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Title: The roles of Conserved Domains in DEMETER-Mediated Active DNA Demethylation *in planta*

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Abstract

DNA methylation plays critical roles in maintaining genome stability, genomic imprinting, transposon silencing, and development. In Arabidopsis genomic imprinting is established in the central cell by DEMETER (DME)-mediated active DNA demethylation, and is essential for seed viability. DME is a large polypeptide with multiple poorly characterized conserved domains. Here we show that the C-terminal enzymatic core of DME is sufficient to complement *dme* associated developmental defects. When targeted by a native DME promoter, nuclear-localized DME C-terminal region rescues *dme* seed abortion and pollen germination defects, and ameliorates CG hypermethylation phenotype in *dme-2* endosperm. Furthermore, targeted expression of the DME N-terminal region in wild-type central cell induces *dme*-like seed abortion phenotype. Our results support a bipartite organization for DME protein, and suggest that the N-terminal region might have regulatory function such as assisting in DNA binding and enhancing the processivity of active DNA demethylation in heterochromatin targets.

Double fertilization during sexual reproduction in flowering-plants is a unique process that underlies the distinctive epigenetic reprogramming of plant gene imprinting. In the ovule, a haploid megaspore undergoes three rounds of mitoses to produce a 7-celled, 8 nuclei embryo sac that consists of egg, central, and accessory cells [1]. During fertilization pollen grain elongates and delivers two sperm nuclei to the female gametophyte to fertilize the egg cell and the central cell, respectively. The fertilized egg cell forms the embryo that marks the beginning of the subsequent generation. Fertilization of the central cell initiates the development of endosperm that accumulates starch, lipids, and storage proteins and serves as a nutrient reservoir for the developing embryo [2, 3]. Endosperm is the major tissue where gene imprinting takes place in plant. Genomic imprinting is the differential expression of the two parental alleles of a gene depending on their parent-of-origin, and is an example of inheritance of differential epigenetic states. In Arabidopsis, MET1-mediated DNA methylation and DME demethylation are two modes of epigenetic regulation critical for imprinted expression of many genes [4, 5, 6, 7, 8]. For example, DEMETER (DME) is required for the expression of *MEA*, *FIS2*, and *FWA* in the central cell and in the endosperm while MET1 is responsible for the silencing of *FIS2* and *FWA* paternal alleles [4, 7]. Gene imprinting is essential for reproduction in Arabidopsis, and seeds that inherit a maternal *dme* allele abort due to failure to activate *MEA* and *FIS2*, essential components of the endosperm PRC2 complex required for seed viability, in the central cell [4, 9].

DME encodes a bifunctional 5mC DNA glycosylase/lyase required for active DNA demethylation in the central cell and the establishment of endosperm gene imprinting in Arabidopsis [5]. Additionally, paralogs of DME, REPRESSOR OF SILENCING 1 (ROS1), DML2, and DML3 are required to counteract the spread of DNA methylation mediated by the RNA-directed DNA methylation (RdDM) machinery into nearby coding genes [10, 11]. The three regions

in the C-terminal half of DME protein (the A, Glycosylase, and the B regions, or as the AGB region hereafter) are conserved among the DME/ROS1 DNA glycosylase clade, and are required for DME 5mC excision activity *in vitro*. Thus, the AGB region comprise the minimal catalytic core for the enzymatic function, catalyzing direct excision of 5mC from DNA and initiating active DNA demethylation that influences transcription of nearby genes [5, 9, 12].

In Arabidopsis, DME-mediated DNA demethylation is preferentially targeted to small, AT-rich, and nucleosome-poor euchromatic transposons that flank coding genes [13]. Consequently, demethylation in the central cell influences expression of adjacent genes only in the maternal genome, and is a primary mechanism of gene imprinting in plant [5, 13, 14, 15]. In addition to small TEs near coding sequences, DME also targets gene-poor heterochromatin regions for demethylation [13]. The mechanism of DME recruitment to its target sites is not known. Studies in ROS1 have uncovered several players required in the ROS1 demethylation pathway [16, 17, 18]. Among them *IDM1* encodes a novel histone acetylase that preferentially acetylates H3K18 and H3K23 *in vitro*, and ROS1 target loci are enriched for H3K18 and K23 acetylation *in vivo* in an IDM1-dependent manner [19]. Thus, IDM1 marks ROS1 target sites by acetylating histone H3 to create a permissible chromatin environment for ROS1 function. The Arabidopsis SSRP1 (STRUCTURE SPECIFIC RECOGNITION PROTEIN1), a component of the FACT (facilitates chromatin transcription/transaction) histone chaperone complex, has been shown to regulate DNA demethylation and gene imprinting in Arabidopsis [20]. Linker histone H1 functions in chromatin folding and gene regulation [21, 22, 23, 24], and was shown to interact with DME in a yeast two-hybrid screen and in an *in vitro* pull-down assay [25]. Loss-of-function mutations in *H1* genes affect the imprinted expression of *MEA* and *FWA* in Arabidopsis endosperm, and impair demethylation of their maternal alleles, suggesting that H1 might participate in the DME

demethylation process by interaction with DME [25].

Computational analysis showed that the DME/ROS1 like DNA glycosylases contain a core with multiple conserved globular domains, and except for the well-characterized glycosylase domain, very little is known about the function of the other domains. Here we show that the C-terminal region of DME necessary for 5-methylcytosine excision activity *in vitro* is sufficient to complement *dme* seed abortion and pollen germination defect, and partially rescue DNA hypermethylation phenotype in endosperm. We present evidence that the region N-terminal to the glycosylase domain can affect endogenous DME activity in a dominant negative manner when ectopically expressed in the nuclei of wild-type central cells. We propose a bipartite structural and functional organization model for the DME/ROS1 family of DNA glycosylases consisting the modular C-terminal AGB region that can substitute for DME's developmental function and the NTD region that might have regulatory functions such as assisting DNA binding and enhancing the processivity of demethylation in heavily methylated genomic regions.

Results

The DME catalytic core region is sufficient to complement *dme* associated developmental defects. Previous studies have revealed that the C-terminal half of DME comprising the three conserved A, Glycosylase, and B regions (the AGB region, as shown in Supplementary Fig. 1a) are required for *in vitro* 5mC excision activity [5], and deletion of the non-conserved linker between domain A and the glycosylase domain (interdomain 1; ID1) does not affect DME *in vitro* enzymatic activity [26, 27]. Thus, the AGB region is thought to be the core catalytic region for DME *in vitro* enzymatic activity. However, it is unknown whether the AGB region alone is

sufficient for DME function *in vivo*. To determine if the AGB region is functional *in vivo*, we tested if expressing the AGB region in the central cell can complement *dme* seed abortion phenotype. A transgene carrying a 3.1-kb *DME* cDNA that encodes the C-terminal half of DME (DME^{CTD}, residue 936-1987) under the control of a native DME promoter was introduced into *DME/dme-2* heterozygous plants by using the floral dipping method [28]. Since DME^{CTD} lacks a nuclear localization signal (data not shown), a classical SV40 nuclear localization signal (PKKKPKV) was introduced in front of the C-terminal fragment (designated as $nDME^{CTD}$, see Supplementary Fig. 1b) to ensure proper nuclear localization. We obtained multiple independent transgenic lines and assessed the transgene's ability to complement *dme-2* seed abortion phenotype.

The self-pollinated *DME/dme-2* plants produce 50% of normal seed that inherited wild type DME maternal allele, and the other 50% of aborted seed that inherited mutant *dme-2* maternal allele. In self-pollinated transgenic plants that carry a single locus of $nDME^{CTD}$ or DME^{FL} (full length DME.2 cDNA, major isoform of DME [29]) transgenes, we observed about 25% aborted seeds among independent transgenic lines, indicating that $nDME^{CTD}$ and DME^{FL} complement *dme* seed abortion phenotype (Fig. 1a, b, Supplementary Table 1). In addition, we also transformed $nDME^{CTD}$ and DME^{FL} into *dme-2/dme-2* homozygous plants (see Materials and Methods for isolation and characterization of *dme-2/dme-2* homozygous lines in Col-*gl*), both constructs produced T1 transgenic plants that displayed expected 50% seed abortion rate (Fig. 1b, Supplementary Table 1). Seed abortion caused by *dme* mutations is in part due to defects in activating imprinted PRC2 subunit genes required for endosperm development [5, 9, 30, 31, 32]. We use qRT-PCR to check if $nDME^{CTD}$ also restores DME target genes expression in the central cell. Indeed, *FIS2* and *FWA* expression is restored in the complemented lines (Fig. 1c). Thus

129 nDME^{CTD} can substitute for the endogenous DME activity for seed viability, and active DME
130 target genes expression.

131 In addition to maternal effects on seed viability [9], mutations in DME also affect pollen
132 function in Col-0. When *DME/dme-2* heterozygous plants are self-pollinated, only about 20-30%
133 of the viable F1 progeny are heterozygous (Supplementary Table 2), due to decreased *dme* pollen
134 germination rate [33]. To test whether nDME^{CTD} can rescue *dme* pollen phenotype, we pollinated
135 wild type Col-0 with pollen derived from transgenic lines that are homozygous for the *dme-2*
136 allele and carry a single locus of the *nDME^{CTD}* transgene (*dme-2/dme-2; nDME^{CTD}/~*). If
137 nDME^{CTD} does not complement *dme-2* pollen germination defects, we expect roughly half of the
138 F1 progeny will carry the nDME^{CTD} transgene (hygromycin resistant) because mutant pollen
139 with or without the transgene would germinate with equal frequency. Instead, we observed 65% -
140 90% of the F1 progeny are hygromycin resistant (Table 1), indicating that nDME^{CTD}
141 complements *dme-2* pollen germination defect. These results show that expressing the C-
142 terminal half of DME protein in the nucleus is sufficient to rescue *dme* visible phenotypes *in*
143 *planta*.
144
145 **nDME^{CTD} partially rescue *dme-2* CG hypermethylation phenotype in the endosperm.** In
146 Arabidopsis seed viability depends on the DME activity in the central cell to activate the
147 MEDEA/FIS2/MSI1/FIE PRC2 complex required for endosperm development. In addition,
148 DME is required to demethylate multiple maternally (*MEGs*) or paternally expressed imprinted
149 genes (*PEGs*) to establish their parent-of-origin specific expression patterns in the endosperm [13,
150 15]. Thus, in *dme* mutant endosperm, discrete genomic loci targeted by DME for demethylation
151 are hypermethylated [13]. Since nDME^{CTD} complements *dme* seed abortion, and activates DME

target gene expression (Fig. 1), we assumed it does so by demethylating the central cell genome and activating PRC2 genes essential for seed development. To test this hypothesis, and to examine the extend of nDME^{CTD} demethylation activity *in vivo*, we manually isolated *nDME^{CTD}*-complemented endosperm (*dme-2/dme-2;nDME^{CTD}/nDME^{CTD}*), determined the DNA methylation profile by whole genome bisulfite sequencing, and compared the complemented methylomes to those of wild-type and *dme-2* endosperm. Methylomes from three independent lines were generated and compared with that of *dme-2* endosperm. We observed although the differentially methylated regions (DMRs) between each independent lines do not completely overlap, the DMRs unique to each line are also demethylated in other lines (Supplementary Fig. 2, 3), suggesting that the number of overlapped DMRs was underestimated due to the cutoff used in defining the DMRs, similar to what's observed in a recent study [34]. We therefore used the combined reads from three independent lines for the subsequent analyses so that all comparisons are confined to the same cutoff criteria (see Materials and Methods). As expected, several DME regulated *MEGs* and *PEGs* are demethylated compared to *dme-2* endosperm, indicating that nDME^{CTD} is correctly recruited to these loci for demethylation (Fig. 2a). We focused our analysis on previously determined differentially methylated sites between *dme-2* and wild-type endosperm (*dme* hyper-DMRs, the DME canonical targets) [13, 15]. Overall, the CG methylation levels in these canonical DME target sites are reduced in the complemented endosperm, indicating that nDME^{CTD} is directed to these endogenous DME target sites for demethylation. However, compared to wt endosperm, these *dme* hyper-DMRs are demethylated to a lesser degree by the nDME^{CTD} (Fig. 2b). Thus nDME^{CTD} only partially rescues the *dme* CG hypermethylation phenotype in the endosperm. The DMRs of *dme* relative to wild-type endosperm or to *nDME^{CTD}*-complemented endosperm partially overlap (Supplemental Fig. 4).

175 However, among the DMRs unique to nDME^{CTD}, we also observed decreased CG methylation in
176 WT endosperm compared to *dme*, indicating that they are also demethylated by the endogenous
177 DME. Similarly, among the DMRs unique to wt endosperm, these regions are also demethylated
178 by the nDME^{CTD}. Thus nDME^{CTD} appears to partially demethylate the majority of the loci
179 targeted by the endogenous DME. These observations also suggest that intact full-length DME
180 protein is required for robust and complete demethylation *in vivo*.

