP pipeline implemented with the methylKit package (v1.36.0), together with GenomicRanges (v1.62.1) and methods (v4.5.0) in R (v4.5.0). DMRs were defined as 50 bp bins containing at least 2, 3, or 4 differentially methylated cytosines (mCGs, mCHGs, or mCHHs, respectively), with an absolute methylation difference of at least 0.40, 0.25, and 0.10, following Khouider et al. (2021). Bins located within 150 bp of each other were merged. Cytosines were required to have a minimum coverage of 10 reads and a maximum coverage of 100 reads. DMRs were annotated according to the nearest Araport11 gene annotation using Bedtools *closest* (v2.31.1), which was used to determine the distance (upstream or downstream) relative to each DMR. IGVTools was used to generate TDF files for visualization of cytosine methylation in the IGV browser.

Mean absolute methylation

Mean DNA methylation levels of candidate genes were investigated at DNA demethylation targets previously identified in the wild type pollen vegetative nucleus (VN) compared to the sperm nucleus (SN) as differentially methylated regions (DMRs), obtained from Supplementary Data 2 (VC vs SC DMRs in Col-0) from Khouider et al. (2021). The dataset was filtered to retain only hypomethylated DMRs annotated as Gene or 5' upstream categories. The filtered regions were reannotated with the Araport11 genome annotation (Cheng et al. 2017) using Bedtools *closest* (v2.31.1) to include recent pollen transcripts and match with the candidate genes list (Cheng et al. 2017). For each line, cytosines located within these DMRs were identified using Bedtools *intersect* (v2.31.1). Mean methylation levels within these regions were calculated as the number of methylated cytosines divided by the total number of covered cytosines and multiplied by 100.

817 Chop-qPCR

818 Candidates methylation states in the controls (wild type, *dme/+ ros1*; *dme/+ ros1 ddm1 nrpd1a*;
819 *dme/+ ros1 ddm1*) and epi pIV lines were assessed through Chop-qPCR. DNA was extracted through
820 the same CTAB protocol described above from flower buds of individual lines. DNA concentrations
821 were measured by NanoDrop quantification and DNA integrity was visually verified by agarose gel
822 electrophoresis. Two methylation-sensitive enzymes were used to cover all targeted loci depending
823 on the restriction sites present in their region. HpaII (C^ACGG) and AclI (C^ACGC) (New England
824 Biolabs), both cleave unmethylated DNA in CG context, but are inactive in case of 5mC presence.
825 gDNA was digested overnight at 37°C in a reaction mix containing 10 ng/μL of DNA, 1X buffer
826 CutSmart and 5 units of each restriction enzyme, in parallel with a mock treatment where the
827 enzymes were replaced by the same volume of water. The reaction was then stopped by heat
828 inactivation at 80°C for 20 minutes. qPCR primers (see primers list) generating 60–200 bp amplicons
829 were designed using Primer3Plus to target regions containing at least one HpaII and/or AclI
830 restriction site and showing differential methylation between WT and *ddm1*. qPCR reactions were
831 performed with SYBR green reagent in duplicates using a QuantStudio 6 Flex system (Applied
832 Biosystems) with 384-well plates. Quantifications were based on the mean Ct value of the two
833 technical replicates unless the Ct difference exceeded 0.5 cycles, in which case the data were
834 discarded. Relative DNA methylation levels were estimated from the difference in Ct values between
835 digested and non-digested samples, assuming 100% PCR amplification efficiency and complete
836 digestion of unmethylated sites, using the formula: methylation (%) = $2^{(Ct \text{ non-digested} - Ct \text{ digested})} \times 100$. Because this calculation assumes perfect PCR efficiency and complete restriction
837 digestion, values can exceed 100% and should be interpreted as relative rather than absolute
838 methylation estimates.
839

840

841 Data availability

842 The sequencing data generated in this study have been deposited in the European Nucleotide
843 Archive (ENA) at EMBL-EBI. Pollen transcriptomes (RNA-seq of FACS-sorted mature pollen, 30
844 samples) are available under accession PRJEB123892; reads are submitted raw and still contain the
845 5' unique molecular identifier (UMI), which must be extracted prior to analysis. Whole-genome
846 nanopore methylomes of the epigenetically segregating lines and their controls (22 samples: 10
847 fertile, 10 sterile and 2 control lines) are available under accession PRJEB123895, as per-sample BAM

848 files basecalled with ONT Dorado, in which cytosine methylation calls in the CG, CHG and CHH
849 contexts are encoded as MM/ML tags. Archive-generated FASTQ files derived from these
850 submissions do not retain these tags, and the submitted BAM files should therefore be used for any
851 methylation analysis.

852 All scripts are accessible at <https://gitbio.ens-lyon.fr/>

853

854 Acknowledgements

855 We would like to thank Pierre Baduel (IBENS, Paris/France) for providing *ddm1-67* mutant line and
856 sharing data as well as Aurélien Petit (IBENS, Paris/France) for help with the DMR analysis. We
857 acknowledge the contribution of SFR Biosciences (Université Claude Bernard Lyon 1, CNRS UAR3444,
858 Inserm US8, ENS de Lyon) cytometry platform, especially Estelle Devèvre and Sébastien Dussurgey
859 for assistance with FACS pollen sorting as well as Sandrine Hughes and Benjamin Gillet for advice and
860 excellent technical support by the PSI sequencing platform. We gratefully acknowledge support from
861 the CBPsmn (Centre Blaise Pascal de Simulation et de Modélisation Numérique; Emmanuel
862 Quemener and Marianne Corvellec. 2013. SIDUS—the solution for extreme deduplication of an
863 operating system. Linux J. 2013) of the ENS de Lyon for the computing resources. We also thank
864 Sophie Terrone and Evelyne Duvernois-Berthet for help with generating Meta-Gene/TE plots and
865 bio-informatic support and feedback as well as Francois Roudier and Annick Dubois (RDP/ENS de
866 Lyon) for helpful discussions. Exchange with, and advice from Filipe Borges (IJPB, Versailles/France)
867 and Martin Bayer (ZMBP, Tübingen/Germany) was an important asset in this work. We acknowledge
868 support from the Center of Advanced Light Microscopy (CALM) at the TUM School of Life Sciences
869 for imaging of PLOU-mCit and PRK4-mSct.

870 Research activity in the group of DB is supported by the Agence Nationale de la Recherche (grants
871 ANR-21-CE12-0036-01 and ANR-21-CE20-0007-02) and an Emergent grant from the ENS de Lyon. The
872 German Research Foundation (DFG) is acknowledged for financial support via grant DR 334/19-1 to
873 TD.

