Title: Subtle perturbations of the maize methylome reveal genes and transposons silenced by DNA methylation

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ABSTRACT

DNA methylation is a chromatin modification that can provide epigenetic regulation of gene and transposon expression. Plants utilize several pathways to establish and maintain DNA methylation in specific sequence contexts. The chromomethylase (CMT) genes maintain CHG (where H = A, C or T) methylation. The RNA-directed DNA methylation (RdDM) pathway is important for CHH methylation. Transcriptome analysis was performed in a collection of *Zea mays* lines carrying mutant alleles for CMT or RdDM-associated genes. While the majority of the transcriptome was not affected, we identified sets of genes and transposon families sensitive to context-specific decreases in DNA methylation in mutant lines. Many of the genes that are up-regulated in CMT mutant lines have high levels of CHG methylation, while genes that are differentially expressed in RdDM mutants are enriched for having nearby mCHH islands, providing evidence that context-specific DNA methylation directly regulates expression of a small number of genes. The analysis of a diverse set of inbred lines revealed that many genes regulated by CMTs exhibit natural variation for DNA methylation and gene expression. Transposon families with differential expression in the mutant genotypes show few defining features, though several families up-regulated in RdDM mutants show enriched expression in endosperm, highlighting the importance for this pathway during reproduction. Taken together, our findings suggest that while the number of genes and transposon families whose expression is reproducibly affected by mild perturbations in context-specific methylation is small, there are distinct patterns for loci impacted by RdDM and CMT mutants.

INTRODUCTION

The epigenome describes the potential for additional heritable information that can be passed on through mitosis or meiosis (Hofmeister *et al.* 2017). DNA methylation is one molecular mechanism that can provide epigenetic information. There is interest in the potential for cryptic

59 information present in genomes that is normally silenced by epigenetic mechanisms but could
60 be activated through epigenetic changes without requiring any genetic change.
61
62 Much is known about the mechanisms that control DNA methylation and the functional roles of
63 DNA methylation in regulating transposon and gene expression in the model plant *Arabidopsis*
64 *thaliana*. However, our knowledge of the regulating mechanisms and function of DNA
65 methylation is much more limited in crop plants. Evidence in rice and maize suggests that major
66 perturbations of DNA methylation disrupt development and the seeds/plants are not viable
67 (Yamauchi *et al.* 2014; Hu *et al.* 2014; Li *et al.* 2014). Forward genetic screens for factors
68 involved in epigenetic phenomena such as paramutation (Dorweiler *et al.* 2000; Hollick *et al.*
69 2005; Alleman *et al.* 2006; Hale *et al.* 2007; Jr *et al.* 2009) or transgene silencing (McGinnis *et*
70 *al.* 2006) have identified several genes that are associated with DNA methylation or chromatin
71 in maize (Hollick 2017). In addition, reverse genetic approaches have been utilized in attempts
72 to document the function of putative methyltransferase genes or other genes associated with
73 DNA methylation (Papa *et al.* 2001; Makarevitch *et al.* 2007; Li *et al.* 2014). To date, these
74 mutants have provided partial reductions in DNA methylation in specific sequence contexts but
75 no mutants with drastic reductions to genomic DNA methylation have been recovered in maize.
76
77 Surveys of natural variation for DNA methylation among diverse lines of maize have revealed
78 many examples of differentially methylated regions (DMRs) (Eichten *et al.* 2011, 2013; Regulski
79 *et al.* 2013; Li *et al.* 2015a). A subset of the genes located near DMRs exhibit a negative
80 correlation between DNA methylation and gene expression (Eichten *et al.* 2013; Li *et al.* 2015a).
81 This is primarily found at genes that have CG or CHG methylation in regions surrounding the
82 transcriptional start site (TSS) and show qualitative (on/off) expression variation among
83 genotypes (Li *et al.* 2015a). This suggests the potential for cryptic information in the maize

genome that is epigenetically silenced in some lines but can be active due to epigenetic changes in other genotypes.

Several maize mutant lines with subtle perturbations of genomic DNA methylation have been previously identified (Li *et al.* 2014). The mutants include *mop1* and *mop3*, two mutants recovered in screens for factors required for maintenance of the paramutated state at the B' locus (Dorweiler *et al.* 2000). The *Mop1* gene encodes an RNA-dependent RNA polymerase related to RDR2 in *Arabidopsis* (Alleman *et al.* 2006) while *Mop3* is predicted to encode the second largest subunit of RNA Pol IV (Sloan *et al.* 2014), which is allelic to *rmr6 (Erhard et al. 2009)*. Mutant alleles for the two chromomethylase genes present in the maize genome, *Zmet2* and *Zmet5* also influence context-specific DNA methylation patterns (Papa *et al.* 2001; Makarevitch *et al.* 2007; Li *et al.* 2014). These genes are likely paralogs resulting from a whole genome duplication event and are orthologous to *CMT3* from *Arabidopsis thaliana*. Previous research has found that *mop1* and *mop3* genotypes have lost CHH methylation at many genomic regions with elevated CHH, and there are changes in CG and CHG at these sites as well (Li *et al.* 2014). However, as these types of regions are quite rare in the maize genome these mutants have minimal effects on genome-wide levels of CG and CHG methylation. The *zmet2-m1* mutant and, to a lesser extent, the *zmet5-m1* mutant, result in reduction of CHG methylation. These mutants also cause reductions of CWA methylation (where W is A or T) in genomic regions with low, but detectable, CWA methylation (Li *et al.* 2014; Gouil and Baulcombe 2016). Attempts to recover double mutants for *Zmet2/Zmet5* were unsuccessful, suggesting at least partially redundant function for these paralogous genes.