181 We next examined the methylome of *dme-2* endosperm complemented by the full length
182 *DME.2 cDNA* (designated as *DME^{FL}*). Unexpectedly, based on the number of DMRs between
183 *dme* and *DME^{FL}*-complemented endosperm and the level of CG methylation within the DMRs
184 (Fig. 2c), DME^{FL} appears to be less active compared to endogenous DME, or to nDME^{CTD}, albeit
185 it being able to complement *dme* seed abortion (Fig. 1b) [9, 35]. Since the *DME^{FL}* transgene only
186 differs from *nDME^{CTD}* by the N-terminal region, reduced activity of DME^{FL} compared to
187 DME^{CTD} cannot be attributed to the lack of introns or 3' flanking sequences that might be needed
188 for robust DME protein production. Indeed, we found both transgenes are expressed at
189 comparable levels in *DME^{FL}*- and *nDME^{CTD}*-complemented lines used in the methylome study
190 (Supplemental Fig. 5), indicating lower activity of DME^{FL} compared to nDME^{CTD} is not due to
191 their differential transcript abundance. Nevertheless, comparison of CG methylation levels in
192 DMR regions unique to DME^{FL}, nDME^{CTD}, or endogenous DME also reveals that unique DMR
193 regions are more or less hypomethylated in WT or in complemented endosperm relative to *dme*
194 endosperm. Thus the methylome difference between wt, *DME^{FL}*-, and *nDME^{CTD}*-complemented
195 endosperm appears to be more in the degree of demethylation, rather than in targeting specificity.
196

Function of the N-terminal region in DME-mediated active DNA demethylation. The *dme-2* allele is caused by an activation-tagging T-DNA insertion in the middle of the A region (Supplementary Fig. 1a)[9]. We found that in floral buds of *dme-2/dme-2* plants, the endogenous *DME* transcripts downstream of T-DNA insertion site is greatly reduced compared to wild-type Col-0 plants, but the level of DME transcripts upstream of the T-DNA insertion site is relatively high (Supplementary Fig. 6). We suspected these transcripts could produce truncated form of DME proteins that might interfere with the DME^{FL} transgene activity. To test this hypothesis, we transformed wild-type Col-0 plants with an engineered GFP-tagged DME NTD (using the genomic DNA sequence upstream of T-DNA insertion site, encoding residues 1-1022, designated as *DME^{NTD}-GFP*) transgene mimicking the *dme-2* T-DNA insertion (Supplementary Fig. 1B). Clear GFP signals are observed in the central cell nuclei of transgenic lines (data not shown). We also observed about one third of transgenic lines showing apparent *dme-2* like seed abortion phenotype, with abortion rates ranging from 10% to ~ 40% (Supplementary Table 3, 4) in the T1 plants, suggesting that expression of DME^{NTD} has a dominant negative effect on endogenous DME protein.

To minimize the possibility and the degree of transgene induced sense co-suppression, we reverse translated DME^{NTD} protein sequence into cDNA sequence using the human codon usage table. As a result, the re-engineered "humanized" version of NTD ($mDME^{NTD}$) codes for the identical protein sequence but with no significant nucleotide sequence similarity to the original cDNA sequence to induce co-suppression (Supplementary Table 5). In addition, a GFP tag was added to the C-terminus ($mDME^{NTD}$-GFP) to monitor its expression (Fig. 3a). We generated 28 independent transgenic lines, and among them 16 lines showed seed abortion rate of 5% - 52% (Supplementary Table 3, 6). The aborted seeds resemble *dme* mutant seeds with abnormal

endosperm, arrested embryo, and shriveled brown seeds (Fig. 3b, c). We selected four lines with high, medium, or no seed abortion rate (Fig. 3d), and assessed the endogenous DME transcript abundance. As shown in Fig. 3e, among lines with different seed abortion rate, the endogenous DME mRNA abundance is similar to that of the vector control line, indicating the severity of seed abortion phenotype is not due to interference of endogenous *DME* transcripts. Furthermore, the rate of seed abortion is positively correlated with the levels of $mDME^{NTD}$-*GFP* mRNA (Fig. 3f), suggesting the degree of seed abortion is likely due to the levels of transgene expression. We next tested whether expression of $nDME^{CTD}$ or DME^{FL} in WT Col-0 can also induce seed abortion phenotype. For each construct, more than 25 independent transgenic lines were examined and none resulted in any seed abortion phenotype (Supplementary Table 3). Thus the dominant negative effect appears to be specific to the DME NTD region.

Evolutionary history and late acquisition of the N-terminal region of DME-like proteins.

We show the C-terminal half of DME is sufficient to complement *dme* mutant developmental phenotypes, and can be recruited to most of the DME target loci. Thus the DME^{CTD} most likely contains intrinsic targeting information. To gain insights from the evolution of the conserved domains in DME, we conducted sequence searches of the NR database with various homologs as query. The core of the DME-like proteins, as previously reported [36], comprises the catalytic glycosylase domain of the HhH (helix-hairpin-helix) modules followed by the FCL ([Fe4S4] cluster loop) motif and a divergent version of an RRM (RNA Recognition Motif) fold domain (Fig. 4). The DNA glycoslase and FCL domains span the A and G regions, whereas the RRM fold domain corresponds to the B region of angiosperm DME homologs. A diversity of domains associate with the basic DME core can be found across various clades. Land plants and

243 charophytes (Streptophyta) possess a permuted divergent version of the umethylated CpG
244 recognizing CXXC domain (containing only one of two structural repeats of the classical CXXC
245 domain) between the FCL and RRM domains. By contrast, one or more copies of the CXXC
246 domain can be found in chlorophyte and stramenopile algae at distinct positions. Some algal
247 DME homologs (from Chlorophyte and stramenopile) also possess other chromatin-modification
248 reader (Tudor and PHD domains), DNA binding (AT-hook motif), and the DnaJ domain which
249 interacts with the chaperone Hsp70 [36, 37]. These accessory domains suggest a potential role for
250 regulating the associated DNA glycosylase activity according to the DNA methylation (via
251 CXXC) or chromatin status (via PHD, Tudor) of the cell in which they are expressed.

252 The N-terminal half of the DME consists of a large portion of unstructured, low complexity
253 sequences (residues 346-947), a stretch of basic amino acid-rich direct repeats (residues 291-
254 345), and a 120 amino-acid N-terminal domain (DemeN) of unknown function (residues 1-
255 120)(see Supplementary Fig. 7 for sequence alignment). The DemeN domain and charged
256 repeats are restricted to the angiosperm lineage and appears to be a late acquisition during land
257 plant evolution.

258 In summary, the evolutionary history of the DME domains can be summarized as follows:
259 bacterial versions of the HhH-FCL pair from a cyanobacterial source fused to an RRM-fold
260 domain and further acquired an insert in the glycosylase domain to give the ancestral form in the
261 plant lineage. This was likely then transferred to the stramenopiles from a secondary chlorophyte
262 endosymbiont of this lineage. Finally, at the base of the streptophyte radiation, DME acquired a
263 permuted CXXC, and later the DemeN domain and the associated charged repeats were acquired
264 in the angiosperm lineage, possibly to facilitate and ensure a robust and thorough demethylation.
265

Discussion

We show for the first time that the core conserved region of the DME protein containing the DNA-glycosylase, FCL, divergent and permuted CXXC and divergent RRM domains is sufficient to rescue visible phenotypic defects caused by *dme* mutation. Although this truncated form of DME protein demethylates the majority of the canonical DME target sites, it does so in a less active and less efficient manner compared to the endogenous protein. We see two possibilities that might explain this lower activity and efficiency: 1) Critical cis-elements residing within introns or in 3'-end flanking sequences that are missing in the transgene might be required for robust transgene expression. 2) The N-terminal region might be required for full DME activity *in vivo*. Unfortunately, our attempt to assess the difference between DME^{FL} and $nDME^{CTD}$ was confounded by the possible interference from truncated NTD proteins due to T-DNA insertion in *dme-2* background. We suspect this might contribute to the reduced DMRs observed in DME^{FL}complemented endosperm. Therefore, we believe it is premature to draw any conclusion based on direct comparisons between DME^{FL}- and $nDME^{CTD}$-complemented endosperm methylomes (Fig. 2c).

Since the C-terminal AGB region is sufficient for DME's seed viability function in *planta*, and can be recruited to most of the canonical DME target sites, the CTD polypeptide most likely contains sufficient targeting information. *in vitro* studies of ROS1 suggest that the B region containing the CXXC and RRM domains is essential for the glycosylase and lyase activities, and might recognize modified DNA[38]. It is possible that the permuted CXXC domain is required to direct the protein to the target sites, or is involved in discriminating methylated vs un-methylated cytosines[39]. This is supported by mutation studies that implicate a potential role for this domain in DME *in vivo* function, but not in vitro enzymatic activity (Huh and Hsieh, unpublished

289 results). Similarly, the role of the enigmatic divergent RNA-recognition motif (RRM) domain is
290 also not fully understood. Mutagenesis screens for residues required for demethylation activity in
291 bacteria identified multiple amino acid residues within the RRM domain[40]. Although the
292 involvement of RNA species in the active DNA demethylation process has not been firmly
293 established, an RRM protein ROS3 required for ROS1 demethylation suggests a potential role of
294 non-coding RNAs in the active DNA demethylation pathway in Arabidopsis[41]. While it is
295 tempting to speculate a role for RNA-binding, the DME RRM might also bind single-stranded
296 DNA with methylated bases.

297 Based on the reduced demethylation activity of nDME^{CTD} on the canonical DME target sites,
298 we suspect the NTD region might be required for full and robust demethylation activity probably
299 to ensure that the imprinting network is properly activated and maintained (e.g., by subsequent
300 PRC2 activity). To achieve this, the DME NTD might function to assist the glycosylase enzyme
301 by tightly binding to DNA template for more complete and thorough demethylation. Supporting
302 such model, *in vitro* study of ROS1 activity on 5mC excision revealed that the basic repeats
303 (3DR, AT-hooks) region binds strongly to DNA template non-specifically, and removal of NTD
304 region impairs the sliding capacity of the protein on DNA template[42], and significantly reduced
305 ROS1 5mC excision activity[43]. We observed reduced degree of demethylation by nDME^{CTD}
306 regardless of target length (Supplemental Fig. 8), suggesting that NTD is needed for complete
307 demethylation in all the target sites.

308 Although DME preferentially targets smaller euchromatic transposons that flank coding
309 genes, it also targets gene-poor heterochromatin regions for demethylation [13]. The biological
310 significance of heterochromatin demethylation by DME is not known, but was speculated to
311 involve reinforcing DNA methylation in egg cell and subsequently in the embryo [13]. These

heterochromatin target sites are densely methylated, and demethylation by DME results in longer DMRs between *dme-2* and wt endosperm. Interestingly, the number of longer DMRs is significantly reduced between *dme-2* and *nDME*^{CTD}-complemented endosperm, suggesting that removal of NTD region also reduces the processivity of demethylation in long target sites (Supplemental Fig. 9a). Since heterochromatin regions are compacted, demethylation in such loci will require substantial chromatin remodeling such as eviction of nucleosomes for DME to gain access to the templates. It is tempting to speculate that the conserved motif in the DemeN domain might recruit other factor(s) via protein interaction to remodel local chromatins to permit DME demethylation. However, based on current data we cannot unequivocally ascribe NTD's function due to lack of proper full length DME transgenic comparison. Nevertheless, our results caution that peculiarity in certain genetic backgrounds (e.g., *dme-2*) might confound data interpretation. Future work on DME functional study could benefit from the generation of a clean loss-of-function background such as deleting the entire DME locus using CRISPR-assisted genome editing techniques.

We envision a possible model where the AGB region is sufficient for directing DME to target loci while NTD region is required for interacting with local chromatin environment, stabilizing binding to chromosomal templates, and assisting demethylating flanking sequences. In the absence of NTD, nDME^{CTD} can still demethylate majority of target sites, but in a less-efficient manner, likely due to the lack of non-specific DNA-binding by the basic AT-hook motifs. We surveyed wt DMRs that are longer than 1.5 kb, and found that these regions are also nDME^{CTD}'s DMRs, but are shorter in length (Supplemental Fig. 9b), possibly due to missing the DemeN domain. If NTD is needed for longer and more robust demethylation, why ectopic expression of NTD causes dominant negative (DN) effects on endogenous protein? Classical examples of

dominant negative mutation often involve protein-protein interactions that are disrupted by mutated or truncated form of one particular partner or subunit. Although we do not have any evidence to suggest DME might homodimerize to become active, any weak physical interaction caused by ectopic NTD expression might induce conformational change that renders DME non-functional. Unfortunately our attempt to assess whether the NTD of DME can interact with each other was confounded by the self-activating activity of DME.2 NTD in yeast two-hybrid assay when fused to the GAL4 DNA binding domain (data not shown). Their possible interaction will need to be assessed by alternative strategies. Another possibility is that NTD binds and titrates out an important interacting partner required to activate DME through conformational change (allosteric interaction). By removing NTD, the AGB region is liberated from such conformational constrain and can demethylate its target sites. It is also possible that the non-specific DNA binding activity of NTD competes with DME for target sites, thereby reducing the overall efficiency of DME. The molecular underpinning of how NTD induces DN effect remains to be elucidated. From an evolutionary viewpoint, the use of an active DME-based demethylation appears to have been acquired early in the plant lineage. The presence of several accessory domains in addition to the conserved core suggests adjustments to the chromatin and methylation environment of the different species. The presence of additional domains such as the DemeN and basic repeats in angiosperms and the permuted CXXC domain in streptophyta lineage might reflect the adjustment to the unique methylation and chromatin environment of the larger Streptophyta and land plant genomes.

Materials and Methods

Molecular Cloning of Constructs Used in this Study.

All general molecular manipulations followed standard procedures (Sambrook et al. 1989). Q5 High Fidelity DNA polymerase (NEB, Ipswich MA, USA) was used for PCR amplifications. PCR products were purified using AMPure XP beads (Beckman Coulter, Indianapolis IN, USA). The sequences of all plasmid constructs were confirmed by sequencing (Eton, Research Triangle Park NC, USA). All PCR primers and double-stranded DNA fragments were synthesized by Integrated DNA Technologies (Coralville IA, USA), and sequences are listed in Supplementary File 1.