874 Declaration of Interests

875 The authors declare no competing interests.

876

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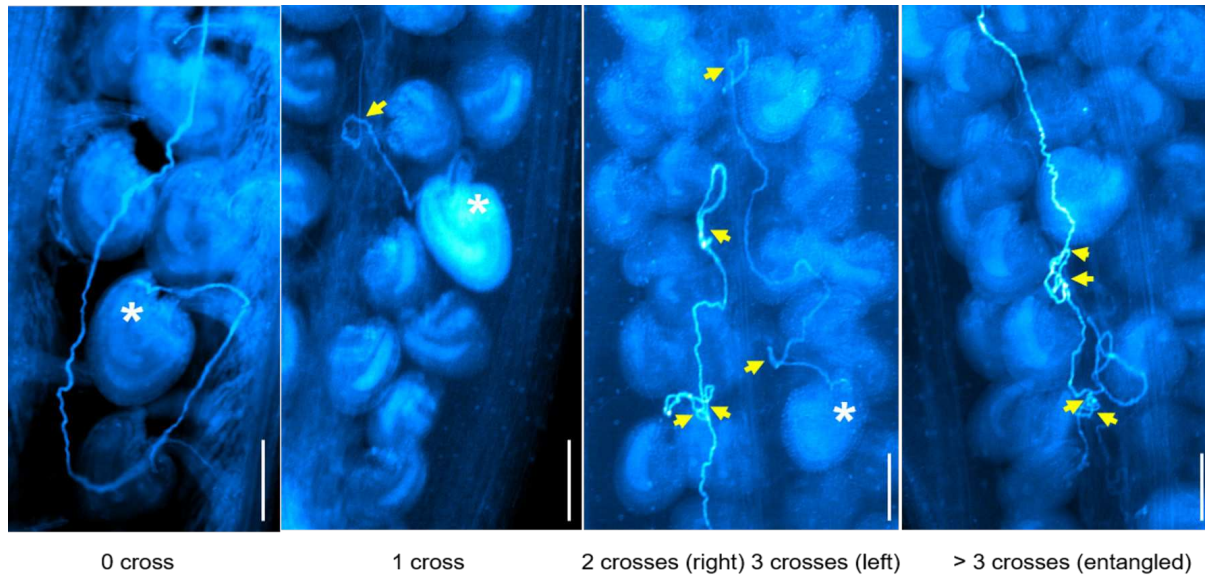
Supplementary Figures

1090 Antagonistic DNA methylation dynamics at a single locus controls pollen tube
1091 guidance in Arabidopsis

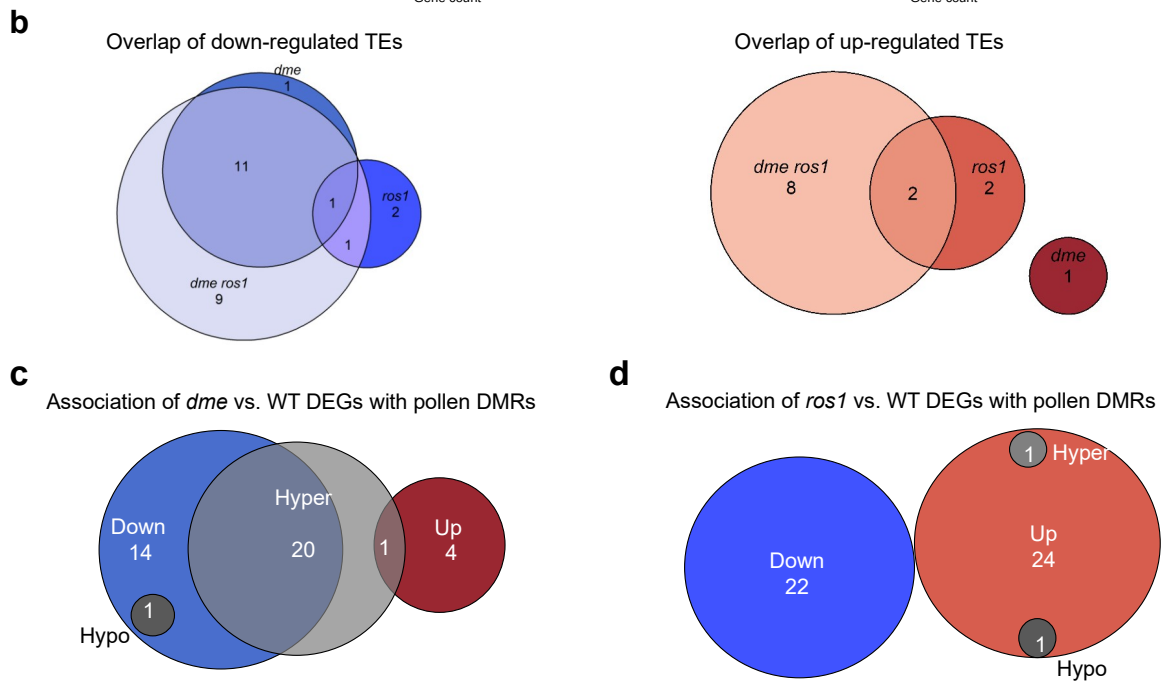
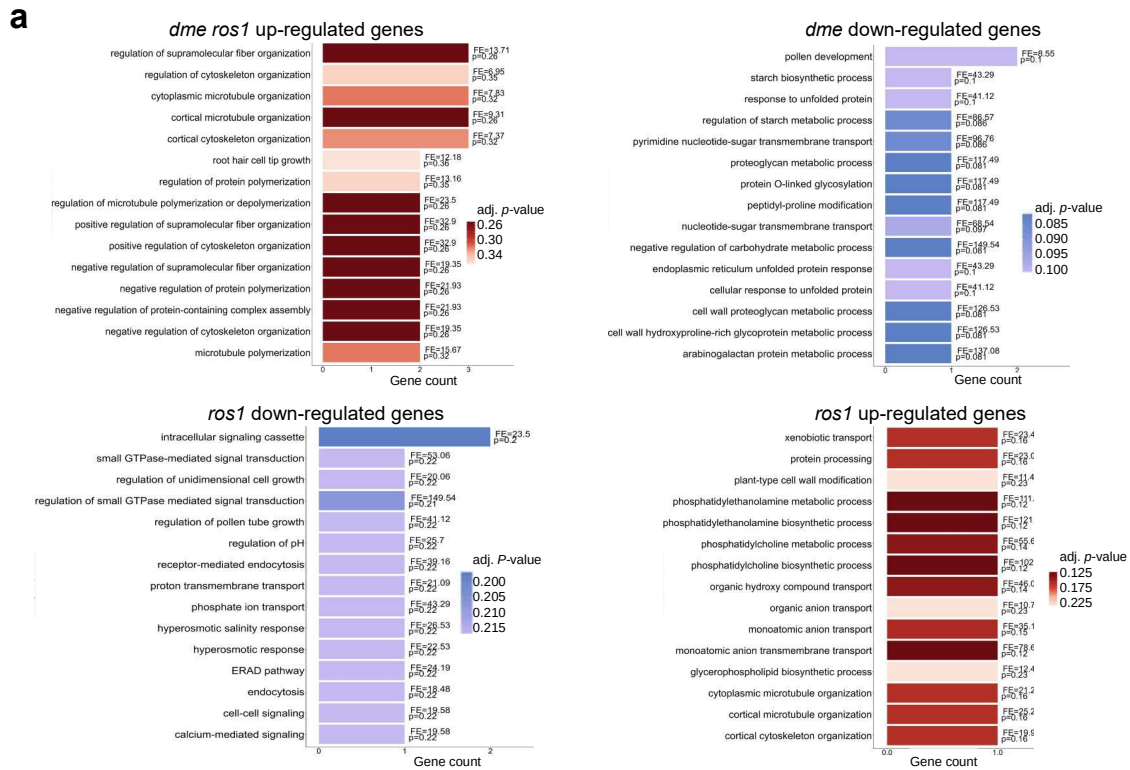
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1093 Laëtitia De Faria, Fiamma Bunello, Yifang Sui, Jérémy Just, Marie Biharé, Marie-France
1094 Gerentes, Andrea Bleckmann, Philipp Denninger, Emanuele de Paoli, Thomas Dresselhaus
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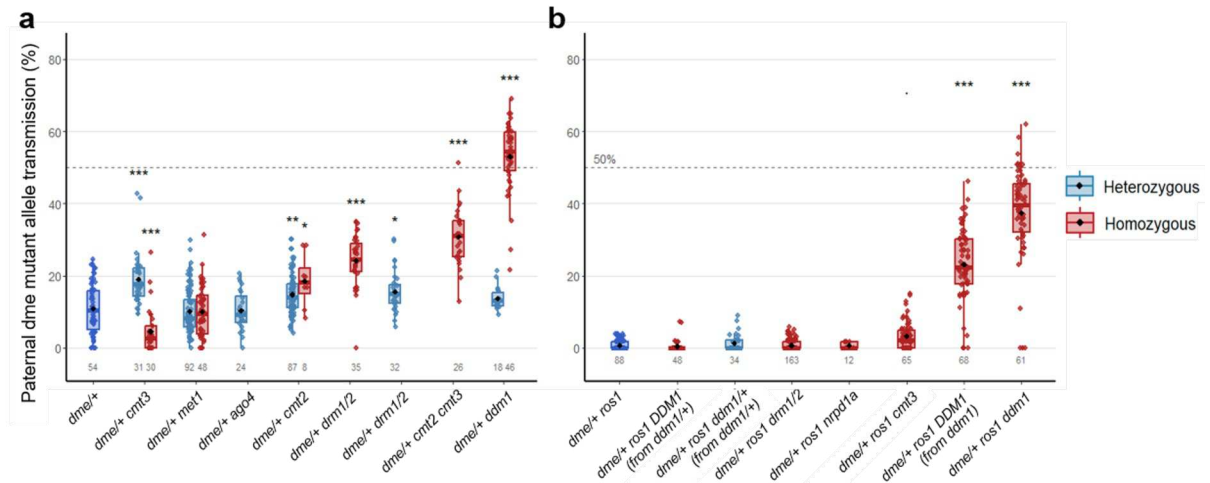
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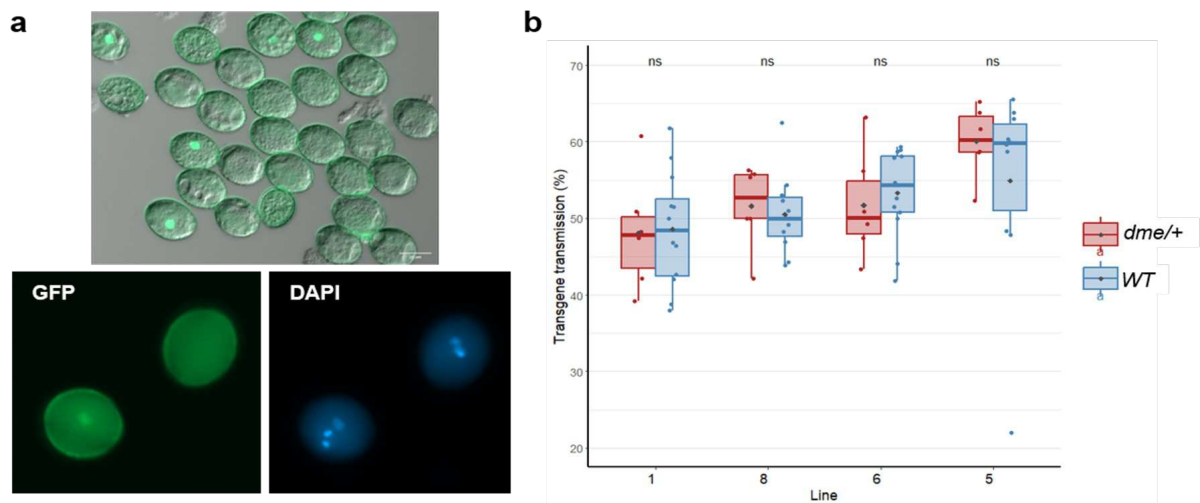
Supplementary Figure 1: Definition of pollen tube trajectory classes. Aniline blue staining of pistils 25 HAP. Yellow arrows indicate the positions where pollen tube trajectories cross, and white asterisks indicate targeted ovules. Scale bars=100 μ m.