Mutants for *mop1*, *mop3*, *zmet2* and *zmet5* are viable with relatively few major phenotypic changes (Dorweiler *et al.* 2000; Papa *et al.* 2001). The *mop1* and/or *mop3* mutations have been shown to play important roles in the regulation of specific maize loci (Dorweiler *et al.* 2000;

110 Alleman *et al.* 2006; Sloan *et al.* 2014), transgenes (McGinnis *et al.* 2006) or transposable
111 elements (Lisch *et al.* 2002; Woodhouse *et al.* 2006). Microarray profiling of gene expression
112 has revealed evidence for altered expression of small sets of genes in studies of *mop1*
113 *(Madzima et al. 2014)* and *zmet2 (Makarevitch et al. 2007)*. There is also evidence from an
114 RNAseq experiment for altered regulation of transposable element expression in apical
115 meristem tissue (Jia *et al.* 2009). Transcriptome analysis of *rmr6*, which is allelic to *mop3*,
116 provided evidence for a potential role in stress response (Forestan *et al.* 2016). The *rmr6*
117 mutation appears to increase the proportion of the genome that is transcribed but has subtle
118 effects at most loci with relatively few genes with significant changes in expression level
119 (Forestan *et al.* 2017). However, there have not been comprehensive studies on the overlap of
120 genes or transposons that are sensitive to mutations in different CMT or RdDM genes in maize.
121
122 Each of the mutant backgrounds used for this study has subtle effects on genomic methylation
123 levels and can produce viable plants. There are several phenotypic abnormalities observed in
124 *mop1* and *mop3* stocks (Dorweiler *et al.* 2000; Barber *et al.* 2012; Sloan *et al.* 2014) although
125 the penetrance in multiple backgrounds has not been well characterized. We sought to
126 determine if the subtle changes in DNA methylation in these mutants would reveal genes or
127 transposons that are sensitive to these shifts in DNA methylation or chromatin. A limited number
128 of genes and transposon families exhibit altered expression in these genotypes. A subset of
129 these genes have high levels of DNA methylation in wild-type that are reduced in the mutant
130 genotypes. Many of these genes exhibit natural variation for DNA methylation and gene
131 expression. This provides evidence that the natural variation at these genes is due to epigenetic
132 rather than genetic variation and highlights cryptic information present in the maize genome that
133 could be accessible through alterations to the epigenome.
134
135 MATERIALS AND METHODS

136 **Biological materials***:* All the mutant and wild type samples used in this study are listed in Table
137 S1 and SRA accession numbers are listed for each dataset. Tissue for RNA and DNA isolations
138 was collected from three biological replicates. Plants were grown in standard greenhouse
139 conditions for 20 days to reach the V3 stage. The 2nd and 3rd leaves were collected individually
140 for each seedling. The 2nd leaf was used to isolate DNA for genotyping, and the 3rd leaf was used
141 for RNA isolation and sequencing. For each biological replicate, 4-6 seedlings were pooled.
142
143 **Library preparation and sequencing***:* Total RNA was isolated using the TRIZol reagent
144 following the manufacturer's protocol. RNA was quantified using RiboGreen and 3 µg total RNA
145 was used to construct libraries using TruSeq strand-specific kit (Illumina) following
146 manufacturer's suggestions. The final library was quantified using PicoGreen and twelve
147 libraries were pooled per Illumina lane. Library quality was checked using Agilent Bioanalyzer.
148 Sequencing was performed on HiSeq2500 using 2 x 50 bp mode.
149
150 **Gene expression analysis***:* Trim_glore was used to trim low-quality base from the 3' end of the
151 reads, as well as to remove adapters. Reads that passed quality control were mapped to B73
152 version 4 genome (Jiao *et al.* 2017) using Tophat2 (Kim *et al.* 2013), allowing at most 1
153 mismatch (-N 1) and the expected inner distance between mate pairs of 200 bp (-r 200). Reads
154 that are properly paired and uniquely mapped were filtered out using samtools (-f 0x0002 –q
155 50). HTSeq (Anders *et al.* 2015) was used to summarize the number of reads mapped to each
156 V4 gene model with the union mode, generating a matrix of count values for each gene in each
157 genotype.
158
159 Raw read counts were input into DEseq2 (Love *et al.* 2014) to perform differential expression
160 analysis. Pair-wise comparisons were made between each mutant and the appropriate wild
161 type. Genes with a FDR value of < 0.05 and $\log_2$(FoldChange) > 1 were called differentially

162 expressed genes. Detailed analysis was restricted to genes with consistent DE calls in at least
163 two mutant contrasts in the same pathway (RdDM or CMT). Genes were considered expressed
164 if at least 3 replicates in the libraries described had an RPM (reads per million) value > 1.
165
166 **TE expression analysis***:* B73v4 (Jiao *et al.* 2017) TE annotation was modified to remove
167 helitrons and the file was resolved using RTrackLayer in R so that each base of the genome
168 was assigned to only a single TE. Exon regions were masked from the TE file using Bedtools
169 (Quinlan and Hall 2010) subtract. Gene annotations were added to this modified TE annotation
170 file, and mapped reads were assigned to features using HTSeq (Anders *et al.* 2015). A custom
171 script was used to read through the HTSeq sam output, assigning unique-mapping reads to
172 individual TE elements and multi-mapped reads to TE families if mapped positions hit only a
173 single TE family. Unique and multi-mapped reads were combined for per-family expression
174 counts, and RPM values were calculated by normalizing to the number of gene reads plus TE
175 family reads in each library. All reads mapped to gene annotations plus TE annotations were
176 excluded from the TE expression analysis. Differentially expressed TE families were determined
177 using DESeq2 (Love *et al.* 2014) using a $\log_2$(FoldChange) cutoff of 1 and FDR adjusted p-value
178 cutoff of 0.05. Detailed analysis was restricted to TE families with consistent DE calls in at least
179 two mutant contrasts in the same pathway (RdDM or CMT).
180
181 **WGBS data analysis***:* The WGBS datasets used in this study are detailed in Table S2 and SRA
182 accession numbers for each sample are provided. One µg DNA was used to prepare libraries
183 for whole-genome bisulfite sequencing using the KAPA library preparation kit. DNA was
184 sheared to a peak between 200-250 bp. End repair was performed to make blunt-ended
185 fragments, followed by adding base A to the 3' end, and adapter ligation. Size selection was
186 performed to enrich library with a size between 250-450 bp. Bisulfite conversion was then
187 carried out using Zymo DNA methylation lightning kit according to user's manual. Finally, library