A binary plasmid vector, pFGAMh, was modified to facilitate the generation of plasmid constructs using the Gibson assembly method. In brief, the replication origins and T-DNA borders originated from pFGC5941 (GenBank Accession: AY310901). A hygromycin resistance gene (HPTII) under the control of the mannopine synthase promoter was installed for selection of transgenic seedlings. A Gateway attR cassette (rfa, Invitrogen, Carlsbad CA, USA), flanked with unique restriction sites XhoI and XbaI-SpeI was placed upstream octopine synthase polyadenylation signal (OCS3'). Plasmid pFGAMh, digested with restriction enzymes XhoI and XbaI, was used to generate plasmids pDME:DME^{CTD}, pDME:nDME^{CTD} and pDME:GFP::DME^{CTD} using the Gibson assembly method. The DME.2 upstream regulatory sequence (DMEpro; 2895 bp upstream of DME.2 translation start codon ATG) was PCR-amplified using primer pair VeDME/P3R and Col-0 gDNA as template. The coding sequence of linker-AGB (with a 6-Ala linker to its N-terminus; 3174 bp), was PCR-amplified using primer pair lnAGBF/CTDVeR and Col-0 cDNA as template. To bridge these two fragments (DMEpro and linker-AGB), one of the following three DNA fragments was used in the assembly reactions. For pDME:DME^{CTD}, a 50-bp fragment was generated by annealing DNA oligos ATGF and ATGR. For pDME:SV40NLS::AGB, a 71-bp fragment was generated by annealing DNA oligos

S40F and S40R followed by two rounds of PCR reactions. For pDME:GFP::DME^{CTD}, a 761-bp fragment was PCR-amplified using primer pair p3GFPF/dmGFPR and plasmid DNA pGFP-JS (Jen Sheen,Massachusetts GeneralHospital, Boston MA, USA) as template.

An intermediate plasmid vector, DME-P3-attR-AGB, was generated by digesting plasmid pDME:SV40NLS::AGB with restriction enzymes AflII and NcoI, and re-assembled with a 2800-bp fragment, which was produced through overlap PCR with 3 primer pairs, upAflII/P3attR, P3attF/attAGBR and attAGBF/dnNcoI, and Col-0 gDNA, attR cassette and Col-0 cDNA as templates. The resulting plasmid DME-P3-attR-AGB bears (1) the same 2895-bp regulatory sequence as the above constructs, (2) an attR cassette flanked by unique restrict sites XbaI and BglII, and (3) AGB coding sequence (3156 bp). To generate pDME:DME^{FL}, plasmid DME-P3-attR-AGB was digested with XbaI and BglII, and assembled with a 2985-bp sequence, which was generated through overlap PCR using primer pairs S1-5e/IN3R and IN3F/S1-5R, and Col-0 gDNA as template. The resulting plasmid pDME:DME^{FL} carries the complete DME.2 coding sequence and intron 2 sequence (6075 bp) immediately downstream of the 2895-bp regulatory sequence with no additional sequences.

The intermediate plasmid vector DME-P3-attR-AGB was digested with restriction enzymes BglII and SpeI (to completely remove the AGB coding sequence), and re-assembled with a 786-bp sequence, which included the coding sequence of GFP (with its start codon ATG changed to TTG) and was PCR-amplified using primers ttGFPF and SpeGFPR and plasmid DNA pGFP-JS as template. The resulting plasmid DME-P3-attR-GFP was used as an intermediate plasmid vector to generate constructs pDME:DME^{NTD}::GFP and pDME:mDME^{NTD}::GFP. Plasmid DME-P3-attR-GFP was digested with XbaI and BglII, and assembled with two DNA fragments: a 3289-bp sequence was PCR-amplified using primers S1-5F and dme2tR2 and Col-0 gDNA as

template and a 158-bp synthetic DNA fragment (FragQ20) (Integrated DNA Technologies, Coralville IA, USA). The resulting construct pDME:DME^{NTD}::GFP included the 2895-bp upstream regulatory sequence, the 3332-bp sequence downstream of translation start codon ATG, the coding sequence of 6-Ala linker, and the coding sequence of GFP. Note the NTD coding sequence included the first 86 bp of intron 4 of gene DME.2, and it was designed to mimic dme-2 T-DNA insertion. To generate pDME:mDME^{NTD}::GFP, the sequence of the first 1012 amnio acid residues of DME.2 protein was converted to DNA sequence using program EMBOSS Backtranseq (http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) and the Homo sapiens codon usage table. The sequence was then analyzed using online programs SoftBerry FSPLICE (http://linux1.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind) and NetPlantGene2 (http://www.cbs.dtu.dk/services/NetPGene/), and manually edited to disrupt potential splicing donor sites or acceptor sites. The mDME^{NTD} sequence (3036 bp) and upstream- and downstream-overlapping sequence are broken into 4 fragments, and synthesized by Integrated DNA Technologies (Coralville IA, USA). The 4 DNA fragments were assembled with plasmid DME-P3-attR-GFP digested with XbaI and BglII, resulting construct pDME:mNTDh::GFP.

Whole-Genome Bisulfite Sequencing and DNA Methylome Analysis

Genomic DNA were isolated from hand dissected, 7-9 DAP *dme-2* endosperm that has been complemented by *DME^{FL}* or *nDME^{CTD}* (*dme-2*/*dme-2*;*DME^{FL}*/*DME^{FL}* or *dme-2/dme-2; nDME^{CTD} nDME^{CTD}*). Whole genome bisulfite sequencing library was constructed as described before [13, 44]. Approximately 20-50 ng of purified genomic DNA was spiked with 0.5ng of unmethylated cl857 *Sam7* Lambda DNA (Promega, Madison, WI) and sheared to about 300bp

using Covaris M220 (Covaris Inc., Woburn, Massachusetts) under the following settings: target BP, 300; peak incident power, 75 W; duty factor, 10%; cycles per burst, 200; treatment time, 90 second; sample volume 50µl. The sheared DNA was cleaned up and recovered by 1.2x AMPure XP beads then followed by end repaired and A-tailing (NEBNext Ultra II DNA Library Prep Kit for Illumina, NEB) before ligation to NEBNext methylated multiplex adapters (NEBNext Multiplex Oligos for Illumina, NEB) according to the manufacturer's instructions. Adaptor-ligated DNA was cleaned up with 1x AMPure XP beads. The purified adaptor-ligated DNA was spiked with 50ng of unmethylated cl857 *Sam7* Lambda DNA and subjected to one round of sodium bisulfite conversion using the EZ DNA Methylation-Lightning Kit (Zymo Research Corporation, Irvine, CA) as outlined in the manufacturer's instructions with 80 min of incubation time. Half of the bisulfite-converted DNA molecules was PCR amplified with the following condition: 2.5 U of ExTaq DNA polymerase (Takara), 5 ul of 10 x Extaq reaction buffer, 25 µM dNTPs, 1 ul of index Primers (10 uM) in 50 uL reaction. The thermocyling condition was as follows: 95 °C for 2 min and then 10 cycles each of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s. The enriched libraries were purified twice with 0.8x (v/v) AMPure XP beads to remove any adapter dimers. High throughput sequencing was performed by Novogene Corporation (USA). For each genotype, sequencing reads from three individual transgenic lines were combined. Sequenced reads were mapped to the TAIR10 reference genomes and DNA methylation analyses were performed as previously described (Supplementary Table 7) [13]. Fractional CG methylation in 50-bp windows across the genome was compared between *dme*, wild-type (GSE38935 [13]), DME^{FL}- $nDME^{CTD}$- complemented *dme-2* endosperm. Windows with a fractional CG methylation difference of at least 0.3 in the endosperm comparison (Fisher's exact test p-value < 0.001) were merged to generate larger differentially methylated regions (DMRs) if they occurred

within 300 bp. DMRs were retained for further analysis if the fractional CG methylation across the whole DMR was 0.3 greater in *dme* endosperm than in wild-type endosperm (Fisher's exact test p-value < 10^{-10}), and if the DMR is at least 100-bp long. The merged DMR lists are in the Supplemental File 2. The *dme* and wild-type endosperm data used in this study were derived from crossed between *Col* (female parent) and *Ler* (male parent) (GSE38935, [13]). To avoid potential ecotype-specific methylation difference, *Ler* hyper-DMRs relative to Col-0 endosperms (GSE52814, [45]) were identified using the same criteria as described above and excluded from further analyses. For making the Venn diagram, merged DMR regions were converted into 50-bp windows. Only windows with methylation scores in all samples were retained for comparison in Venn diagram and boxplot analysis.

Plant Materials and Complementation Assays

We found we can easily obtained *dme-2/dme-2* Col-*gl* plants from *DME/dme-2* heterozygotes if we rescued seeds prior to desiccation on MS sucrose plates. This is consistent with the report that *fis* endosperm cellularization defect and embryo arrest can be rescued by culturing the developing seeds in sucrose media because *fis* seeds have reduced hexose level [46]. Using this method we generated multiple homozygous lines, and we did not detect any difference between individuals in terms of normal seed rate or visible phenotype. The adult *dme-2/dme-2* plants are morphologically indistinguishable from wild-type Col-*gl* plants but produce ~0.1% viable mature seeds. These *dme-2/dme-2* plants are not due to genetic mutation or heritable aberrant epigenetic effects that escape requirement of DME activity during gametogenesis because their subsequent progeny are phenotypically normal and produces same level (~0.1%) of normal seeds.

The *DME/dme-2* heterozygous or *dme-2/dme-2* homozygous lines in Col-*gl* background were subjected to Agrobacterium-mediated floral dipping transformation procedures [28]. Seeds were sterilized by 30% bleach solution and screened for T1 transgenic plants on a 0.5x MS nutrient medium with 1% sucrose, 0.8% agar and 40 µg/ml hygromycin. Germinated seedlings were transferred to soil and grown in the growth room under 16 hours of light and 8 hours of dark cycles at 23°C. Siliques from T1 transgenic plants were dissected 14-16 days after self-pollination using a stereoscopic microscope (SteREO Discovery.V12, Carl Zeiss, Wetzlar, Germany). The numbers of viable and aborted seeds in transgenic lines were statistically analyzed with the $\chi 2$ test. The probability that deviates from a 1:1 or 3:1 segregation ratio for viable and aborted seeds was also calculated.

RNA extraction, cDNA synthesis and quantitative PCR analysis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, USA) and treated with TURBO DNase (Ambion, Austin TX, USA) according to the manufacturers' instructions. For cDNA synthesis, 5mg of total RNA was reverse-transcribed using Superscript III Reverse Transcriptase and oligo(dT) primer (Invitrogen). The cDNA was treated with RNase H (Invitrogen) at 37oC for 20min and diluted tenfold with H2O. For each 15-µl qPCR reaction, 1µl of diluted cDNA was used. The quantitative PCR was run on ABI 7500 Fast Real-Time PCR System (http://www.appliedbiosystems.com) using FastStart Universal SYBR Green Master Mix (Roche, http://www.roche.com). The quantitative PCR primers are listed in Supplementary File 1. The Ct values were normalized against *ACT2* (*At3g18780*) mRNA or *UBC* (*At5g25760*) mRNA. The abundance of mRNAs was expressed as relative to controls, with control values set to 1. The error bars represent the standard deviation of 4 biological replicates.

495

496 **Protein domain analysis and phylogenetic inference**

497 We utilized a domain-centric computational strategy to study DME and its related proteins.
498 Specifically, we identify DME homologs by using the iterative profile searches with PSI-BLAST
499 [47] from the protein non-redundant (NR) database at National Center for Biotechnology
500 Information (NCBI). Multiple sequence alignments were built by the Promals [48] program,
501 followed by careful manual adjustments. Consensus secondary structures were predicted using
502 the PSIPRED [49] JPred program [50]. Conserved domains were further characterized based on the
503 comparison to available domain models from pfam [51] and sequence/structural features. The
504 PhyML program [52] was used to determine the maximum-likelihood tree using the Jones–Taylor–
505 Thornton (JTT) model for amino acids substitution with a discrete gamma model (four categories
506 with gamma shape parameter: 1.096). The tree was rendered using MEGA Tree Explorer [53].

507

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517 **Author contribution.**

C.Z., Y.-H.H., X.-Q.Z., J.H.H. and T.-F.H. designed the research. C.Z., Y.-H.H., X.-Q.Z. performed the experiments. D.Z., L.M.I, and L.A. performed the evolutionary analysis. C.Z., Y.-H.H., and T.-F.H. wrote the article. T.-F.H., C.Z., Y.-H.H., W.X., J.H.H. interpreted and commented the article.

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Figure Legends

Figure 1 Complementation of *dme* seed abortion phenotype by the truncated DME nAGB. (a) Siliques were dissected and photographed 14 days after self-pollination. In *dme-2/dme-2* silique greater than 99% of seeds are aborted. A single copy of $nDME^{CTD}$ transgene reduces seed abortion rate to 50%; and in the *dme-2/dme-2;* $nDME^{CTD}/nDME^{CTD}$ silique, all the *dme-2* seeds

726 are rescued and developed normally. Scale bar = 0.5 mm. (b) Complementation of *dme-2* seed
727 abortion phenotype by *nDME^{CTD} and DME^{FL}*. (c) The *nDME^{CTD}* transgene restores DME target
728 genes *FWA* and *FIS2* expression. WT: Col-0; *nDME^{CTD}*: *dme-2/dme-2; nDME^{CTD}/ nDME^{CTD}*;
729 *dme-2*: *dme-2/dme-2*. Total RNA was isolated from stage F1 to F12 floral buds.
730
731 **Figure 2 Endosperm methylome analysis.** (a) Genome browser snapshots of CG DNA
732 methylation at selected imprinted gene loci. Top two tracks are coding genes (magenta) and TEs
733 (orange) with Tair10 chromosome coordinates. For the bottom seven tracks, each track
734 represents fractional CG methylation levels for different genotype: black trace, *dme-2* endosperm;
735 dark green trace, WT endosperm; dark blue trace, *DME^{FL}*-complemented endosperm; dark purple
736 trace, *nDME^{CTD}*-complemented endosperm; light green trace, WT endosperm subtracted from
737 *dme-2* mutant endosperm; light blue trace, *DME^{FL}*-complemented endosperm subtracted from
738 *dme-2* endosperm; light purple trace, *nDME^{CTD}*-complemented endosperm subtracted form *dme-*
739 *2* endosperm. DNA CG hypomethylation at selected maternally expressed (*FIS2* and *SDC*) and
740 paternally expressed (*SUV7*, *YUC10, and PHE1*) imprinted genes is restored in *DME^{FL}*- and
741 *nDME^{CTD}*-complemented endosperm. (b) Boxplot of CG methylation levels among canonical
742 DME target sites in *dme-2* mutant (grey), WT (white), *DME^{FL}*- (blue), or *nDME^{CTD}* - (red)
743 complemented endosperm. (c) Venn Diagram (top panel) of CG hyper-DMRs in 50-bp windows
744 between *dme-2* endosperm relative to WT, *DME^{FL}*-complemented or *nDME^{CTD}*-complemented
745 endosperm. Boxplot (bottom panel) of CG methylation levels in *dme-2* mutant (grey), WT
746 (white), *DME^{FL}*- (blue) or *nDME^{CTD}*- (red) complemented endosperm in WT only (left panel),
747 *DME^{FL}* only, or *nDME^{CTD}* only (right panel) DMRs.
748

Figure 3 Expression of DME NTD region in wild-type central cell induces *dme*-like seed abortion phenotype. **(a)** Confocal microscopy image of ovule in F12 floral bud shows the expression of mDME^{NTD}-GFP in the central cell. Scale bar, 20 µm. (**b-c**) Ectopic expression of *DME^{NTD}* in WT central cell induces *dme-2* like seed abortion phenotype in silique (**b**) and in developing seeds (**c**). Total RNA was isolated from stage F1 to F12 floral buds from independent lines with different seed abortion ratios (**d**) to assess transgene and endogenous DME expression. (**e**) Endogenous DME transcript levels in independent transgenic lines are comparable to the control line, but the transgene expression level varies among these independent lines with different seed abortion rates. Error bars indicate SD. NS, $p > 0.2$ (Ctrl vs 23), $p > 0.5$ (Ctrl vs 15), $p > 0.3$ (Ctrl vs 25), $p > 0.4$ (Ctrl vs 8), not significant (two-tailed t test). (**f**) Correlation analysis shows that the transcript abundance of the transgene, but not that of the endogenous DME transcripts, correlates with seed abortion rates (by linear regression).