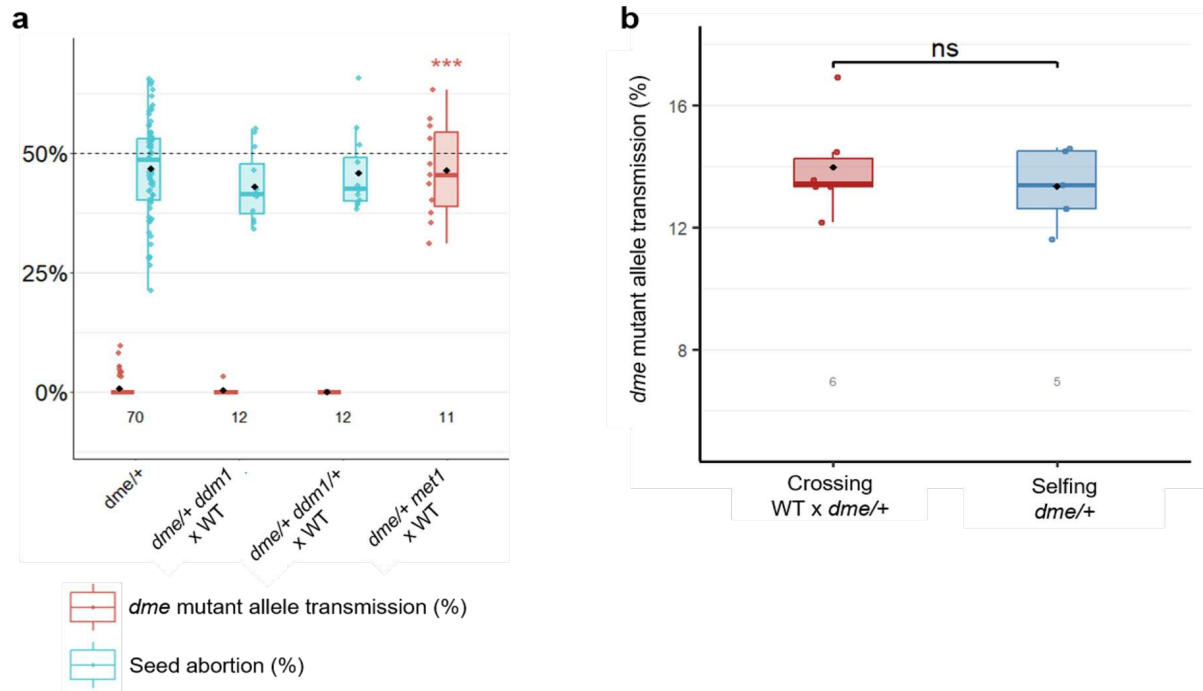
Supplementary Figure 2: DME and ROS1 act synergistically to activate pollen-related genes and some TEs. **a** GO-term enrichment analyses of differentially expressed genes (DEGs): up-regulated genes in the *dme* *ros1* double mutant, down-regulated genes in *dme*, and both up- and down-regulated genes in *ros1* versus wild type (WT), respectively. Enriched categories correspond to Biological Processes. Gene count indicates the number of DEGs associated with each GO term. Bar colors represent adjusted *p*-values, and FE indicates fold enrichment. Statistical significance was assessed using Fisher's exact test followed by Bonferroni correction. **b** Venn diagram giving the number and overlap of up- and down-regulated TEs in *dme*, *ros1* and *dme* *ros1* compared to WT pollen. **c** Venn diagram giving the number and overlap of up- and down-regulated genes in *dme* mutant with differentially methylated regions (DMR) in the sperm cell vs. vegetative cell in CG context. **d** Venn diagram giving the number and overlap of up and down regulated genes in *ros1* mutant with demethylation in the vegetative cell (DMR in sperm cell vs. vegetative cell in CG context).