188 was enriched using PCR amplification. Library quality was checked using the Agilent
189 Bioanalyzer. Library quantification was performed with qPCR before sequencing. Sequencing
190 was performed on HiSeq2000 with paired end 100 cycles.
191 Analysis was performed as previously described (Li *et al.* 2015a; Song *et al.* 2016). Read quality
192 was checked with FASTQC, adapters and low-quality bases at the 3' end of each read were
193 trimmed using Trim_glore. The high quality reads were mapped to B73 V4 genome (Jiao *et al.*
194 2017) using BSMAP (Xi and Li 2009) allowing at most 5 mismatches. Only properly paired
195 reads with unique mappings were kept and used for calling DNA methylation. Methylation calls
196 were performed using the methratio.py script from BSMAP. Finally, DNA methylation in each
197 context (CG, CHG, CHH) was summarized for each 100-bp non-overlapping tile of the 10 maize
198 chromosomes.
199
200 **DMR calling**: DMRs were called using previously described criteria (Li *et al.* 2015a). Briefly,
201 each 100-bp tile with > 6 CG/CHG sites, > 2X coverage and > 60% difference for CG/CHG were
202 compared. CHH DMRs were called using the same coverage and site number criteria, but with
203 a requirement for <5% CHH in one genotype and >25% CHH in another genotype, reflecting the
204 low level of CHH methylation in the maize genome.
205
206 **mCHH Islands***:* High CHH bins were called genome-wide by requiring CHH methylation over
207 25% with at least 10 informative counts per bin. Genes and TEs were considered associated
208 with a mCHH island if when at least one high methylation bin was identified within the gene or in
209 the 2 kb region surrounding the gene.
210
211 **Data availability***:* All data used in this study are deposited at the NCBI Sequence Read Archive
212 (SRA). Accession numbers for all libraries are listed in Table S1.
213

214 RESULTS

215 **Alteration of gene and transposon expression in maize mutants with perturbed**
216 **methylomes:**

217 RNAseq was used to perform transcriptome profiling for several maize lines carrying mutations
218 in genes encoding CMT (this study) or RdDM components (Gent *et al.* 2014; Li *et al.* 2015b).
219 Together these factors are expected to be responsible for the majority of CHG and CHH
220 methylation in the maize genome. For CMT genes, three biological replicates of seedling leaf
221 tissue were profiled for mutations in two different genes, with multiple alleles utilized for one of
222 the genes (Table S1). In addition, for the RdDM genes we analyzed seedling leaf tissue for
223 *mop1* and *mop3* (Li *et al.* 2015b), along with immature ear tissue for *mop1* (Gent *et al.* 2014).
224 The genetic background, read number and accession information for each sample is provided in
225 Table S1.
226

227 The expression of individual genes was estimated from the RNAseq data for each sample.
228 Differentially expressed (DE) genes in each mutant line (relative to the appropriate control) were
229 identified using DESeq2 followed by a requirement for a minimum of 2 fold-change and an FDR
230 value of less than 0.05 (Table S2). The observed differences in gene expression in the mutant
231 lines could be direct effects of the mutation on expression, indirect effects caused by direct
232 targets, or could be the result of introgressions of linked loci that contain cis-regulatory variation.
233 The number of genes in each 2 Mb bin with differential expression was assessed throughout the
234 genome (Figure S1). For mutations that were identified in one background and then
235 backcrossed into another background (*zmet2-m1, zmet2-m2, zmet5, mop1*), there were often a
236 cluster of DE genes surrounding the locus of the mutation itself. These regions often included
237 similar numbers of up- and down-regulated genes. For the other mutation (*mop3*) that was not
238 backcrossed into another genetic background, there is less evidence for expression changes at

239 linked genes (Figure S1). Based on these results we omitted DE genes located within 40 Mb of
240 the mutation in subsequent analyses.
241
242 A principle component analysis was performed using all DE genes to cluster samples used in
243 this study (Figure 1). This reveals that the major sources of differences are tissue and genetic
244 background, as demonstrated, respectively, by the relatively larger differences for *mop1-ear* and
245 *mop3* which was not introgressed into B73 (Figure 1A). When comparing only leaf libraries in
246 the B73 background, few genotypes were substantially different from the wild-type controls,
247 suggesting limited changes to transcript levels induced by each mutation (Figure 1B). The
248 number of differentially expressed genes in each mutant genotype was highly variable (Figure
249 1C). In most cases the homozygous mutant individuals exhibit more up-regulated genes than
250 down-regulated genes, which is compatible with the concept that the CMT and RdDM genes
251 normally provide silencing activities.
252
253 Transposable elements (TEs) comprise a large portion of the maize genome, and typically have
254 high levels of CG and CHG methylation, with CHH methylation peaks at the edges of some TE
255 families. There are two classes of TEs, Class I (retrotransposons) and Class II (DNA
256 transposons), which transpose either through a copy-and-paste mechanism requiring an RNA
257 intermediate (retrotransposons) or through a cut-and-paste mechanism (DNA transposons)
258 (Wicker *et al.* 2007). Within each class are several orders divided into superfamilies,
259 distinguished by structural and protein-coding features. Families within each superfamily are
260 defined by sequence identity, and each family can contain any number of individual TE
261 elements (Jiao *et al.* 2017). Individual TE elements are defined at a single location within a
262 genome and are associated with a family, superfamily and class. We sought to document how
263 minor perturbations to the methylome impacted expression of TEs. Due to the highly repetitive
264 nature of TE sequences, we assessed per-family levels of expression by mapping RNA-seq