Figure 4. Evolution of plant DME-like proteins. A phylogenetic tree was reconstructed using the PhyML program. Only node supporting values >0.80 from ML bootstrap analyses are shown. The representative domain architectures of DME homologs in major plant clades are shown along the tree, demonstrating domain fusions during evolution. Domain abbreviations: DemeN, N-terminal domain of DEME-like proteins in angiosperms; DnaJ, DnaJ molecular chaperone homology domain (Pfam: PF00226); FCL, [Fe4S4] cluster loop motif (also called Iron-sulfur binding domain of endonuclease III; Pfam: PF10576); HhH-GL, HhH-GPD superfamily base excision DNA repair protein (Pfam: PF00730); PHD, PHD finger (Pfam: PF00628); RRM, RNA recognition motif (Pfam: PF00076); Tudor, Tudor domain (Pfam: PF00567).

Supplemental Information

Figure Legends

Fig. S1. Diagrams of DME protein structure and transgene constructs.

(a) DME protein domain architecture. The positions of conserved domains along DME protein. Numbers represent amino acid position relative to the translation start sites. DME.1 is shorter than DME.2 by 258 amino acids due to alternative splicing, missing the very N-terminal DemeN domain. DemeN is a domain of unknown function conserved among angiosperm DME-like protein. 3DR is the stretch of basic rich amino acid direct repeats, resembling AT-hook motifs, and serves as a nuclear localization signal; per-CXXC is the permuted CXXC zinc finger motif; RRM is the RNA recognition motif; FCL is a [Fe4S4] cluster loop following the HhH module. The *dme-2* allele harbors a T-DNA insertion in region A at amino acid position 1012. ID1 and ID2 are variable, low complexity sequences between the glycosylase domain and the conserved B region. (b) Transgene constructs used in this study. DMEpro refers to the upstream regulatory sequence (2895 bp upstream of the translation start codon ATG) of DME.2. SV40NLS: PKKKRKV. A polypeptide linker comprising 6 alanine residues is placed between any protein fragment fusions.

Fig. S2. DNA methylomes of three independent *nDME^{CTD}*-complemented *dme-2* endosperm.

(a) Venn diagram showing partial overlap of *dme* CG hyper-DMRs relatives to each nAGB-complemented endosperm (*nDME^{CTD}-1* to *nDME^{CTD}*-3). (b) Boxplot of CG methylation levels among canonical DME target sites in *dme-2* mutant (black), *nDME^{CTD}*-1 (pink), *nDME^{CTD}-2* (magenta), or *nDME^{CTD}-3* (red) complemented endosperm, in *nDME^{CTD}-1* specific (left panel), *nDME^{CTD}-2* specific (middle panel), and *nDME^{CTD}-3* specific DMRs. These results show that the

combined DMRs are more or less hypomethylated in each independent line compared to *dme-2* endosperm.

Fig. S3. DNA methylomes of three independent DME^{FL}-complemented *dme-2* endosperm. (a) Venn diagram showing partial-overlap of *dme* CG hyper-DMRs relatives to each *DME^{FL}*-complemented endosperm (*DME^{FL}-1* to *DME^{FL}-3*). (b) Boxplot of CG methylation levels among canonical DME target sites in *dme-2* mutant (black), *DME^{FL}*-1 (light blue), *DME^{FL}-2* (medium blue), or *DME^{FL}-3* (dark blue) complemented endosperm, in *DME^{FL}-1* specific (left panel), *DME^{FL}-2* specific (middle panel), and *DME^{FL}-3* specific DMRs. These results show that the combined DMRs are more or less hypomethylated in each independent line compared to *dme-2* endosperm.

Fig. S4. The DMRs of *dme* relative to WT endosperm or nDME^{CTD}- complemented endosperm. Venn Diagram (top) and Boxplot analysis (bottom) of CG hyper-DMRs in 50-bp windows between *dme-2* endosperm relative to *nDME^{CTD}*-complemented or WT endosperm. CG methylation levels of DMRs unique to *nDME^{CTD}*-complemented endosperm are also demethylated in the WT endosperm (left panel). Similarly, DMRs unique to WT endosperm are demethylated in *nDME^{CTD}*-complemented endosperm (right).

Fig. S5. DME^{FL} and nDME^{CTD} transgenes are expressed at comparable levels among independent complementation lines. *DME^{FL}* and *nDME^{CTD}* expression levels are comparable between the four of the six complementation lines used in the methylome study. Total RNA was isolated from stage F1 to F12 floral buds. The results show that there is no significant difference

818 in expression level between these two transgenes(t-test, p>0.4).

819

820 **Fig. S6. The effects of T-DNA insertion on endogenous DME transcript abundance in *dme-***
821 ***2/dme-2* plants.** Total RNA was isolated from stage F1 to F12 floral buds. Equal amount of total
822 RNA from WT and *dme-2/dme-2* were used for reverse transcription and quantitative PCR. Six
823 paired of primers (PN1-PN6) correspond to the N-terminal region before the T-DNA insertion
824 site, and three pairs of C-terminal region primers (PC1-PC3) were used to assess endogenous
825 DME transcript level in *dme-2/dme-2* mutant plants. The position of each primer pair is indicated
826 in the DME diagram where T-DNA insertion site is shown.

827

828 **Fig. S7. Alignment of angiosperm DME-like proteins showing the conserved DemeN**
829 **domain and the basic rich 3DR repeats.** Bioinformatics analysis using available DME-like
830 sequences identified a ~ 120-amino-acid-long conserved region at the very N-termini among
831 DME-like proteins in angiosperms. This sequence is characterized by a highly conserved
832 WxPxTPxK motif that might function in protein-protein interactions. Further toward the C-
833 terminus is a stretch of basic amino acids rich region that serves as a nuclear localization signal.
834 This sequence consists of three direct repeats (3DR) reminiscent of the AT-hook motifs that may
835 bind DNA.

836

837 **Fig. S8.** Boxplot of CG methylation levels among canonical DME target sites in different DMR
838 length category, in *dme-2* mutant (black), wild-type (white), or $nDME^{CTD}$-complemented
839 endosperm

840

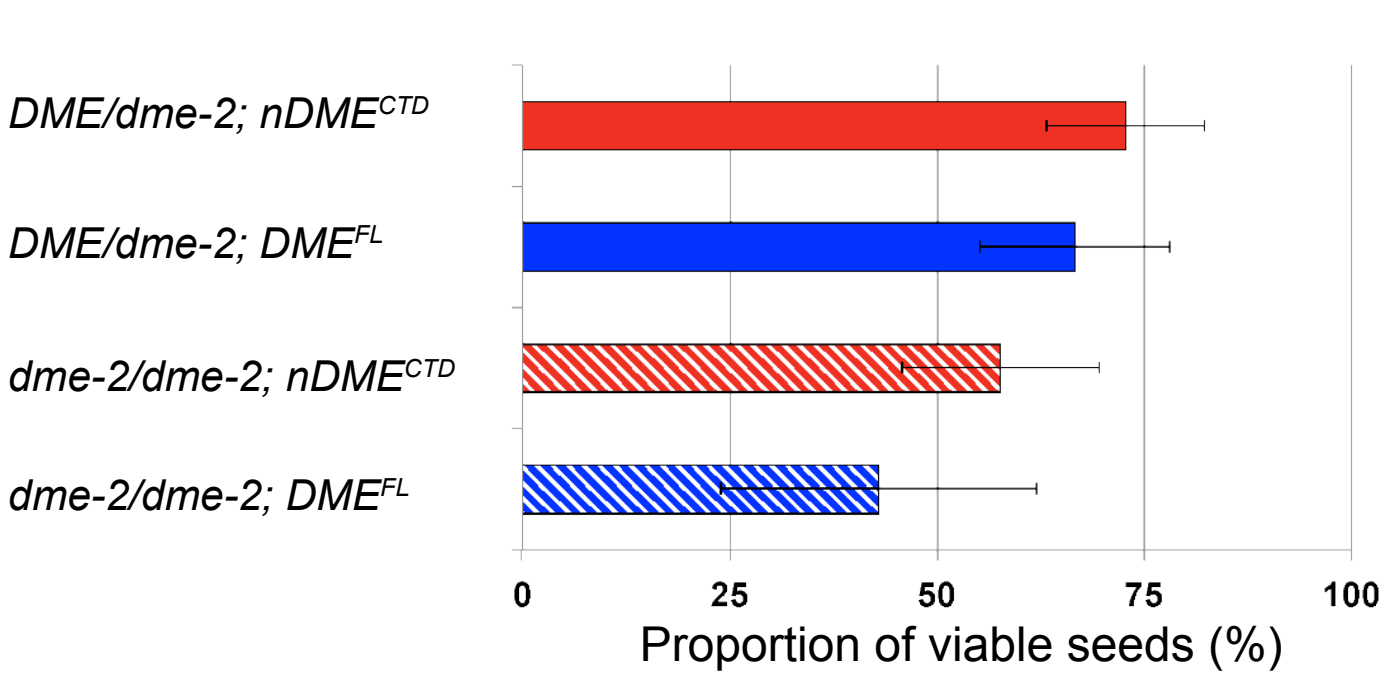
841 **Fig. S9.** (a) Merged DMR length distribution in WT and *nDME*^{CTD}-complemented endosperm. (b)
842 Genome Browser examples of long WT DMRs. Tracks are as labeled. The DMR regions are
843 indicated as horizontal bars according to their length in each sample (bottom two tracks). Even
844 though *nDME*^{CTD} complemented endosperm lack longer DMRs, these regions are also shorter
845 DMRs in *nDME*^{CTD}-complemented endosperm.
846
847
848
849

Table 1. Rescue of the reduced paternal *dme-2* allele transmission by the *nDME^{CTD}* transgene.

Female	Male parent	F1, DME/*dme-2*	F1, *DME/dme-2*; *nDME^{CTD}*	*nDME^{CTD}* transmission rate (%)	*p* for 1:1†
Col-0	*dme-2/dme-2; nDME^{CTD}* /~ Line 1	32	62	66	2.0E-3
Col-0	*dme-2/dme-2; nDME^{CTD}* /~ Line 2	3	50	94.3	1.1E-10
Col-0	*dme-2/dme-2; nDME^{CTD}* /~ Line 3	8	34	81	6.0E-5
Col-0	*dme-2/dme-2; nDME^{CTD}* /~ Line 4	9	44	83	1.5E-6

† Probability that that the deviation from the indicated segregation ration (1:1 inheritance of paternal genome with or without *nDME^{CTD}* transgene in the F1 generation) is due to chance.