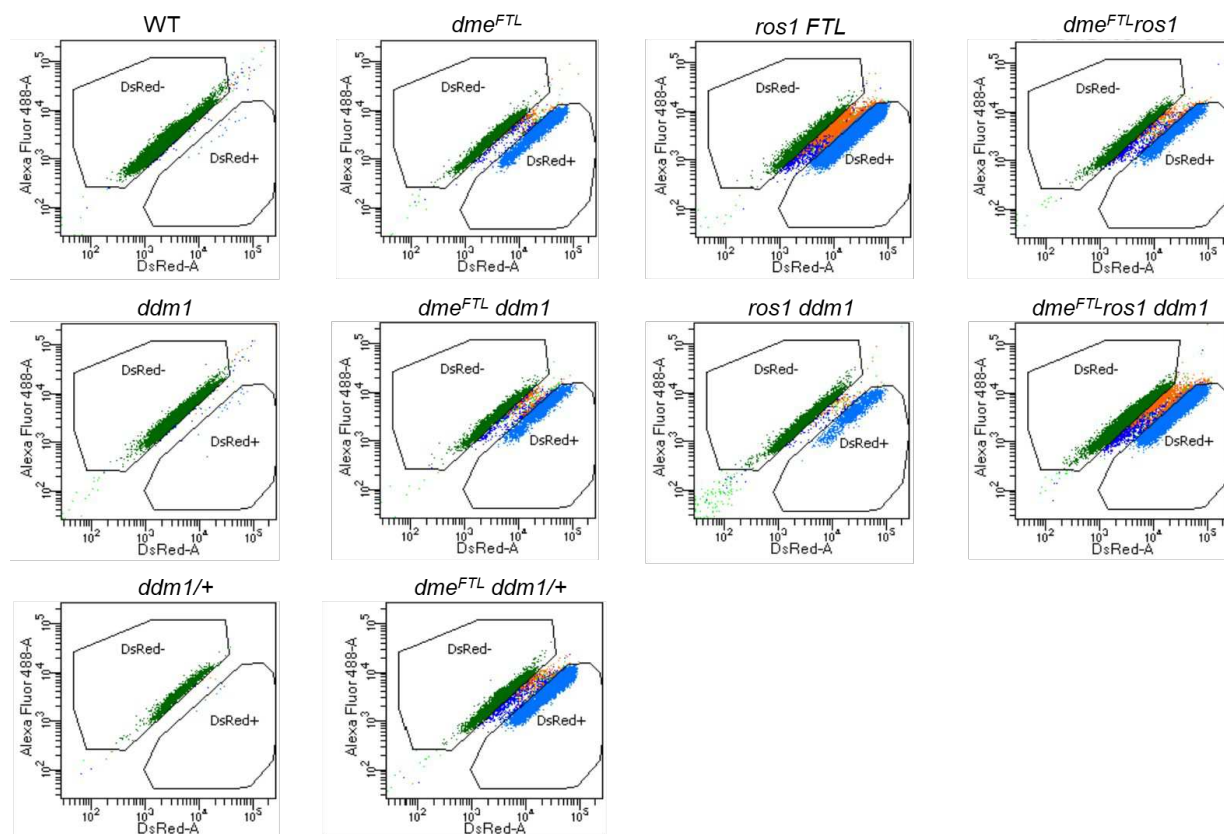
Supplementary Figure 3: Specific rescue of pollen fertility in *dme/+* and *dme/+ ros1* mutants by the *ddm1* mutation. **a & b** Transmission frequency of the *dme* mutant allele in crosses between wild type maternal plants and pollen donors from **a)** *dme/+* and **b)** *dme/+ ros1* in combination with mutants as indicated. The color code discriminates between either heterozygous (blue) or homozygous (red) states for these mutants, to monitor potential gametophytic effects. Boxplots display the median (central line) and interquartile range (box limits), with whiskers representing the distribution of the data. black diamonds indicate mean transmission rates, and each dot corresponds to an individual biological replicate, the number of which is given below each box-plot. Pairwise comparisons of *dme* transmission rates relative to the *dme/+* control crosses were performed using a Dunnett's test. Statistical significance is indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.



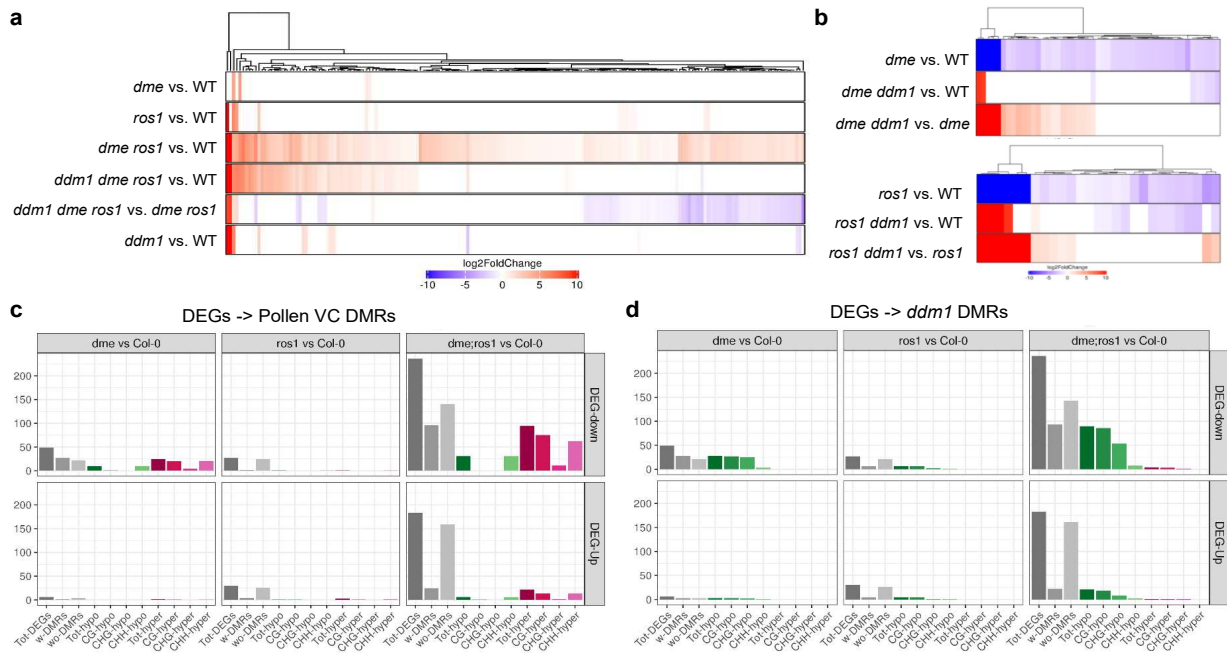
Supplementary Figure 4: Ectopic expression of *DDM1* in the pollen vegetative cell does not affect male fertility. **a** Fluorescence microscopy of transgenic pollen expressing a *DDM1*–GFP fusion protein under the control of the *LAT52* promoter, conferring vegetative cell-specific expression in mature pollen. DAPI staining confirms the localization of *DDM1*–GFP in the vegetative nucleus. **b** Box plot visualization of *pLAT52::DDM1*–GFP transgene transmission rates in four independent single-locus transgenic lines in WT (blue) and *dme/+* (red) backgrounds. Transmission rates close to 50% indicate the absence of a fertility defects. Genotyping of the transgene presence/absence was performed by counting the seeds expressing a red fluorescent reporter of the transgene expressed in seeds (FAST; (Shimada et al. 2010). Differences in transmission rates between backgrounds were not significant (ns) based on a Wilcoxon test.