265 reads to the genome, reporting up to 20 best hits for each read using Tophat2. Per-family read
266 counts were determined by summing unique mapping reads (to a specific element) and multi-
267 mapping reads that align to only a single TE family. Overall, the total portion of RNAseq reads
268 that map to TE families is not significantly higher in the mutants than in wild-type plants
269 suggesting a lack of genome-wide activation of TEs in these mutants (Figure S2). In order to
270 assess expression of individual TE families in each genotype, per-family expression was
271 normalized by dividing the family counts by the total number of reads in the library assigned to
272 either TE families or genes, generating an RPM estimate. Using this approach we were able to
273 detect expression of 1,694 TE families in at least one of the genotypes used for this study. A
274 relatively small number of DE TE families ($\log_2 FC > 1$, FDR < 0.05) were identified in each
275 mutant (Table S3; Figure 1D). Consistent with the role of DNA methylation in silencing TEs,
276 more families were identified as up-regulated rather than down-regulated in mutants compared
277 with WT controls. However, the majority of the TE families expressed in these libraries do not
278 exhibit significant changes in expression level in CMT or RdDM mutants in maize.
279
280 There is a significant overlap in the number of genes and TEs that exhibit consistent changes in
281 gene expression in at least two samples of CMT mutants (*zmet2-m1/zmet2-m2/zmet5*) or RdDM
282 mutants (*mop1/mop3*) (Figure S3). In order to understand the reproducible effects of these
283 pathways on expression, we focused our analyses on the set of 237 genes and 104 TE families
284 that exhibit consistent up- or down-regulation in multiple CMT or RdDM mutants. Hierarchical
285 clustering of the expression level of these genes or TE families in all samples reveals evidence
286 for many consistent changes in expression within a pathway (Figure 1E-F). Within CMT
287 mutants, many genes show shared expression changes between *zmet2-m1* and *zmet2-m2*,
288 though there is a smaller subset of genes primarily shared between *zmet2-m2* and *zmet5*
289 (Figure 1E). Although both *zmet2-m1* and *zmet2-m2* are predicted to encode loss of function
290 alleles, there are some genetic differences in the behavior of these alleles (Papa *et al.* 2001;

291 Makarevitch *et al.* 2007; Li *et al.* 2014). The *zmet2-m1* mutation exhibits partial dominance that
292 may reflect dominant negative action of the protein that could be produced from this allele (Papa
293 *et al.* 2001). Plants that are homozygous for *zmet2-m1* have the greatest loss of CHG
294 methylation and this could result from influence of the *ZMET2* protein product on functional
295 *ZMET5* protein. The *mop1* and *mop3* seedling leaf samples have a number of examples of
296 consistent up-regulation but fewer examples of consistent down-regulation, consistent with the
297 greater number of up-regulated than down-regulated genes in RdDM mutants in general.
298
299 **Some genes that are up-regulated in CMT mutants exhibit high CHG methylation levels:**
300 The differential expression observed in each mutant background could result from direct
301 changes in DNA methylation or chromatin at these loci or could result from indirect effects due
302 to secondary effects from genes that are direct targets. Given that many of these mutants are
303 expected to affect DNA methylation levels, we might expect that wild-type plants would contain
304 high levels of DNA methylation for genes that exhibit increased expression in the mutants. The
305 context-specific DNA methylation profiles were assessed in wild-type B73 for genes that were
306 up- or down- regulated compared with all expressed genes (Figure 2A). Genes that are
307 differentially expressed in RdDM mutants exhibit slightly higher levels of CHH methylation
308 upstream of the transcription start sites relative to all expressed genes. The genes that are
309 down-regulated in CMT mutants do not show unusual patterns of DNA methylation. In contrast,
310 genes that are up-regulated in the CMT mutants exhibit distinct patterns of CG and CHG
311 methylation within gene bodies relative to other expressed genes (Figure 2A). Among the 112
312 genes up-regulated in CMT mutants, approximately half have high (>50%) and half have low
313 (<20%) methylation in the CG and CHG contexts (Figure 2B-C). In contrast, only ~4% of all
314 expressed genes have high CHG methylation in the same region. While a small number of
315 genes with high methylation overlap annotated TEs, most of the genes in this subset do not,
316 suggesting that this genic methylation is not solely due to nearby TEs. In wild-type samples,

317 genes with high CHG methylation in the gene body also have high CG methylation (Figure 2D).
318 However in *zmet2-m1* mutants, CHG methylation for these genes is reduced, with few examples
319 of a corresponding reduction in CG methylation (Figure 2D-E). As in the examples
320 (*Zm00001d045627* and *Zm00001d021982*) shown in Figure 2F, these genes have high levels of
321 CG and CHG methylation in wild-type B73, and the reduction in CHG methylation in *zmet2-m1*
322 mutants is associated with increased expression, suggesting that CMT-dependent silencing of
323 these genes depends on CHG but not CG methylation.
324
325 **Genes that are up-regulated in RdDM mutants are enriched for being near mCHH islands:**
326 A large number of maize genes (~60%) have been associated with the presence of a region of
327 elevated CHH in the promoter region, termed a mCHH island (Gent *et al.* 2013; Li *et al.* 2015b).
328 Genes with mCHH islands are enriched for high expression and the mCHH island often occurs
329 at the edge of the TE nearest these genes (Li *et al.* 2015b). These mCHH islands may form
330 important boundaries that could protect TE heterochromatin from the influence of genes (Li *et*
331 *al.* 2015b) and may also be important for long-distance interactions (Rowley *et al.* 2017). The
332 methylation within these mCHH islands requires *mop1* and *mop3* (Li *et al. 2014, 2015b)*. We
333 sought to determine if the genes that exhibit altered expression in *mop1* and *mop3* are enriched
334 for the presence of mCHH islands. Genes that are up- or down-regulated in the RdDM mutants
335 are enriched for the presence of mCHH islands, but this is only significant for the up-regulated
336 genes with 87.8% having a mCHH island within 2kb of the gene, compared to 64.6% of all
337 expressed genes (Figure 2G). The fact that both RdDM up- and down-regulated genes are often
338 near mCHH islands could be due to the fact that the mCHH island may provide long-range
339 interactions (Rowley *et al.* 2017) that could have either positive or negative influences on gene
340 expression.
341