Figure 1



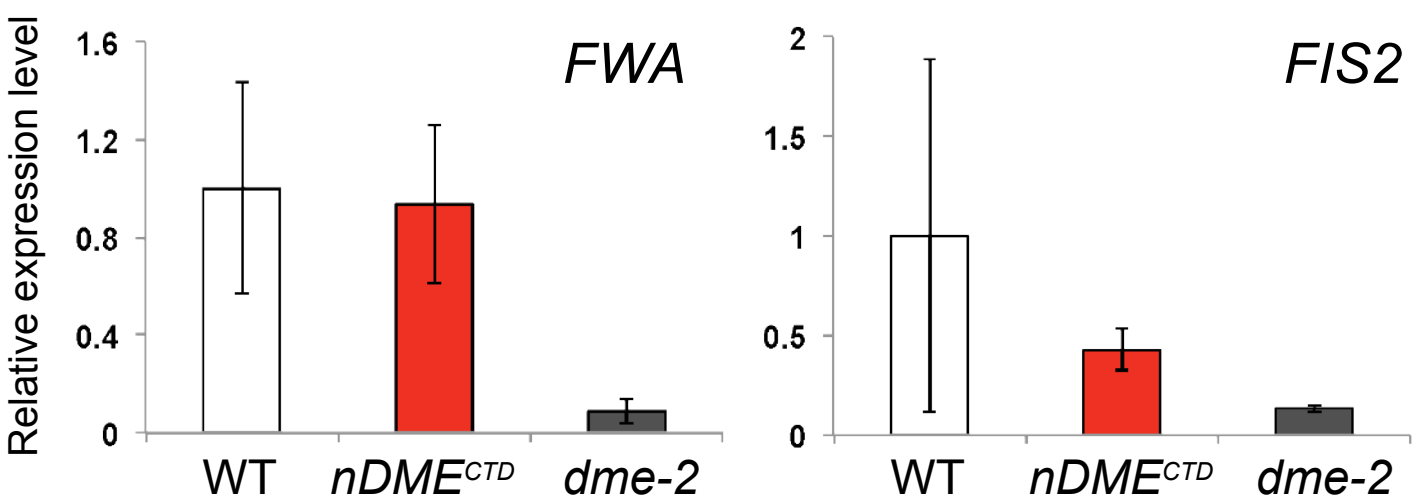


Figure 2

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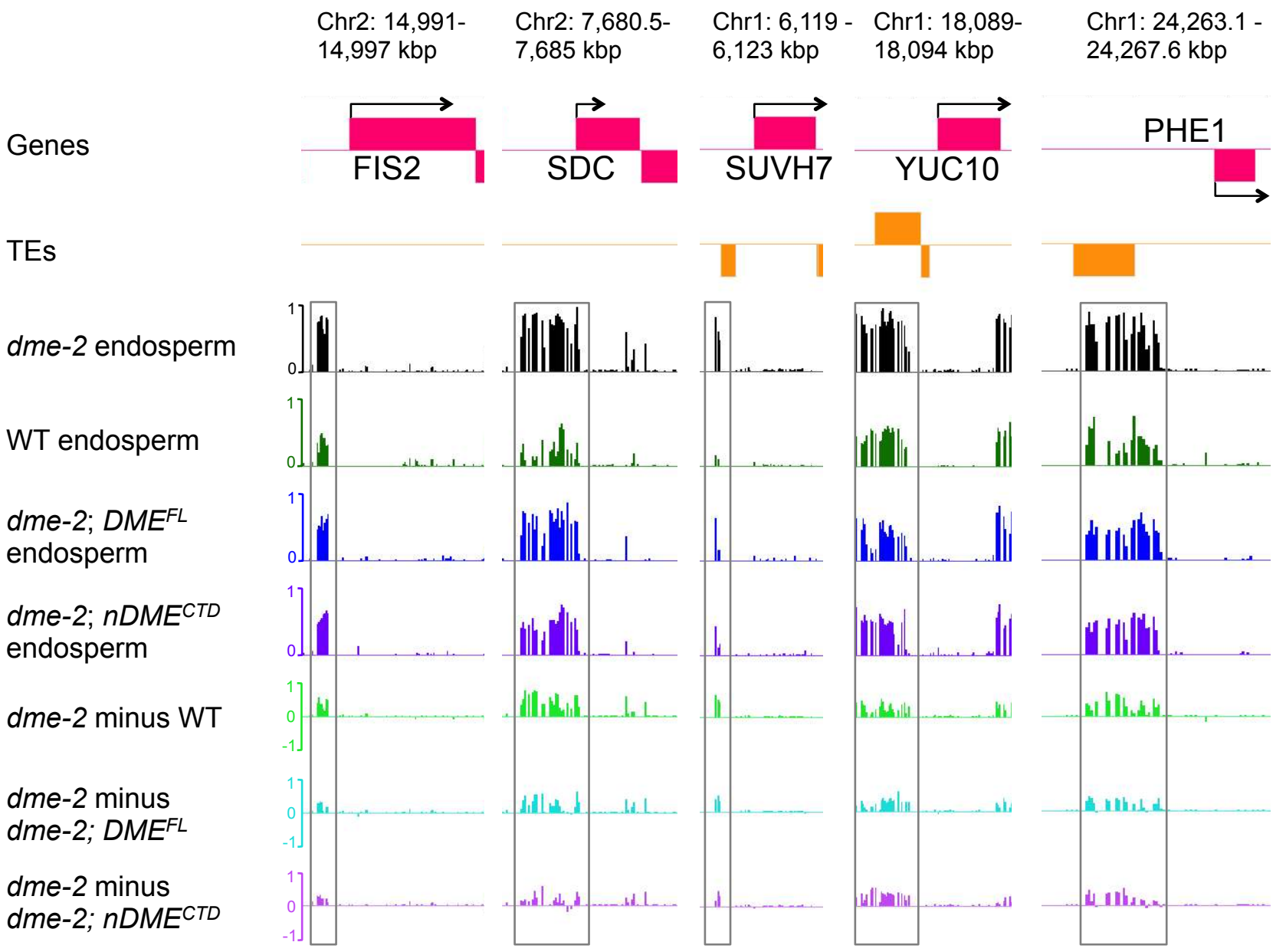

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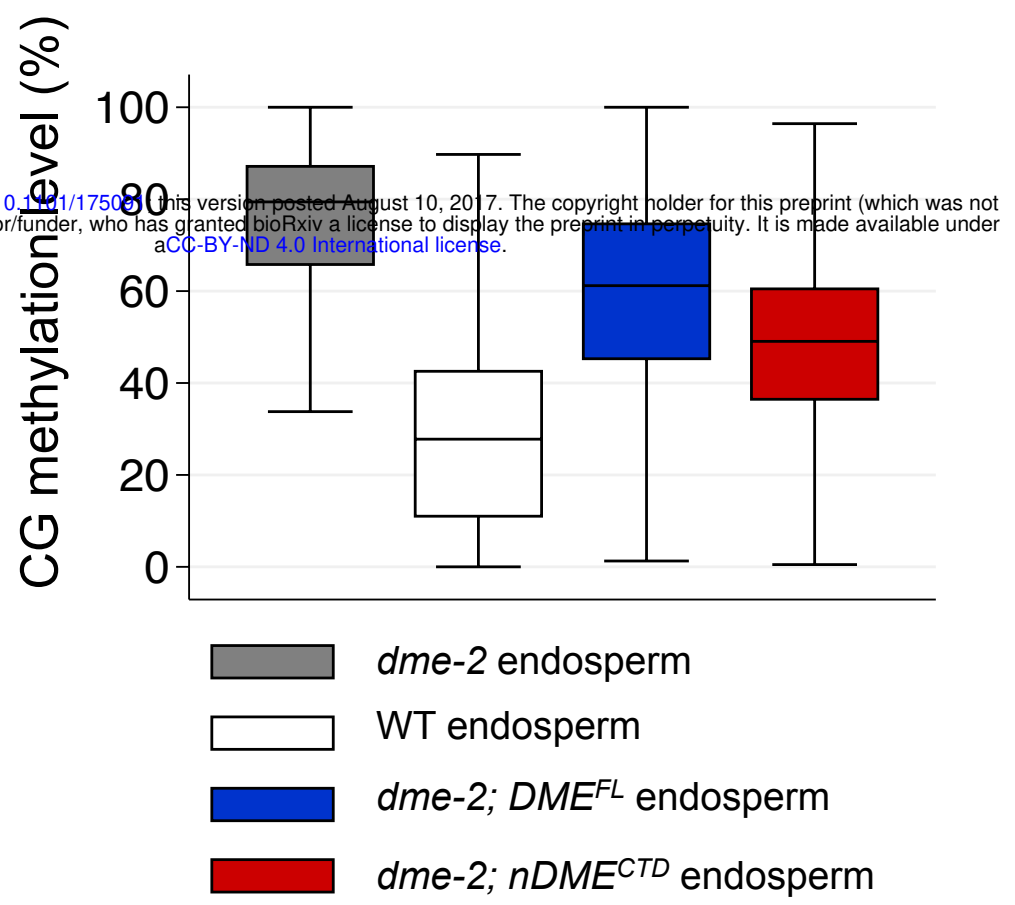

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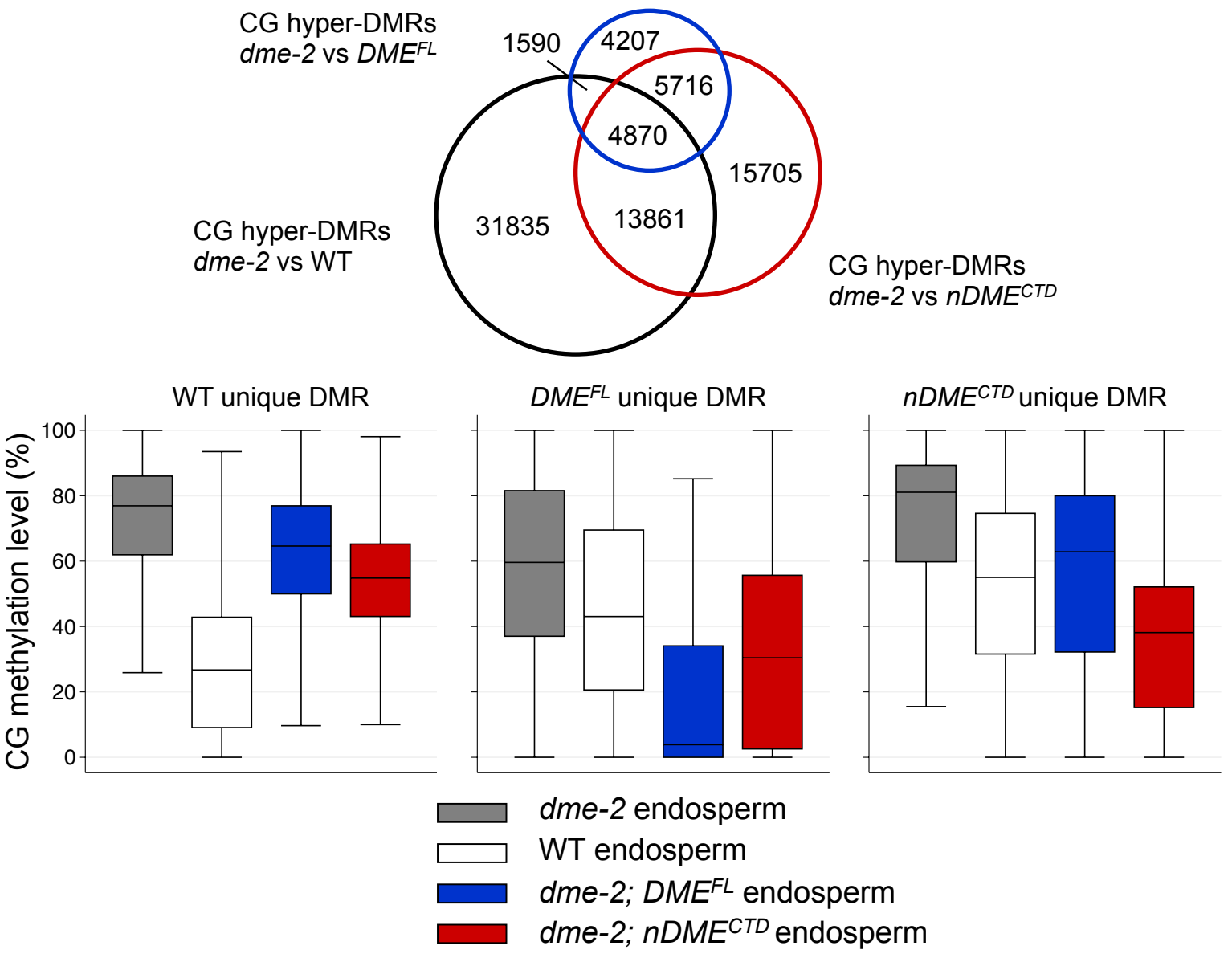

Figure 3

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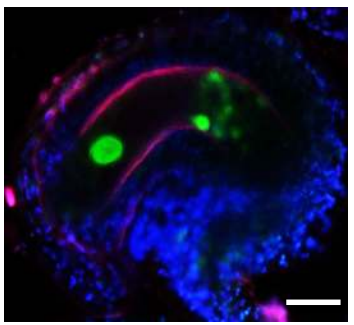

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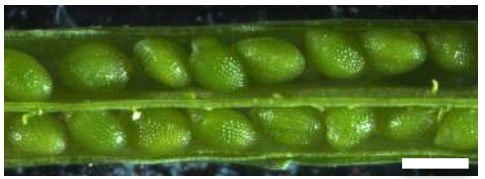
DME/DME



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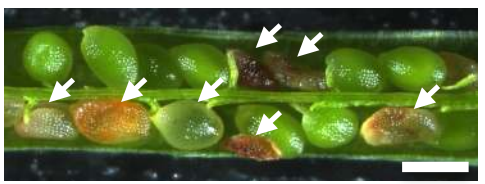
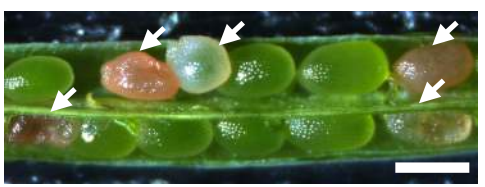