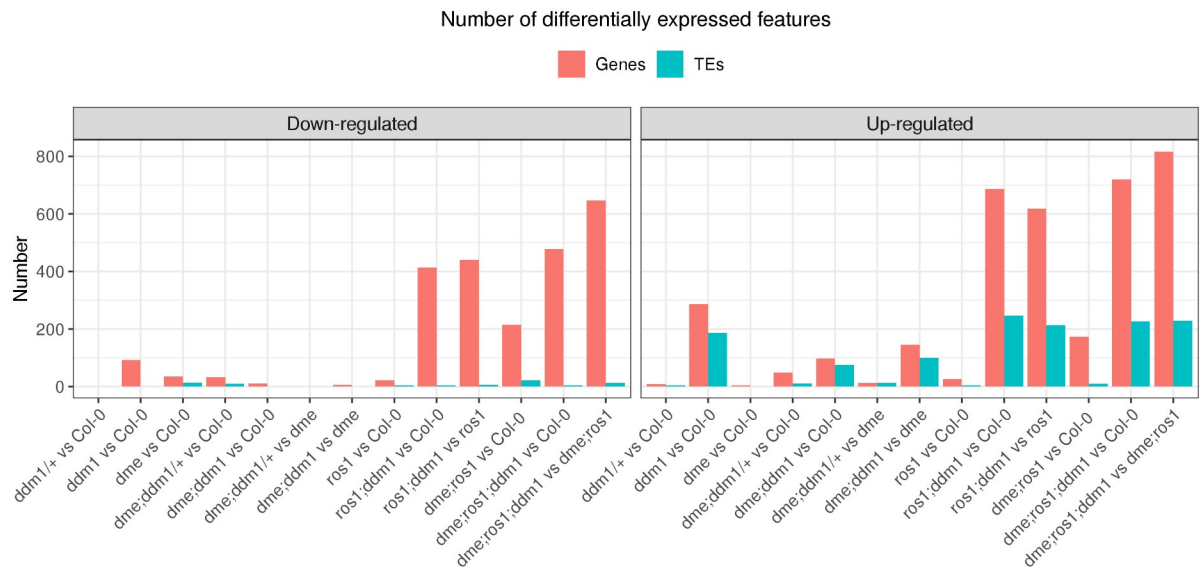
Supplementary Figure 5: The *ddm1* mutation confers paternal, but not maternal complementation of the *dme* mutation, whereas *met1* specifically rescues maternal *dme*. **a** Transmission frequency of the *dme* mutant allele in crosses between wild type (WT) pollen donors and *dme/+* maternal plants. The presence of either homozygous or heterozygous *ddm1* mutations does not increase *dme* mutant allele transmission through the female side and leads to abortion of approximately half the seed set. On the contrary, *dme/+* maternal fertility is rescued by *met1*, as has been previously described (Xiao et al. 2003). Box-plots show the median (central line) and the interquartile range (box limits), with whiskers representing the data distribution. Diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of biological replicates is indicated above each genotype. The dashed horizontal line indicates the expected Mendelian allele segregation (50%). **b** Transmission frequency of the *dme* mutant allele in crossings between maternal WT plants and *dme/+* pollen donors compared to the transmission rate in selfed *dme/+* plants (ns = non significant, t-test).



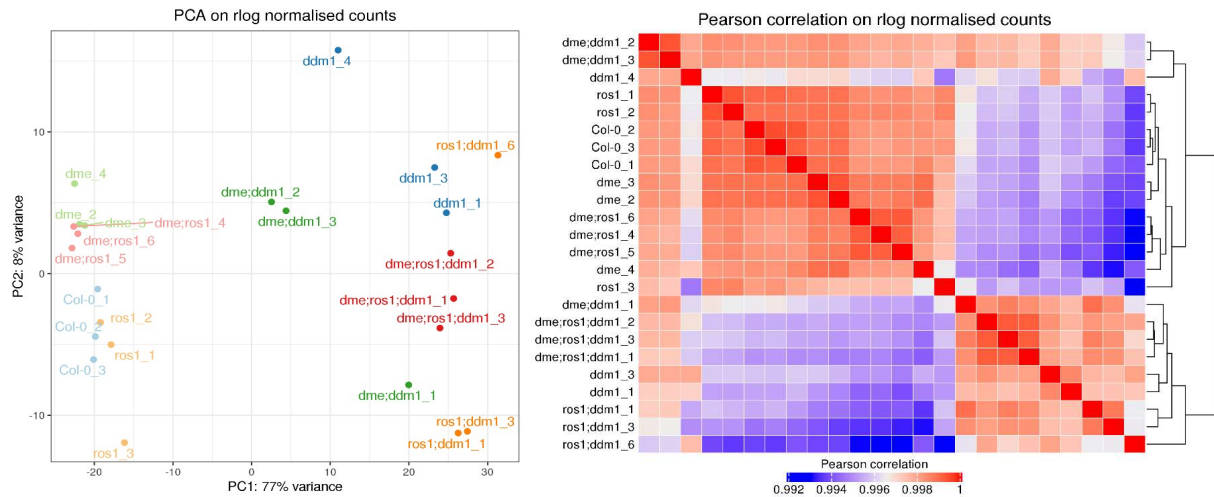
Supplementary Figure 6: Representative FACS profiles of mature pollen with (FTL= dsRed+ in blue) and without fluorescent tag (= dsRed- in green) for all genotypes used in this study to obtain *dme* mutant-specific RNA from heterozygous *dme*/+ lines. Vertical axis (AlexaFluor. 488-A) represents the extent of autofluorescence of the pollen grains and the horizontal axis dsRed-specific fluorescence (dsRed-A). The encircled populations were sorted and collected for further treatment.



Supplementary Figure 7: Heatmap of deregulated genes and their association with pollen demethylation and DDM1-dependent DNA methylation. **a** Heat-map of log2-fold change of transcript levels of annotations up-regulated in *dme ros1* for the indicated genotypes in comparison with WT pollen. Colour code is given at the bottom of the graph. **b** Heat-map of down-regulated genes in *dme* (above) and *ros1* compared to WT pollen for the comparisons indicated on the left side. **c** Deregulated genes in the respective comparison with and their association with differential methylated regions (DMRs) detected between demethylase mutant vs. WT vegetative cell (VC) in pollen for the respective sequence context. Hypo-methylation is indicated in green, hyper-methylation in red. **d** Association of deregulated genes with DMRs detected in *ddm1* vs. WT plants with the same colour-code as in **c**.