342 In some cases, the mCHH island itself may result in transcriptional regulation. Work in
343 *Arabidopsis* has noted a positive feedback loop involving DNA methylation levels and
344 expression of the demethylase enzyme *ROS1* such that reduced levels of DNA methylation
345 result in lower *Ros1* expression but increased methylation is associated with elevated *Ros1*
346 expression (Williams *et al.* 2015). Reduced expression of maize DNA glycosylases has also
347 been observed in several transcriptome datasets of maize RdDM mutants (Williams *et al.* 2015;
348 Erhard *et al.* 2015). We find that one maize gene with sequence homology to *Ros1*,
349 *Zm00001d038302*, showed significantly reduced expression in the *mop1* and *mop3* mutants
350 and has a strong mCHH island in several inbred lines (Figure S4). This provides evidence to
351 support a requirement for RdDM and CHH methylation in the proper control of this gene in
352 maize.
353
354 **Genes regulated by CMT are enriched for natural DMRs:**
355 The genes that are sensitive to mutations in CMT or RdDM components may reflect examples
356 of natural variation for epigenetic regulation. Indeed, an earlier study has found that many of the
357 genes influenced by *Zmet2* exhibit variable expression patterns in different maize inbreds
358 (Makarevitch *et al.* 2007). We used WGBS data from B73 and 17 other diverse maize inbreds to
359 document natural variation for DNA methylation among maize inbreds. Differentially Methylated
360 Regions (DMRs) were identified in all three contexts (CG, CHG, and CHH) between B73 and
361 the other inbreds. More than 200,000 DMRs were called in the CG and CHG contexts, with over
362 50,000 DMRs in the CHH context. Each maize gene was classified based on whether there was
363 a DMR within 200 bp of the transcription start site for each of the three sequence contexts. A
364 relatively small portion (~3-7%) of maize genes have CG, CHG or CHH DMRs near the
365 promoter (Table S2). We proceeded to assess whether naturally variable DMRs were more
366 prevalent near genes that exhibit altered expression in CMT or RdDM mutants (Figure 3A).
367 Genes that are up- or down-regulated in CMT mutants exhibit a significant enrichment for CG

368 and CHG DMRs in their promoter regions. RdDM up-regulated genes are significantly enriched
369 for having CHH and CHG DMRs and also show an enrichment (though not significant) for CG
370 DMRs near the promoter (Figure 3A).
371
372 This suggests that many of the genes with altered expression in CMT or RdDM mutations may
373 have pre-existing natural variation for DNA methylation that would affect expression levels in
374 maize populations. RNAseq data from leaf tissue for ten of the inbred lines with WGBS data
375 was utilized to determine whether there was a significant association (p.value < 0.05, pearson
376 correlation) between context-specific methylation level at the DMR and gene expression levels.
377 We found that nearly 50% of the genes that exhibit altered expression in CMT or RdDM mutants
378 that are located near DMRs had natural variation for gene expression levels that was
379 significantly associated with DNA methylation levels. Two examples of CMT up-regulated genes
380 that exhibit significantly correlated expression and CHG methylation at the bin overlapping the
381 TSS among diverse lines are shown in Figure 3B-C. In wild-type maize inbred lines we see two
382 classes with respect to expression level and CHG methylation levels at the DMR near the TSS.
383 In one group of lines, including B73, the DMR is highly methylated and the gene is
384 transcriptionally silent. In the other group of genotypes (and in B73 *zmet2-m1* mutant lines -
385 blue dots) the DMR has low methylation and the gene is expressed. Although we were
386 interested in performing a similar analysis for natural variation in TE methylation and expression
387 we were not able to assess this due to the highly polymorphic nature of TEs among different
388 maize lines and the lack of de novo assemblies for other genotypes.
389
390 **Properties of TEs with altered expression:**
391 There are 104 TE families with altered expression in mutants that perturb RdDM or CMT
392 components in maize. These included 32 families up- and 7 families down-regulated in at least
393 two contrasts of RdDM mutants, and 48 families up- and 24 families down-regulated in at least

394 two contrasts of CMT mutants (Figure 1D). There are examples of both class I (specifically Long
395 Terminal Repeat or LTR) and class II (specifically Terminal Inverted Repeat or TIR) TE families
396 that exhibit altered expression in both RdDM and CMT mutants (Figure 4A). Most families with
397 varied expression were small (< 10 members), consistent with the genome-wide distribution
398 (Figure 4B). The relative age of the LTR families that have altered expression was assessed to
399 determine if they were particularly young or old families (Figure 4C). The relative ages of LTR
400 transposons can be approximated by comparing the sequence similarity of the two LTR
401 sequences. Since LTR sequences must be identical upon initial integration, a higher LTR
402 similarity denotes younger TE insertions. LTR families that are up-regulated in RdDM mutants
403 are enriched for younger LTR elements when compared with the distribution of ages present
404 genome-wide. We also tested the mean GC content of TEs within families to test whether
405 families depleted in cytosines are more susceptible to subtle perturbations in methylation, as is
406 the case for the *ONSEN* family in *Arabidopsis* (Cavrak *et al.* 2014). TE families up-regulated in
407 RdDM mutants do have a slightly lower GC content on average, though it is not clear if this
408 change alone is sufficient to cause the expression changes (Figure 4D).
409
410 We sought to further document the properties of these TE families through analysis of their
411 expression in nearly 100 developmental tissues or stages of B73. During typical development,
412 approximately 3,400 TE families are expressed in at least one tissue or stage. There are 5 TE
413 families up-regulated in RdDM mutants and 18 TE families up-regulated in CMT mutants that
414 are not expressed in any tissue or developmental stage assessed. The other families of TEs
415 that are up-regulated in RdDM mutants (25 families) or CMT mutants (27 families) were
416 assessed to determine if they exhibit distinct patterns of expression. Interestingly, approximately
417 one third of the TE families that are up-regulated in RdDM mutants show higher expression in
418 the endosperm than other tissues (Figure 5A). In contrast, the TE families up-regulated in CMT
419 mutants do not show any evidence for higher expression in a particular tissue type. The