DME/DME ; DME^{NTD} /~



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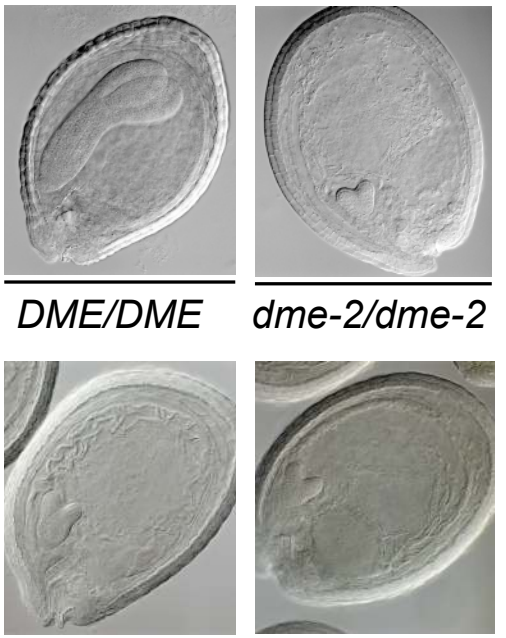
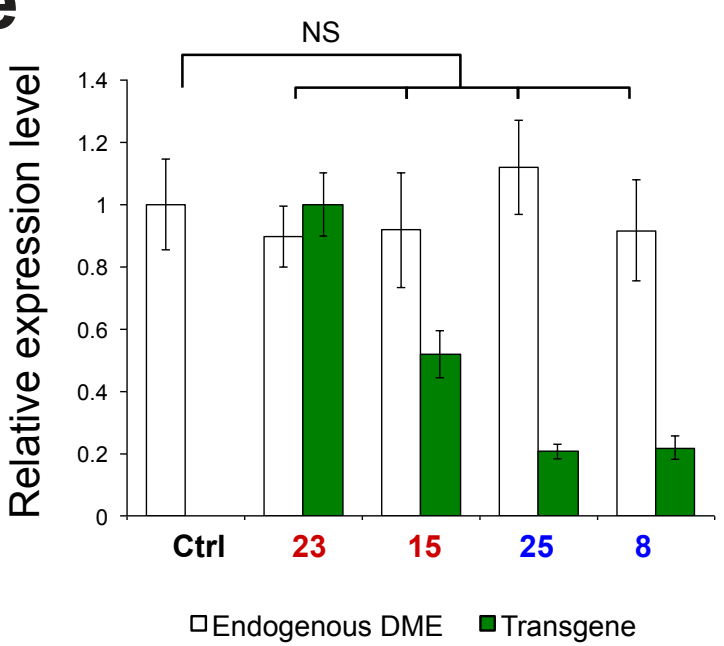


d

Sample	Proportion of aborted seeds (%)
Control	0
Line 23	52
Line 15	27
Line 25	0
Line 8	0

e



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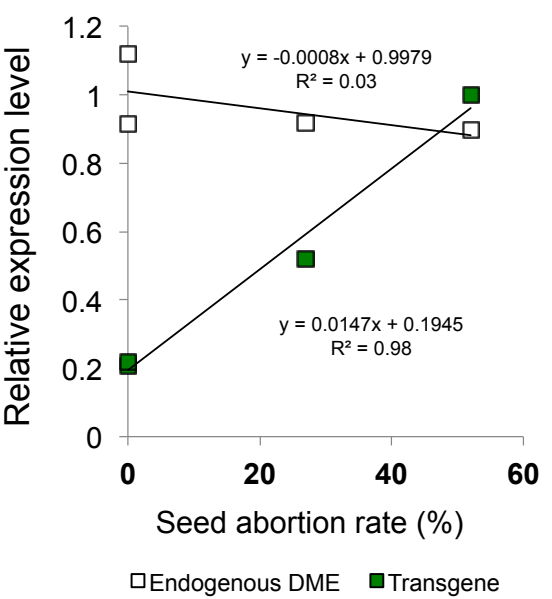
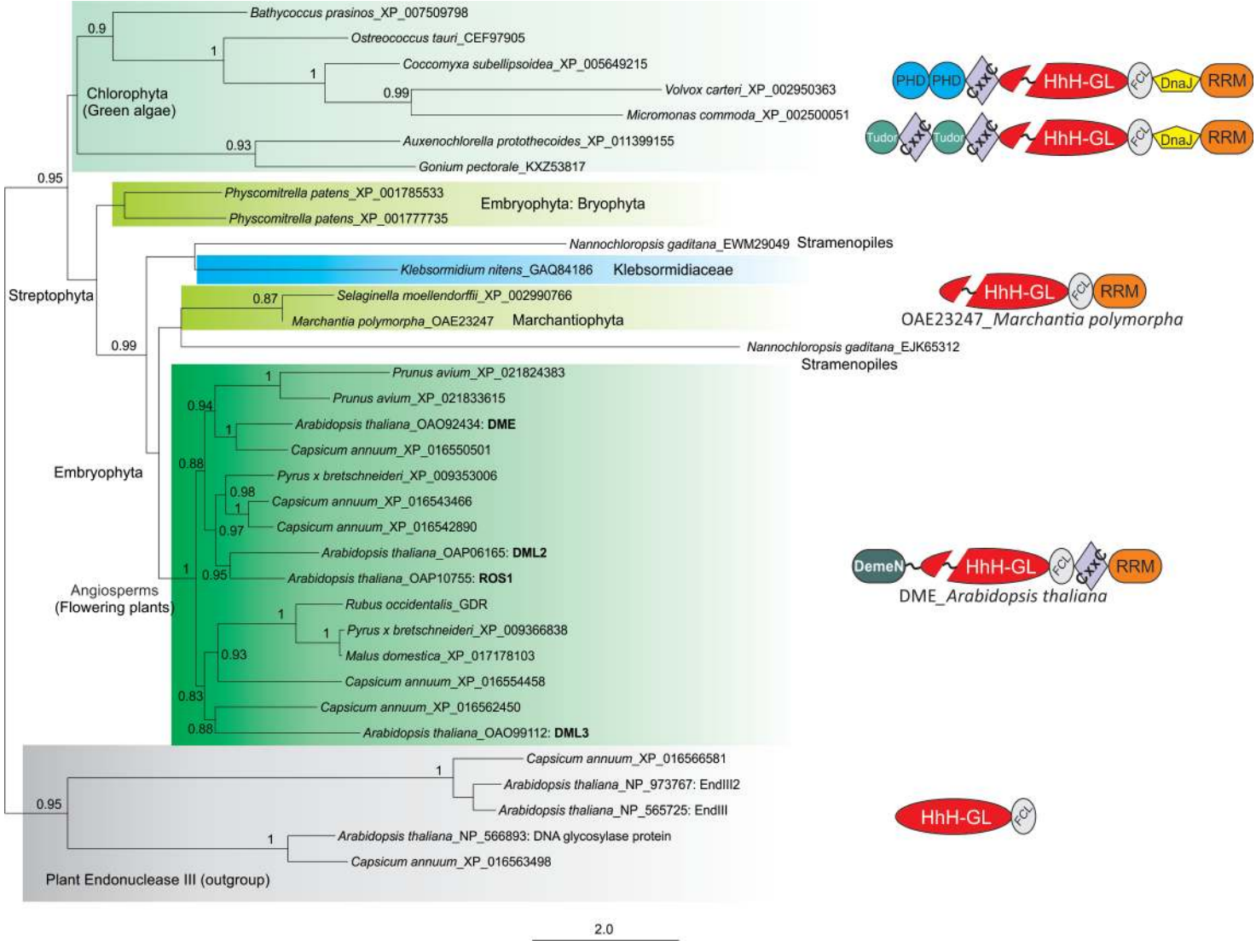


Figure 4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Auxenochlorella protothecoides_XP_011399155
Gonium pectorale_KXZ53817
Chlorophyta
(Green algae)
Physcomitrella patens_XP_001785533
Physcomitrella patens_XP_001777735
Embryophyta: Bryophyta
Nannochloropsis gaditana_EWM29049
Stramenopiles
Klebsormidium nitens_GAQ84186
Klebsormidiaceae
Selaginella moellendorffii_XP_002990766
Marchantia polymorpha_OAE23247
Marchantiophyta
Nannochloropsis gaditana_EJK65312
Stramenopiles
Streptophyta
Embryophyta
Angiosperms
(Flowering plants)
Prunus avium_XP_021824383
Prunus avium_XP_021833615
Arabidopsis thaliana_OAO92434: DME
Capsicum annuum_XP_016550501
Pyrus x bretschneideri_XP_009353006
Capsicum annuum_XP_016543466
Capsicum annuum_XP_016542890
Arabidopsis thaliana_OAP06165: DML2
Arabidopsis thaliana_OAP10755: ROS1
Rubus occidentalis_GDR
Pyrus x bretschneideri_XP_009366838
Malus domestica_XP_017178103
Capsicum annuum_XP_016554458
Capsicum annuum_XP_016562450
Arabidopsis thaliana_OAO99112: DML3
Capsicum annuum_XP_016566581
Arabidopsis thaliana_NP_973767: EndIII2
Arabidopsis thaliana_NP_565725: EndIII
Arabidopsis thaliana_NP_566893: DNA glycosylase protein
Capsicum annuum_XP_016563498
Plant Endonuclease III (outgroup)
PHD
PHD
CxxC
HhH-GL
FCL
DnaJ
RRM
Tudor
Tudor
CxxC
CxxC
HhH-GL
FCL
DnaJ
RRM
HhH-GL
FCL
RRM
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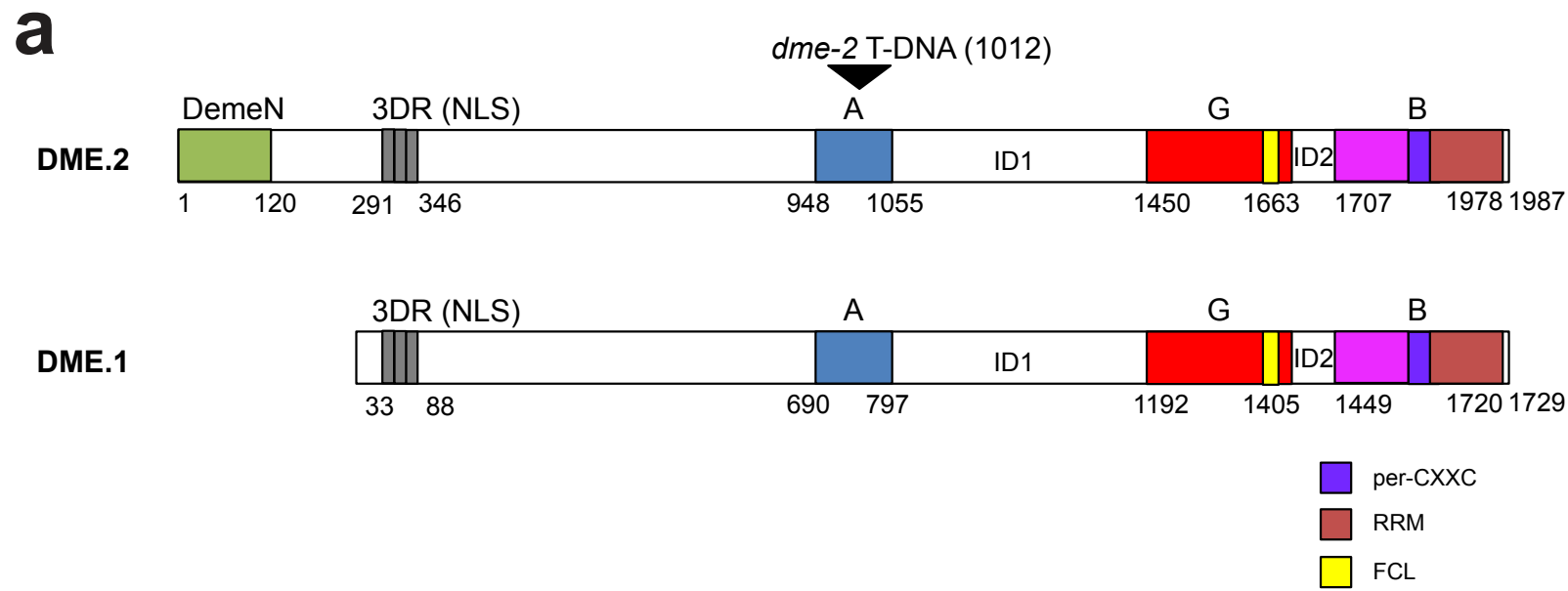
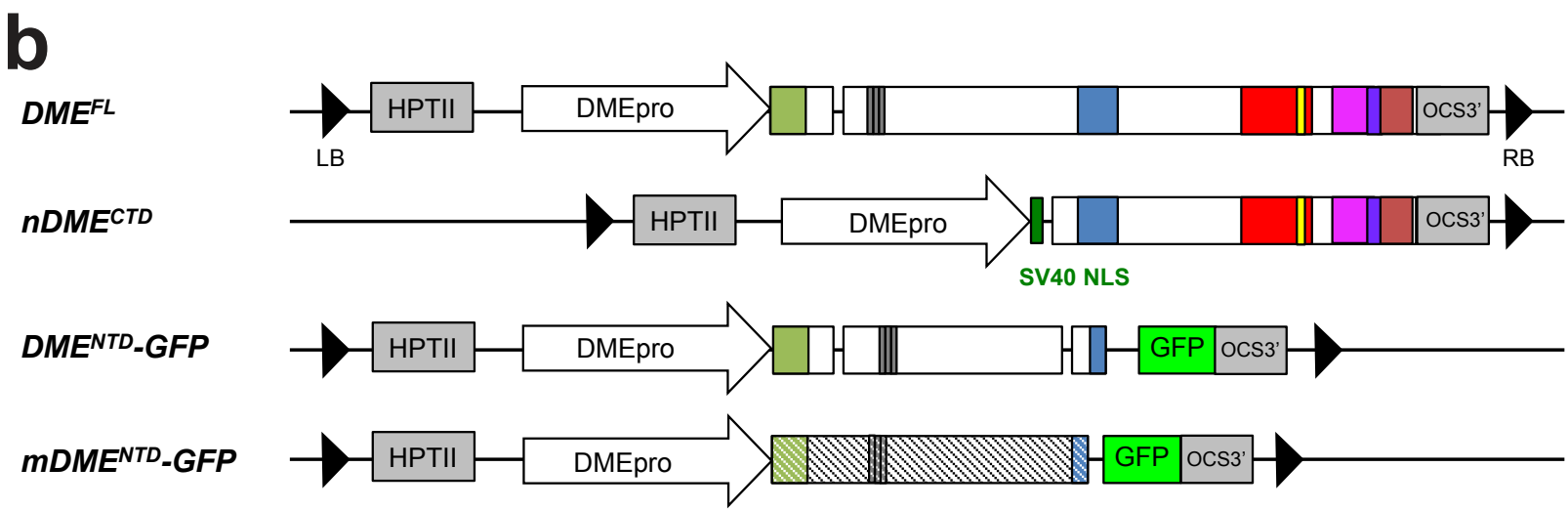
Supplementary Figure S1