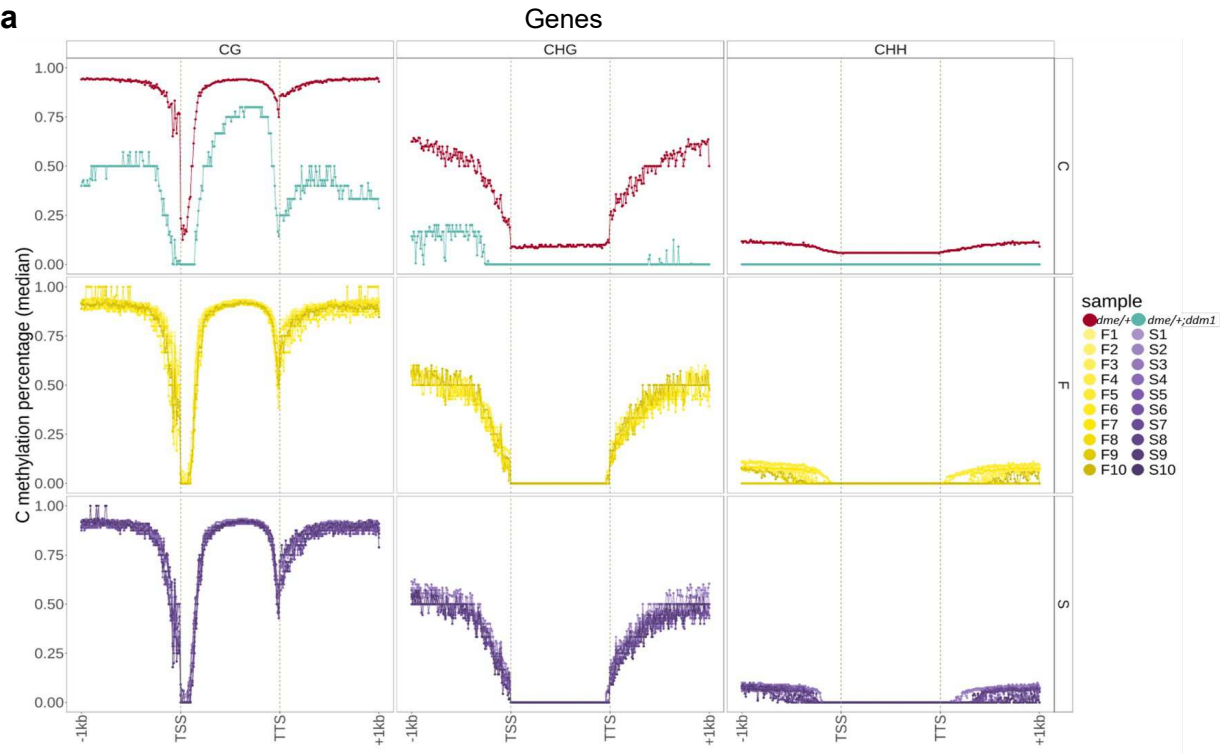


Supplementary Figure 8: Effect of *ddm1* mutation on the number of differentially expressed features in demethylases mutants. Bar plot representing the number of differentially expressed annotation classes (genes, TEs) in the respective comparisons as indicated below the graphs. Down-regulated annotations are shown on the left side, up-regulated annotations on the right side.

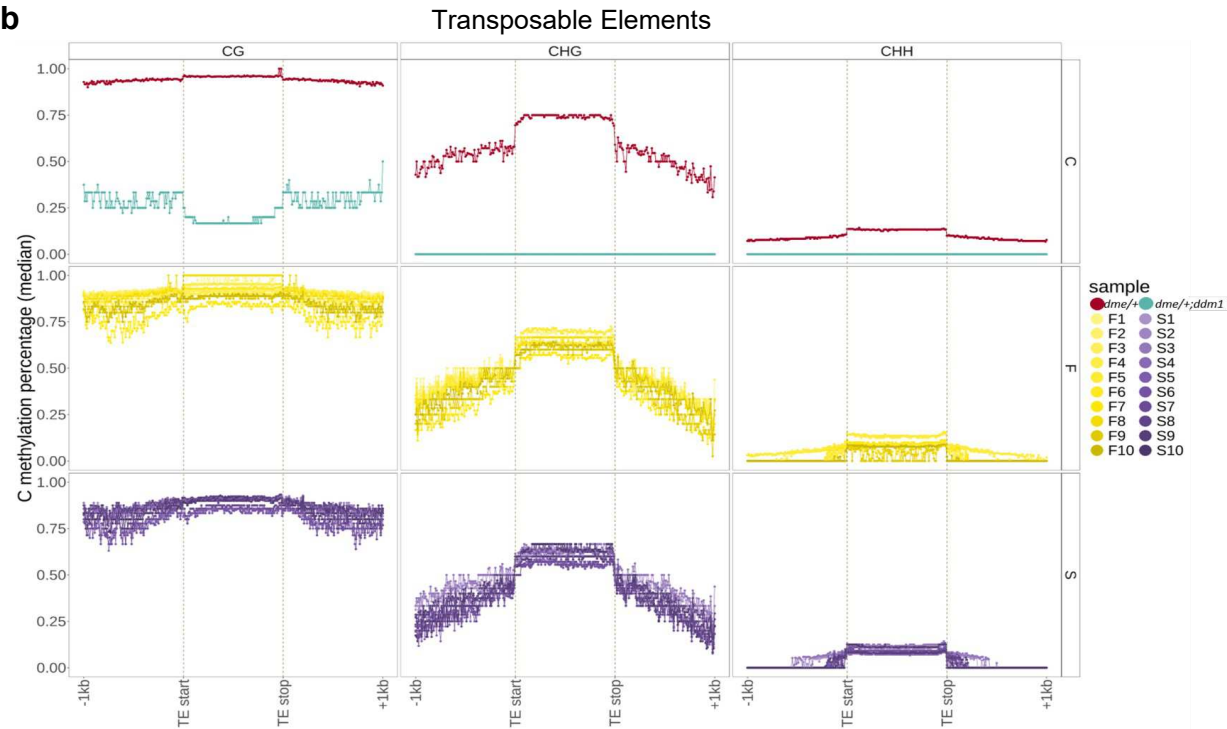


Supplementary Figure 9: Principal component analysis and Pearson correlation of RNA-seq data generated from mutant pollen of *dme*, *ros1*, *dme ros1* and their combination with *ddm1*. Numbers indicate the replicate. Left: Principal component plot visualizing maximum differences between transcriptome samples and their replicates. Component 1 (PC1) accounts for 86% and PC2 for 5% variance between the samples. Color code is assigned to respective genotype with- (darker colored) and without *ddm1* (lighter colored) as *ddm1*. Right: Pearson correlation heat-map of RNA-seq data relating each sample with each other. A value of 1 means no variables between samples; the smaller the value the more variable are the samples.