420 enrichment for endosperm expression in TE families up-regulated in RdDM mutants does not
421 extend to genes up-regulated in the mutants and cannot be simply attributed to lower
422 expression of the *Mop1* and *Mop3* genes in these tissues (Figure 5). This result highlights the
423 potential for some TEs to escape RdDM-based silencing in endosperm, where dynamic
424 changes to DNA methylation may reinforce TE silencing in the embryo (Martínez and Slotkin
425 2012; Gehring 2013; Wang *et al.* 2015; Dong *et al.* 2017). Meanwhile, both genes and TE
426 families susceptible to mis-regulation in CMT mutants are less often expressed across
427 development, consistent with the greater developmental stability of CHG methylation over CHH
428 methylation (Kawakatsu *et al.* 2016, 2017; Narsai *et al.* 2017; Bouyer *et al.* 2017).
429
430 **Evidence for locus-specific and coordinated changes in expression of TEs:**
431 While per-family analysis is useful in capturing additional expression dynamics of repetitive
432 transposable elements, the expression of individual elements can be influenced by a variety of
433 location-specific attributes such as methylation levels and proximity to genes as well as family-
434 level attributes such as binding motifs and nucleotide content. We were interested in
435 documenting the relative behavior of different elements within the same family to understand
436 whether the changes in expression of TEs were occurring in an element-specific or family-wide
437 manner. Coordinate changes in expression could indicate the importance of RdDM or CMT for
438 family-wide regulation while element-specific changes could reflect influences at particular loci.
439 A set of TE families with <10 elements that had altered expression and for which at least 50% of
440 the reads could be uniquely assigned to specific element were identified and used for analysis
441 of coordinate versus locus-specific expression (Tables S3, S4). The unique mapping reads for
442 these families were used to evaluate element-specific expression. Half of the testable TE
443 families had expression of a single member of the family indicating locus-specific changes
444 (examples in Figure 6A, C). In the other half of the TE families there was evidence for
445 expression changes for multiple elements of the same family (Figure 6B, D). This suggests at

446 least some level of coordinate regulation of multiple members of the family by CMT or RdDM
447 pathways. However, even in examples of coordinate expression a single element accounted for
448 the vast majority of unique reads mapping to the family. Examples of both locus-specific and
449 coordinate changes in expression for both CMT and RdDM mutants were found but we were not
450 able to assess enough families to determine if there was any enrichment for the type of
451 regulation for these two silencing pathways.
452

453 DISCUSSION

454 Maize has been a model system for the discovery of several epigenetic phenomena such as
455 imprinting (Kermicle 1970; Kermicle and Alleman 1990), paramutation (Brink 1956; Chandler
456 2007; Hollick 2017) and transposon silencing (Chandler and Walbot 1986; Chomet *et al.* 1987).
457 An unresolved question is whether epigenetic regulation plays important roles in quantitative
458 trait variation beyond handful of well characterized loci. Our ability to document the full role for
459 epigenetic regulation and DNA methylation has been limited by our inability to recover plants
460 with major reductions in the level of DNA methylation (Li *et al.* 2014). Forward genetic screens
461 have uncovered a number of components of the RNA-directed DNA methylation (RdDM)
462 machinery as playing critical roles in maintenance of silenced paramutant states (Dorweiler *et*
463 *al.* 2000; Jr *et al.* 2009) or transgene silencing (McGinnis *et al.* 2006). These mutants have
464 substantial effects on CHH methylation in maize but have minimal effect on genome-wide levels
465 of CG or CHG methylation (Li *et al.* 2014). Reverse-genetic analyses have identified loss-of-
466 function alleles for a number of other genes predicted to play important roles in DNA
467 methylation but the only single mutants with significant effects on genome-wide DNA
468 methylation are the CMT genes of maize, *Zmet2* and *Zmet5 (Li et al. 2014)*. In this study we
469 have documented how these subtle perturbations of the maize methylome affect the
470 transcriptome in order to find genes subject to epigenetic regulation.
471

472 The effects of mutations in RdDM or CMT genes in maize are quite limited. Our evidence
473 suggests that there is little effect on the overall transcriptome of these plants. This might be
474 expected given the limited effect on overall plant phenotype for each of these mutations. A
475 recent study found that *rmr6* (allelic to *mop3*) mutants exhibited transcription changes from a
476 larger portion of the genome but much of this was associated with increased transcriptional
477 'noise' at lowly expressed regions (Forestan *et al.* 2017). However, there are sets of genes with
478 clear changes in expression in each of the mutant lines in our study. Similarly, a directed
479 analysis of differential expression in *rmr6* found a smaller set of genes with significant changes
480 (Forestan *et al.* 2017). While relatively few genes exhibit major changes in expression there are
481 a significant number of genes that exhibit similar expression changes in multiple RdDM or CMT
482 mutants. These findings are compatible with the concept that there are a small number of genes
483 in the maize genome that have epigenetic regulation that is solely dependent upon RdDM or
484 CMT mediated regulation. It is likely that a much larger number of genes are redundantly
485 regulated by the RdDM and CMT pathway along with MET1 mediated CG methylation.
486
487 Many of the genes that are up-regulated in CMT mutants exhibit high levels of CHG
488 methylation. The CMT mutants reduce this methylation and allow for increased expression.
489 Previous studies noted that the genes sensitive to *zmet2-m1* mutations varied in different maize
490 inbreds (Makarevitch *et al.* 2007). This prompted us to investigate whether the genes that are
491 up-regulated in CMT mutants might exhibit natural variation for DNA methylation levels. Many of
492 the genes that are up-regulated in CMT mutants have CHG DMRs nearby and many of these
493 exhibit variable levels of expression among maize genotypes that is negatively correlated with
494 CHG methylation levels. This suggests epiallelic diversity for targets of CMT-mediated gene
495 silencing. If these changes in expression lead to phenotypic variation, plant breeders are likely
496 able to select for preferred epigenetic states. However, it would also be possible to introduce
497 novel epigenetic variation through reductions of CHG methylation.