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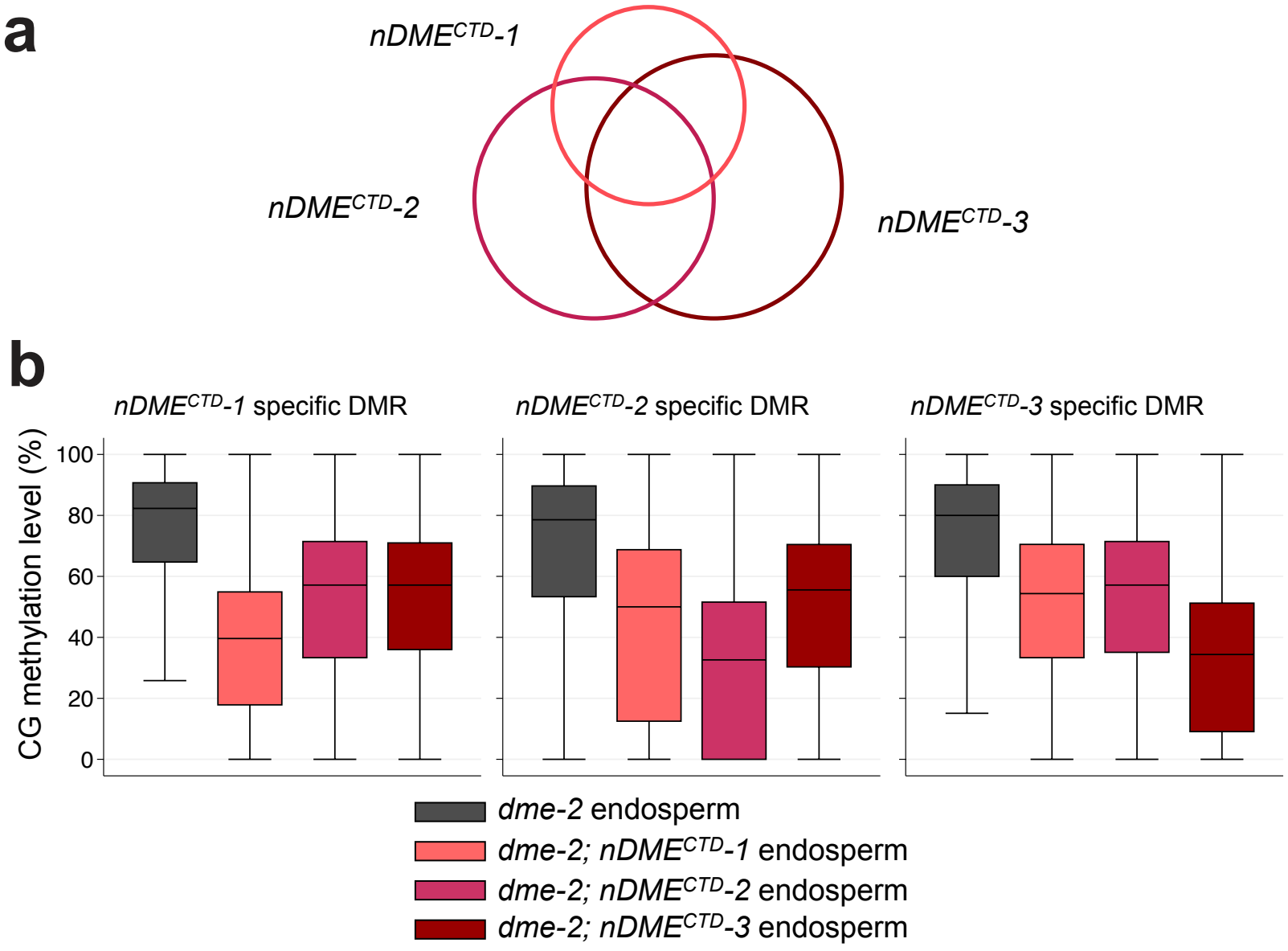
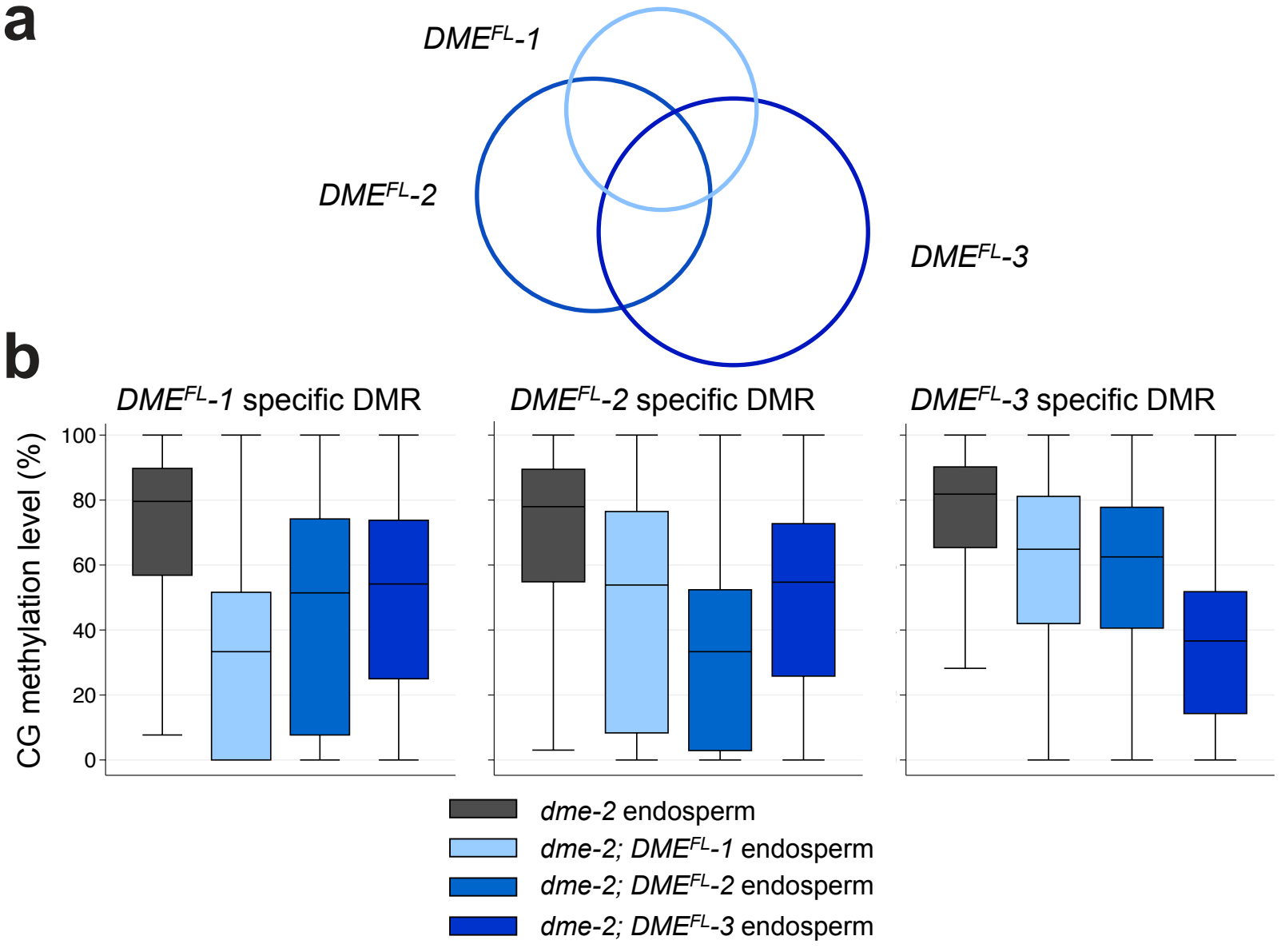
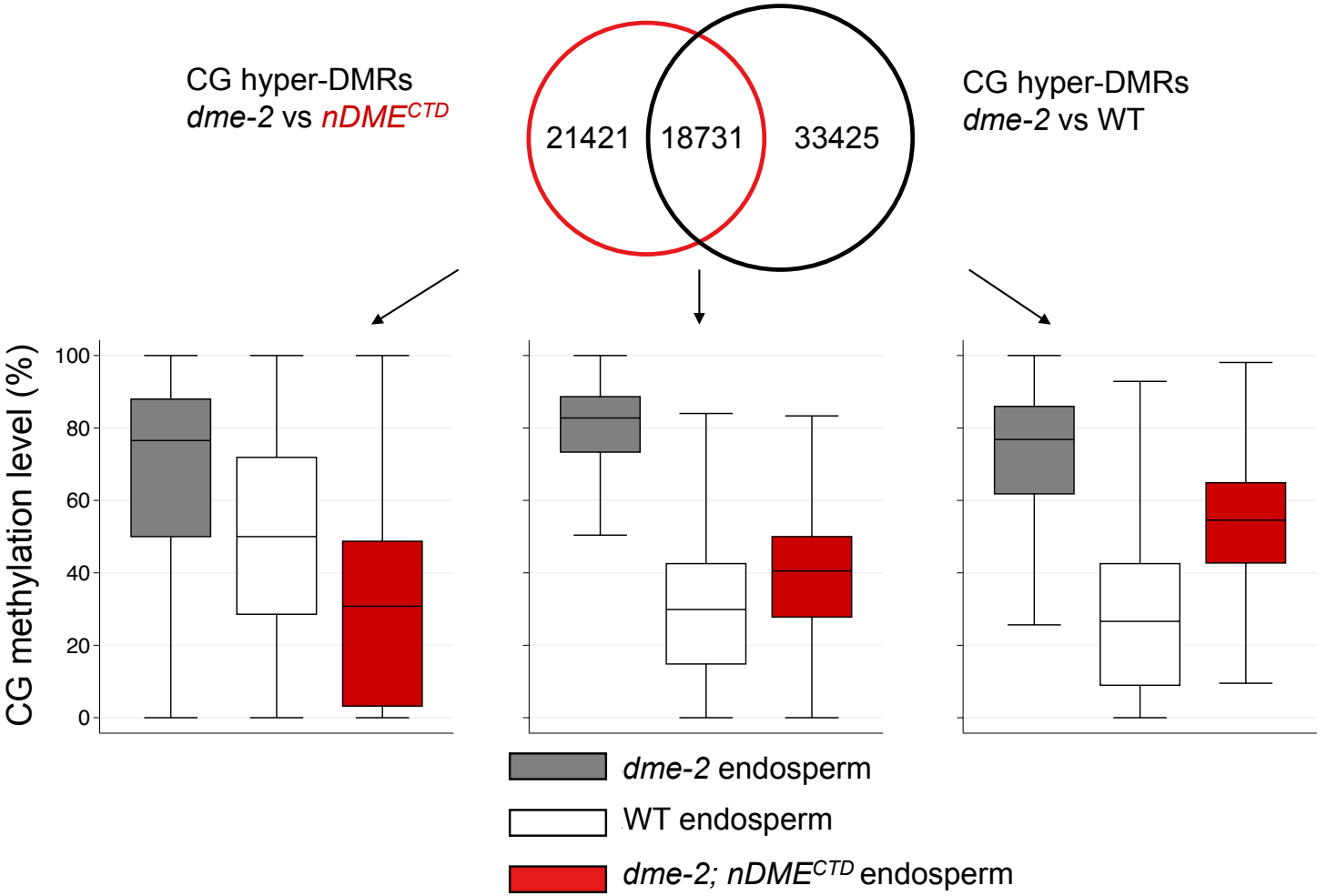