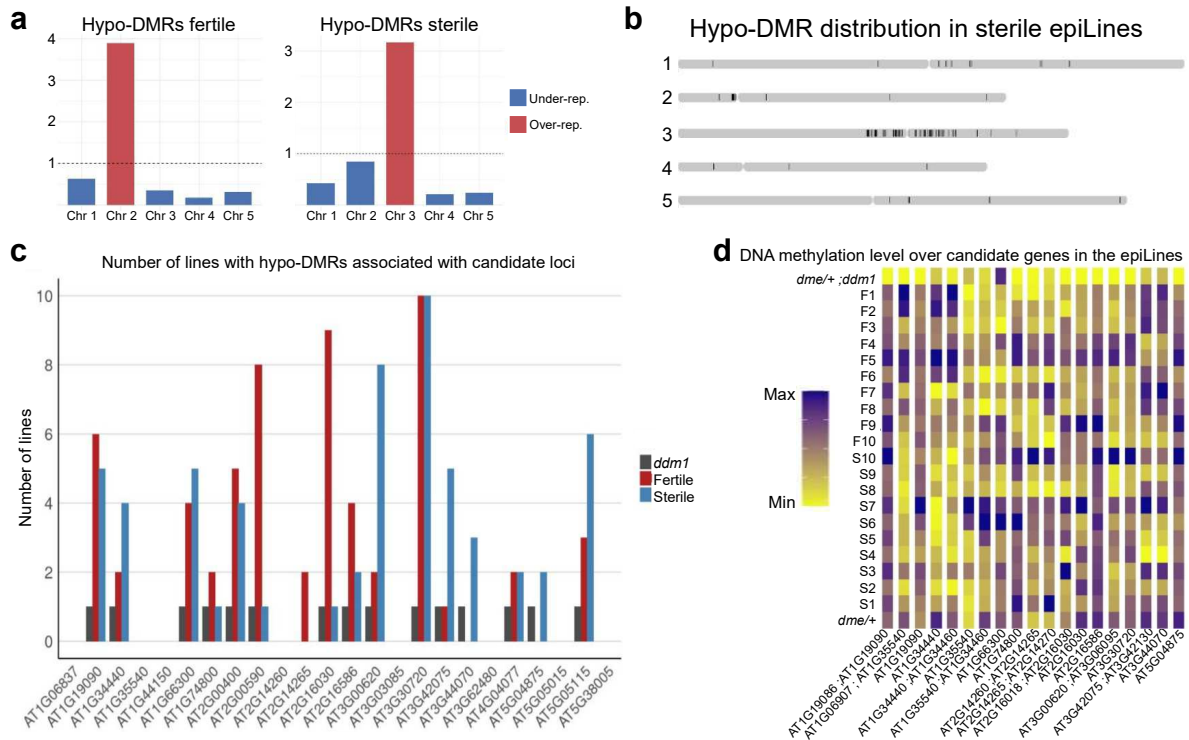
1174 **a**



1175 **b**

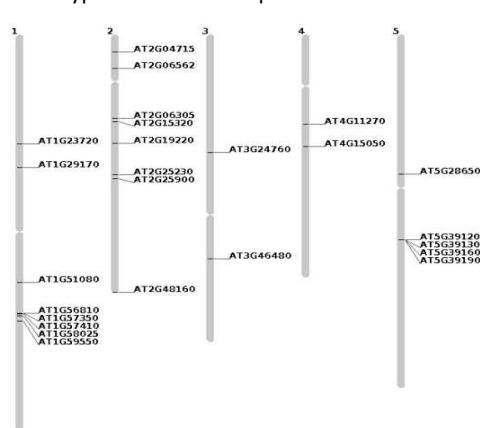


1176 **Supplementary Figure 10: *dme*/+ fertility increase is not the result of a global hypomethylation. a**
1177 **Metagenome analysis showing median DNA methylation levels in the three sequence contexts for the two controls**
1178 **(*dme*/+ and *dme*/+ *ddm1*) and for fertile and sterile lines. Profiles include 1 kbp upstream of genes, and 1 kbp**
1179 **downstream regions. b Meta-TE analysis showing median DNA methylation levels in the three sequence**
1180 **contexts for the two controls (*dme*/+ and *dme*/+ *ddm1*) and for fertile and sterile lines. Profiles include 1 kb**
1181 **upstream of TE, and 1 kb downstream regions.**

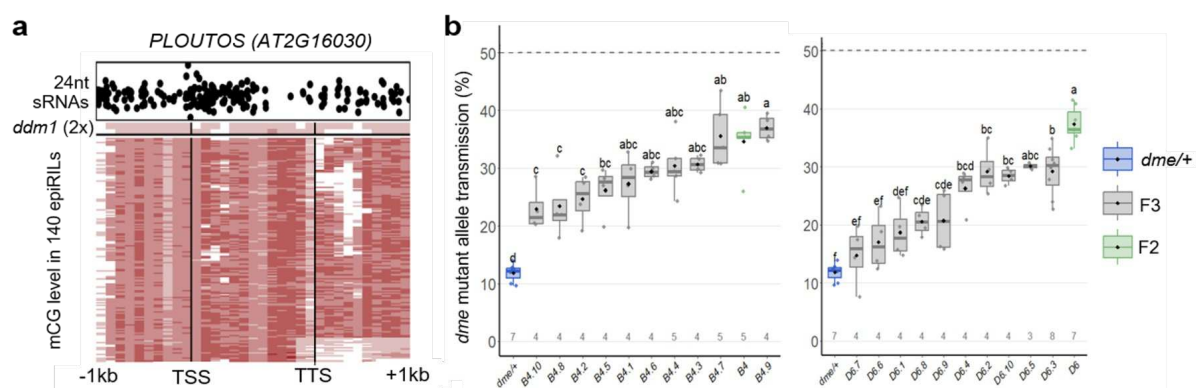


Supplementary Figure 11: Distribution of hypomethylated DMRs in fertile and sterile epiLines. **a** Chromosomal distribution of hypomethylated DMRs identified in all fertile and sterile epiLines across the five *Arabidopsis thaliana* chromosomes compared to the expected distribution based on chromosome size. Bars represent observed-to-expected ratio of DMRs per chromosome. Values above 1 indicate enrichment (red), whereas values below 1 indicate depletion (blue). **b** Chromosomal distribution of hypo-DMRs present in all sterile epiLines. **c** Number of hypomethylated lines per candidate gene in fertile and sterile epiLines. **d** Heatmap of DNA methylation levels in the three contexts in demethylation target regions of candidate genes across samples. For each annotation, methylation values were rescaled between 0 and 1, where 0 represents the minimum and 1 the maximum methylation observed for that annotation. Lines were ordered according to their fertility, from the most fertile (F1) to the most sterile (S1).