498

499 DNA methylation is often considered to play a primary role in maintaining genome integrity by
500 silencing transposable elements. Indeed, there are clear examples of release of transposon
501 silencing in mutants affecting DNA methylation in *Arabidopsis* (Miura *et al.* 2001; Mirouze *et al.*
502 2009) and maize (Lisch *et al.* 2002; Jia *et al.* 2009). However, generating a complete
503 understanding of transposon expression is complicated by the highly repetitive nature of
504 transposable elements. In order to survey expression using RNAseq most researchers focus on
505 unique mapping reads to ensure that expression is accurately attributed to the proper genomic
506 locus. In this study we elected to primarily focus on TE families rather than individual elements
507 and we utilized an approach that allowed for the combined use of unique and multiple-mapping
508 reads to assess TE family expression. We did find evidence that a number of TE families
509 require RdDM and/or CMT for silencing. There were few distinguishing features about these
510 TEs relative to others making it unclear why the silencing of these families was easily released
511 in these mutants. For several families we were able to document evidence for specific release of
512 silencing of a single member of the family while in other cases we found that multiple members
513 of the same family were reactivated.
514
515 This study defines a set of genes and TE families that are regulated by DNA methylation. The
516 silencing of these genes and TEs relies solely upon RdDM or CMT based epigenetic regulation.
517 These loci provide important insights into the mechanisms that allow for epigenetic regulation
518 and the natural variation for epigenetic regulation in maize.
519

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FIGURES

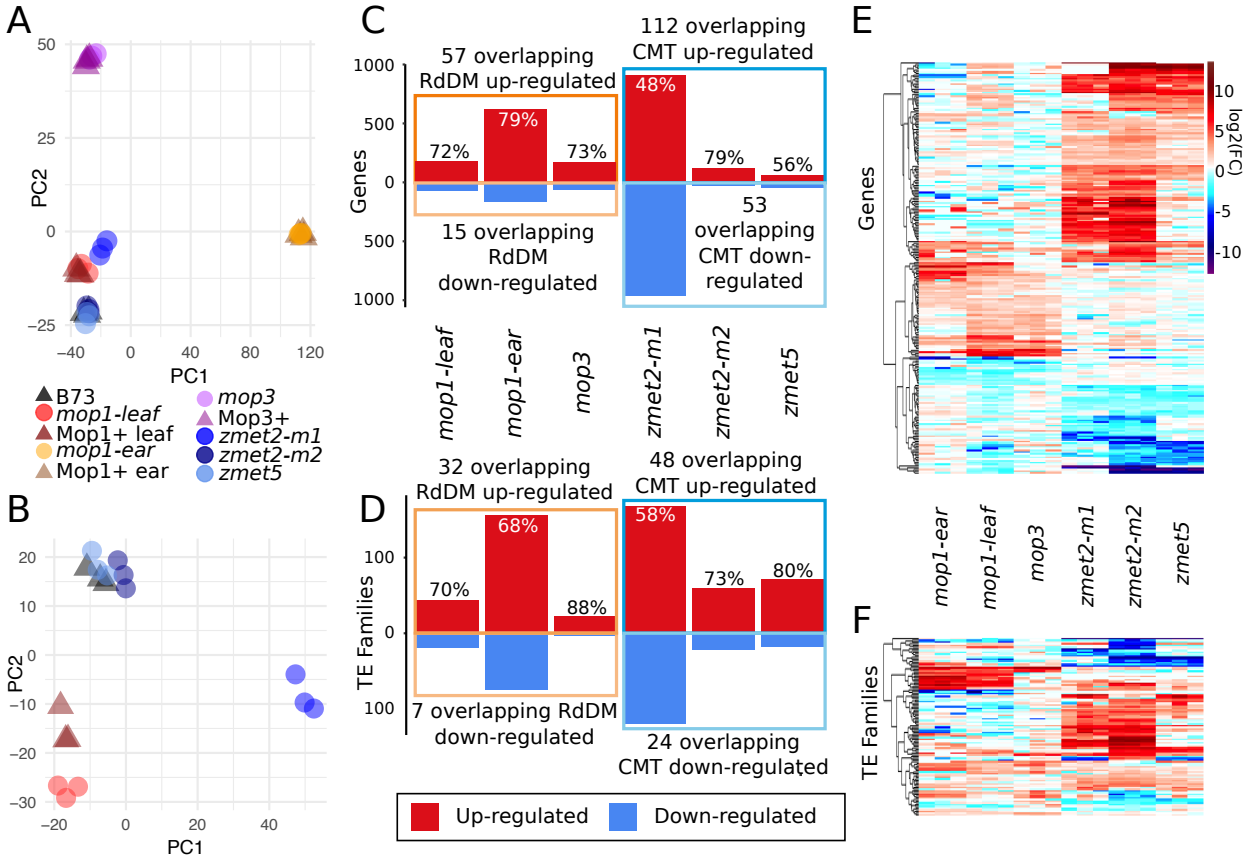


Figure 1: Summary of differentially expressed genes in mild methylation mutants. A-B: A principal component analysis (PCA) was performed using $\log_2$(RPM+1) expression values for genes that are DE in at least one mutant line relative to the appropriate control. (A) The full set of samples used for this study were assessed and we found that samples in other genetic backgrounds (*mop3* and *Mop3*) or tissues (*mop1* ear and *Mop1* ear) have the highest level of variation. (B) A second PCA was performed using only samples in the B73 genetic background assessed in leaf tissue. WT samples are denoted with triangles and mutants with circles. C-D: The number of up- (red) and down-regulated (blue) genes (C) and TEs (D) is shown for each mutant relative to the appropriate wild-type control. The percent of DE genes or TE families that are up-regulated is marked above each bar, and the number of genes or TEs with consistent changes in two or more mutants from the same pathway are labeled. E-F: Each of the genes (E)