Supplementary Figure S3



Supplementary Figure S4



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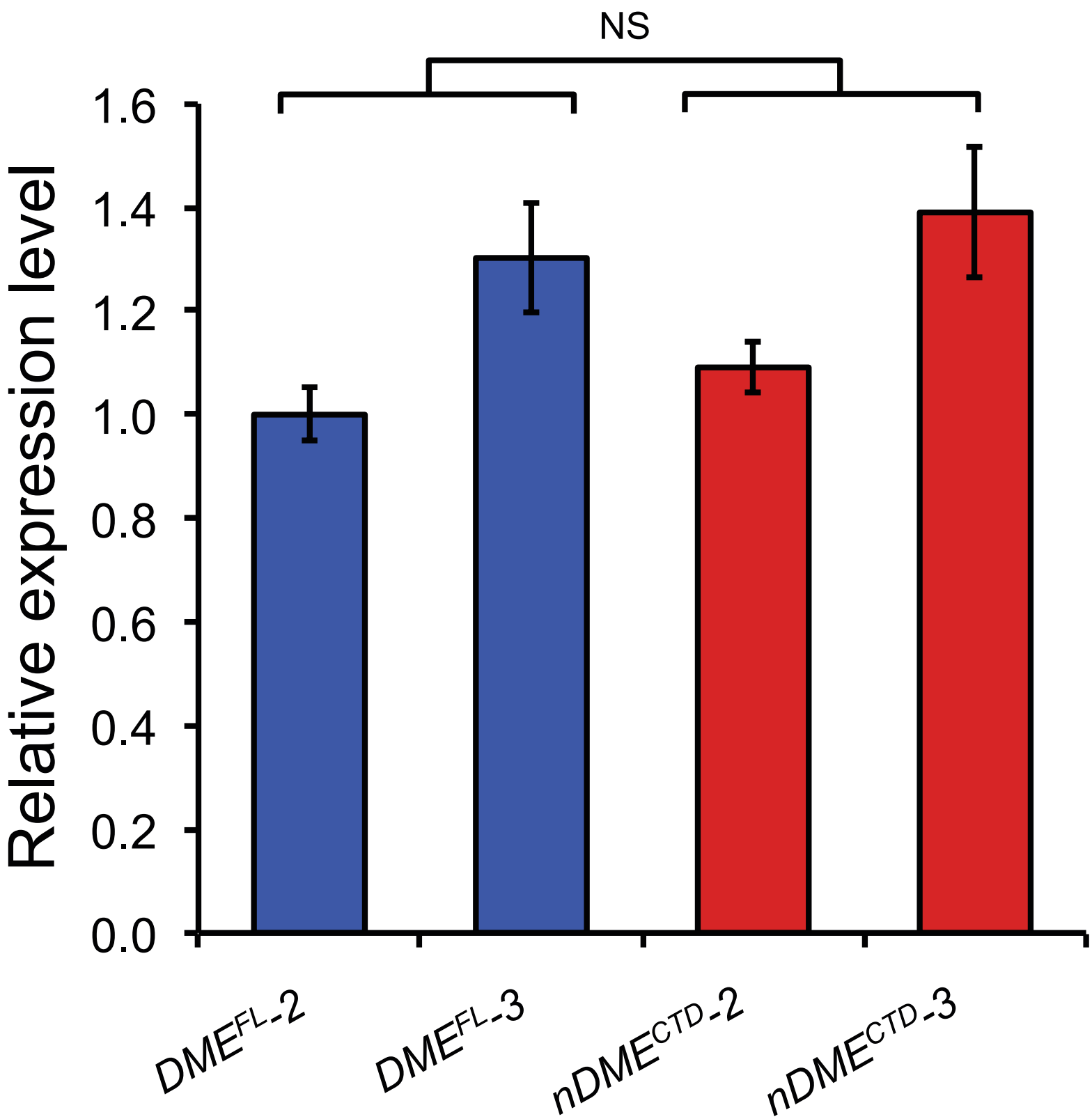
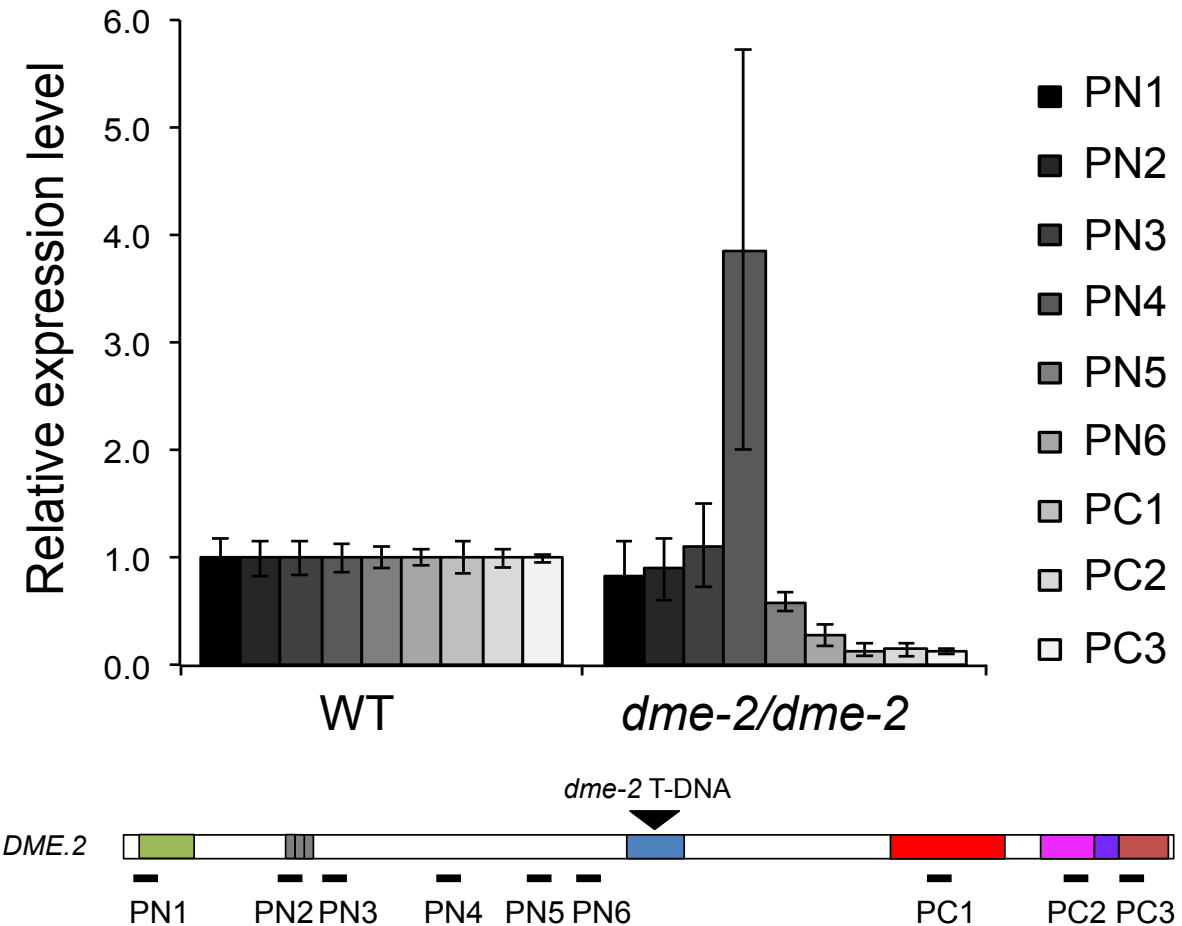
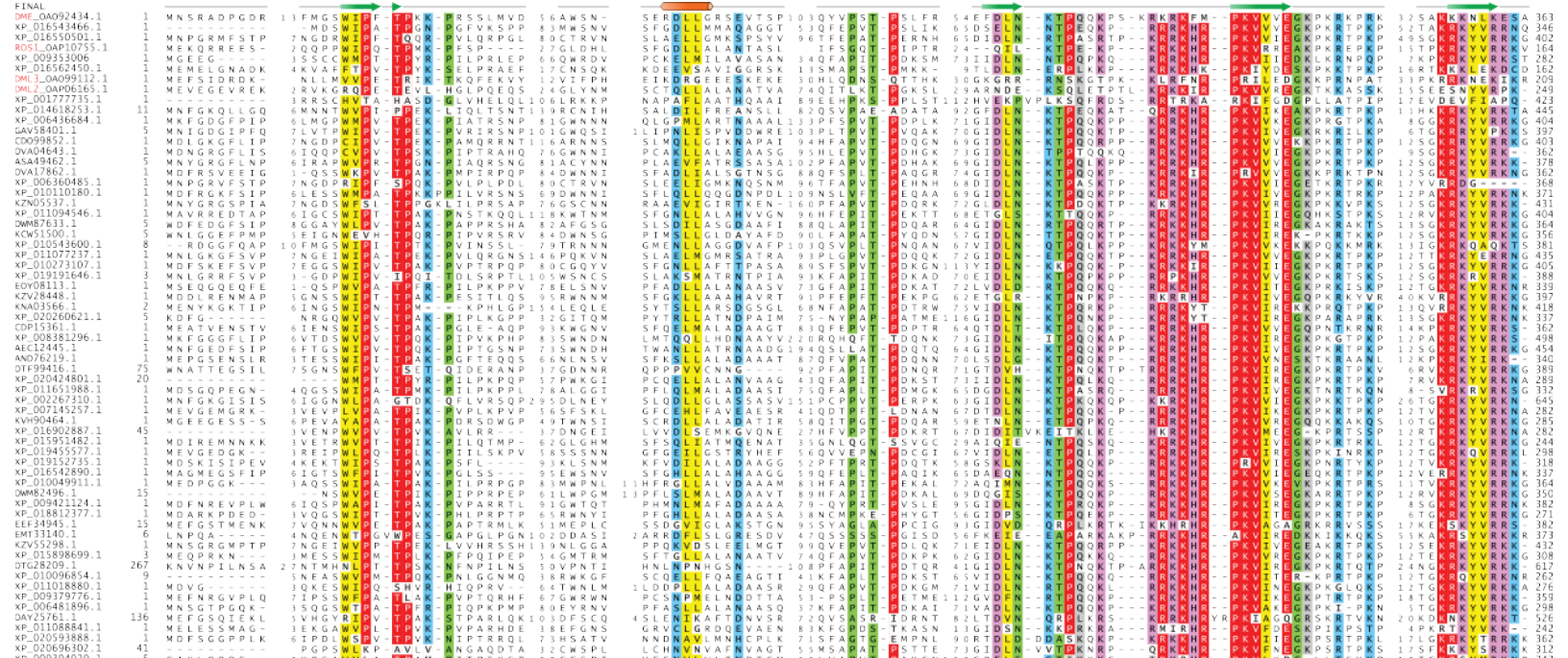


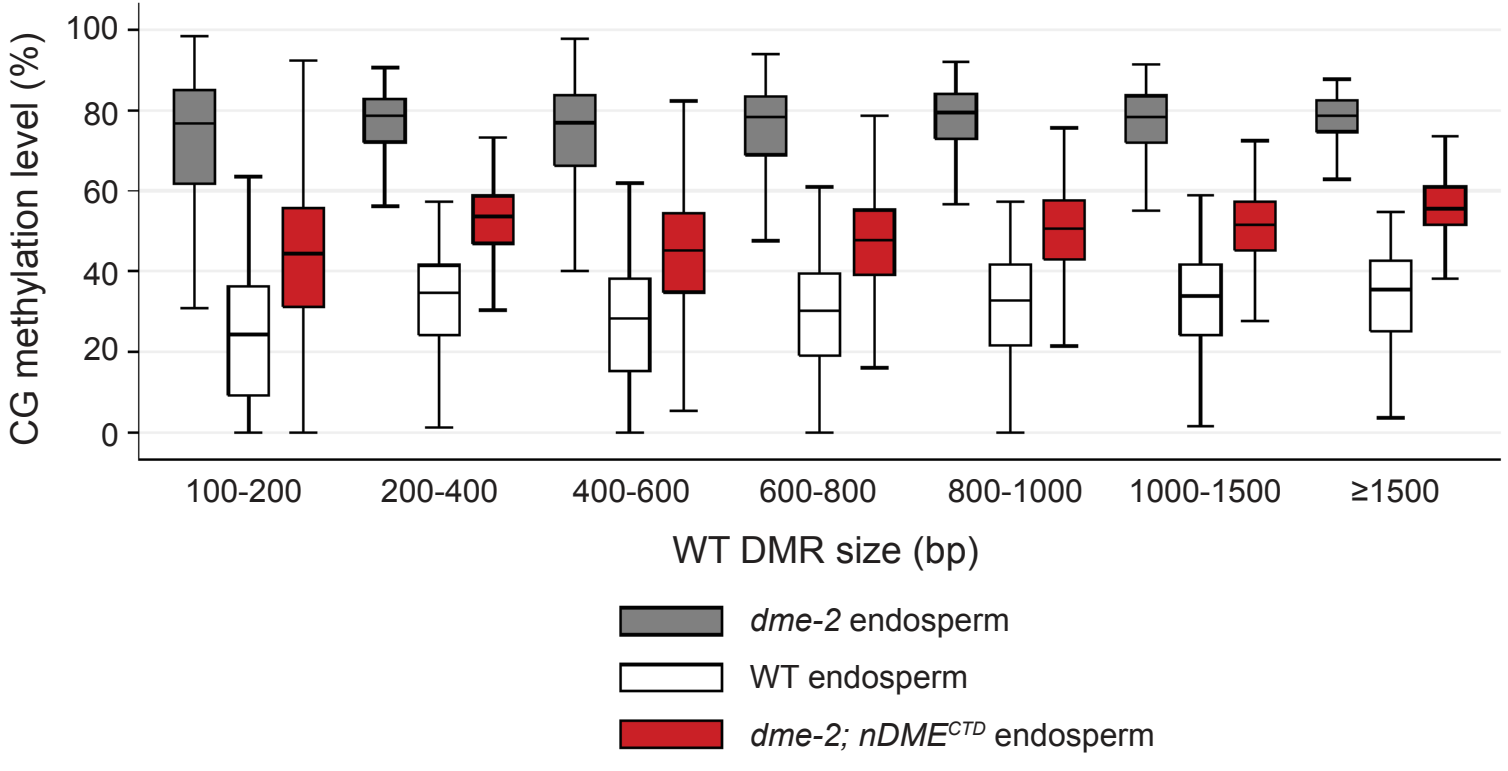
Supplementary Figure S6



Supplementary Figure S7

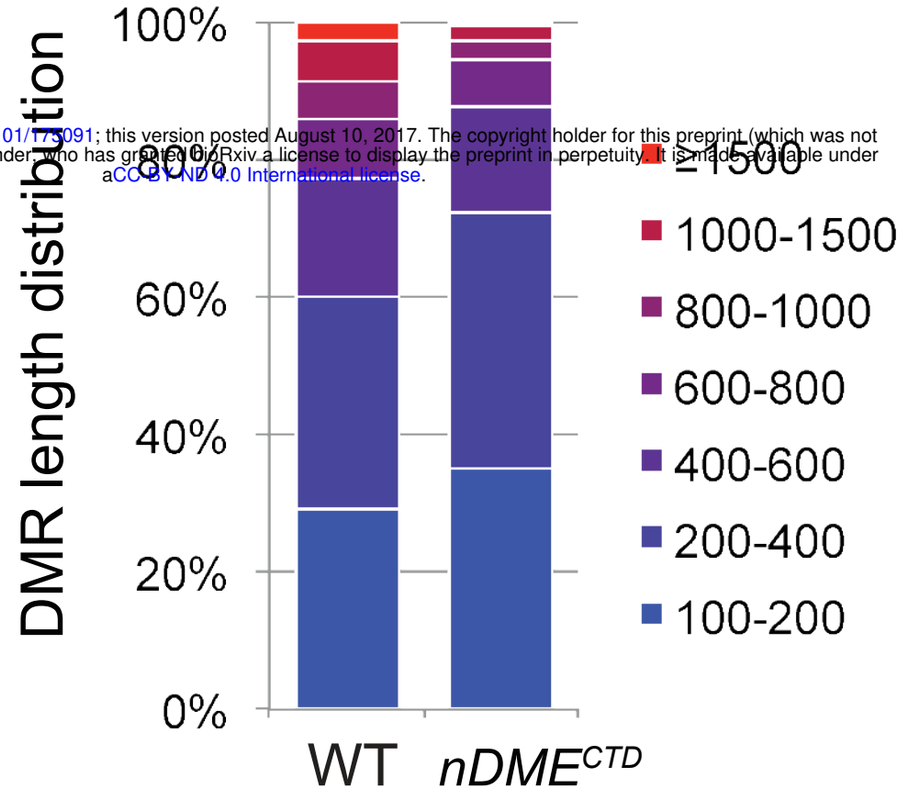


Supplementary Figure S8



Supplementary Figure S9

a



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