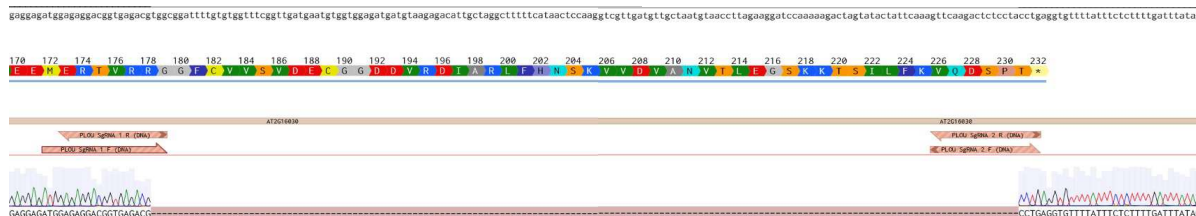
Hyper DMRs fertile epiLines



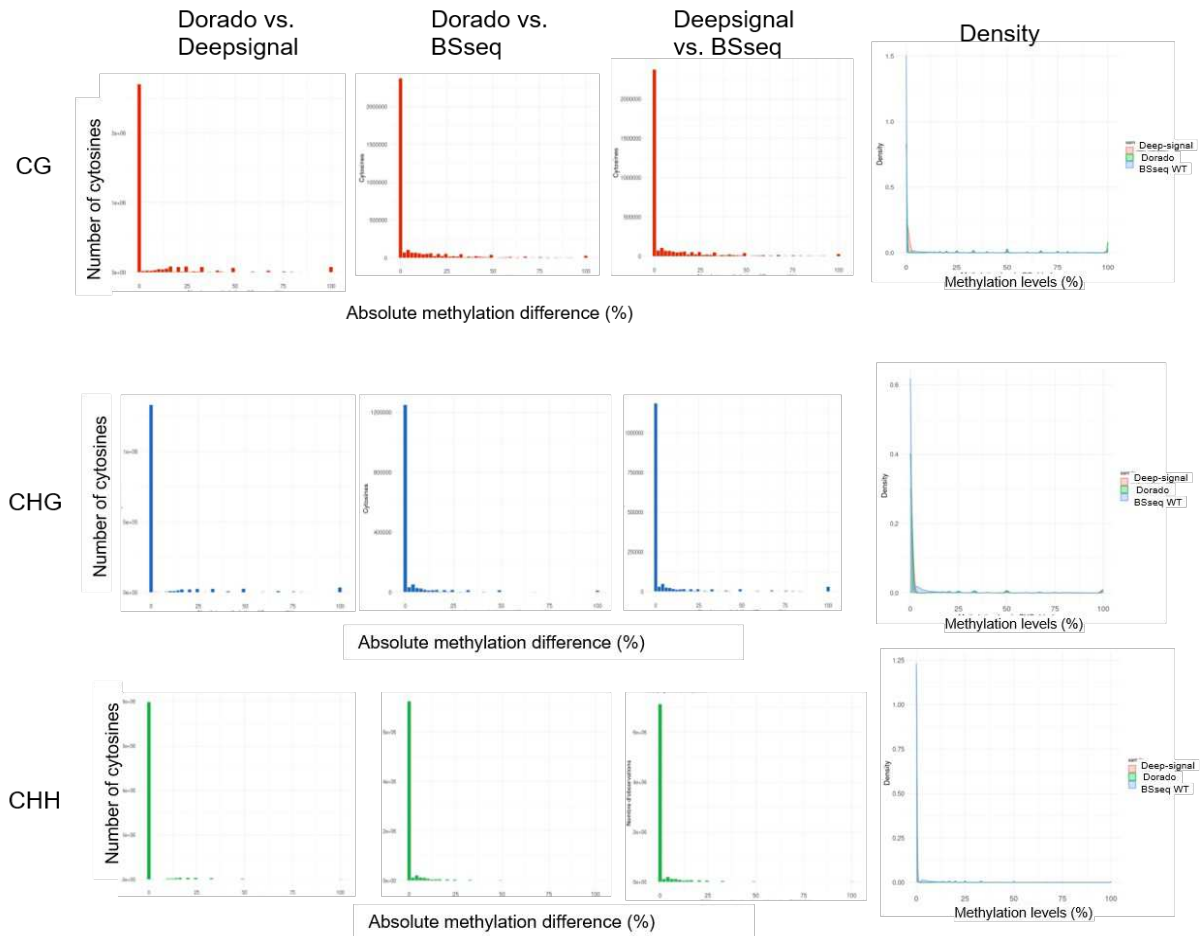
Supplemental Figure 12: Distribution of hypermethylated DMRs in fertile and sterile epiLines along chromosomes. Localisation of annotations associated with hyper-DMRs commonly detected within fertile (left) and sterile (right) epiLines vs. the control line (*dme/+*), respectively.



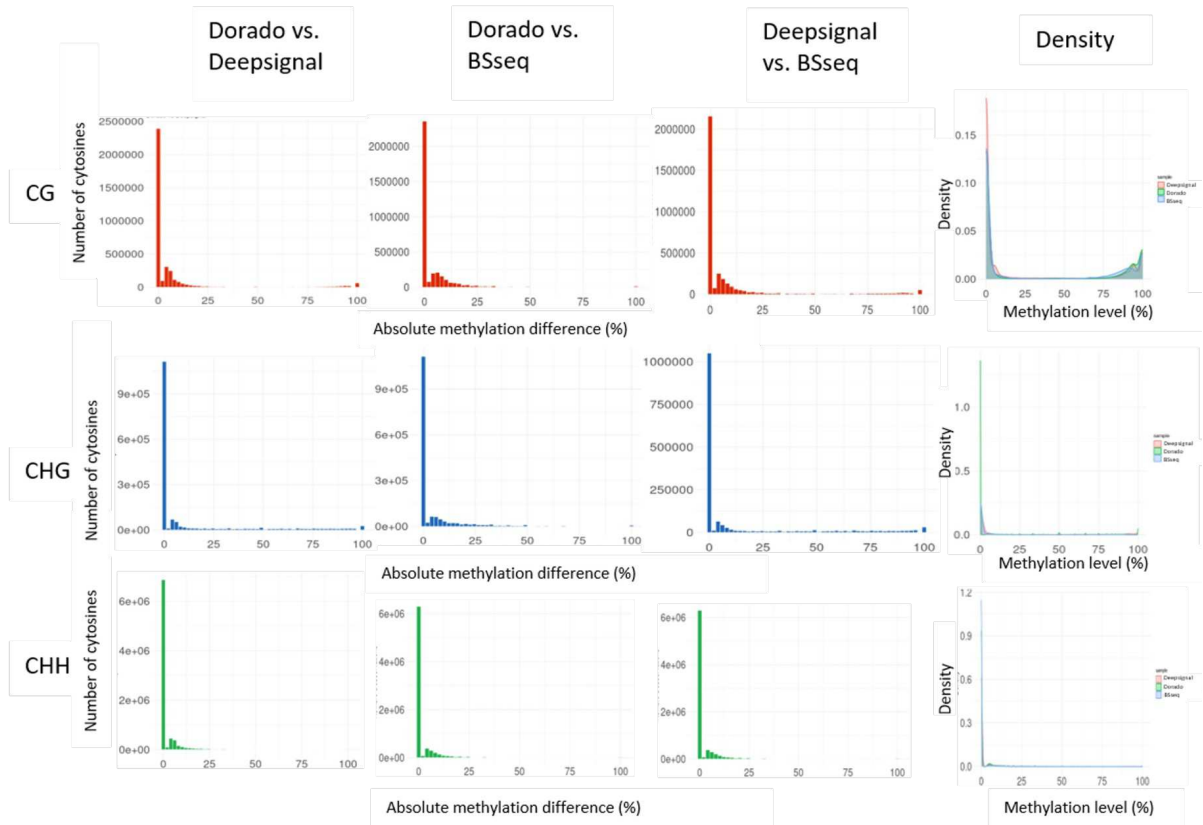
Supplementary Figure 13: Remethylation of over *PLOU* in the EpiRILs and reduced fertility restoration in the successive generation of two epiLines. **a** CG-methylation heatmap and presence of 24nt small RNAs (sRNAs) over the *PLOU* locus in the F8 generation of 140 individual epiRILs (Zhang, Nature Comm 2021) as well as two *ddm1* samples (upper rows). Darker colour refers to higher methylation level in the respective 100bp bins. Dots represent sRNAs matching DNA sequence. **b** Transmission frequency of the *dme* mutant allele in F2 individuals B4 and D6 (shown in green) and their respective F3 progeny derived from the epiLine population. Pollen fertility in the F3 generation never exceeds that of F2 and instead shows a progressive decline. Boxplots show the median (central line) and the interquartile range (box limits), with whiskers representing the data distribution. Diamonds indicate the mean transmission rate. Each dot represents an individual biological replicate. The number of biological replicates is indicated below each line. Statistical analysis was performed using a one-way ANOVA followed by Tukey's HSD test ($\alpha = 0.01$). The dashed horizontal line indicates the expected Mendelian transmission rate (50%).



Supplementary Figure 14: CRISPR/Cas9 deletion of 179 bp in the unique exon of *PLOU*. DNA sequence browser screenshot of Sanger-sequenced chromatogram from PCR-amplicons covering the *PLOU* coding region from a homozygous CRISPR/Cas9-generated *plou* mutant line. Guide sgRNA position over the sequence is indicated as arrows.



Supplementary Figure 15: Comparison of methylation levels detected in *dme/+* ONT data by Dorado and DeepSignal and compared to WGBS data in Col-0 WT four-day-old seedlings (GSE85975). Histograms display the number of cytosines based on the absolute methylation difference detected for each cytosines between Dorado and DeepSignal, Dorado and BSseq, and DeepSignal and BSseq data in each context. Density plots (right) compares the distribution of methylation levels detected with the two tools and in BSseq data.



Supplementary Figure 16: Comparison of methylation levels detected in *ddm1*-67 ONT data by Dorado and DeepSignal and compared to BSseq data in *ddm1*-2 vegetative tissues (GSE62206). Histograms display the number of cytosines based on the absolute methylation difference detected for each cytosines between Dorado and DeepSignal, Dorado and BSseq, and DeepSignal and BSseq data in each context. Density plots (right) compares the distribution of methylation levels detected with the two tools and in BSseq data.