680 or TE families (F) that are DE in at least two CMT or RdDM mutants were used to perform
681 hierarchical clustering using the Euclidean method and the $\log_2$ of the fold-change relative to
682 wild-type is visualized with a heat map.
683
684

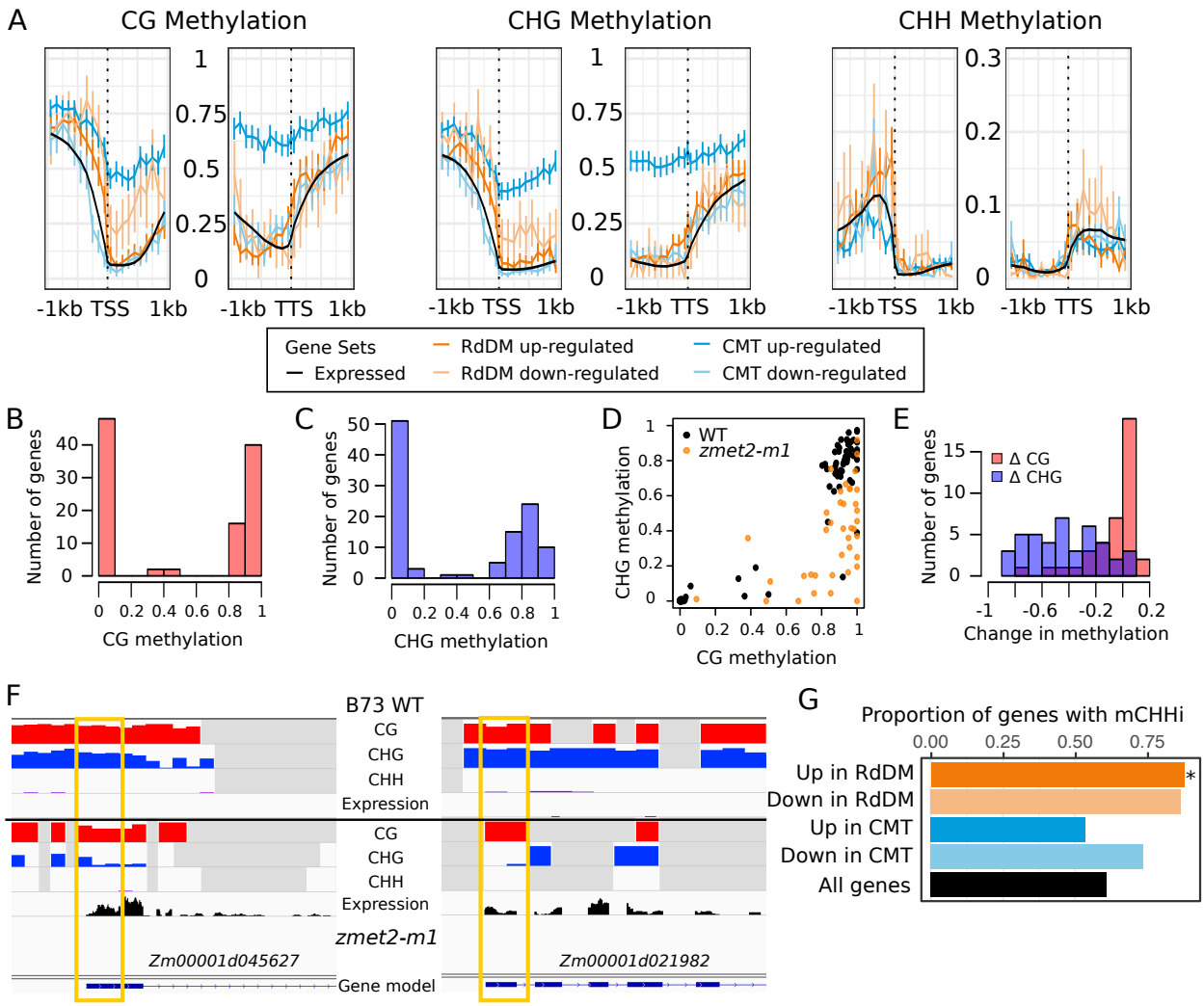


Figure 2: Methylation profiles of DE genes. A. The metaprofile of DNA methylation levels in wild-type B73 seedling leaf tissue was assessed for different sets of DE genes. The DNA methylation levels surrounding the TSS and TTS were plotted for all expressed genes and genes DE in CMT (blue) and RdDM (orange) mutants. The three panels show the levels of CG, CHG, and CHH methylation, with the y-axis showing DNA methylation levels. Error bars represent standard error. B-C Histogram of the number of genes with different methylation levels in the first 400 bp downstream of the TSS in the CG (B) and CHG (C) contexts, showing a bimodal distribution of methylation values. D. Wild-type methylation levels in the CG and CHG contexts are correlated. *zmet2-m1* mutant methylation data is shown for those genes with high

695 (>50%) CHG methylation in wild-type (orange dots), showing a loss of CHG but not CG
696 methylation in the mutant. E. Histogram of the difference between mutant and WT methylation
697 in the CG (red) and CHG (blue) contexts for those genes up-regulated in CMT mutants that
698 have WT CHG methylation >50%. F. IGV view of two up-regulated genes in CMT mutants:
699 *Zm00001d045627* and *Zm00001d021982,* which have high CG and CHG methylation in WT
700 and reduced CHG methylation near the TSS (yellow box) in *zmet2-m1* mutants. G. The
701 proportion of genes within 2 kb of mCHH islands (mCHHi) is shown for different sets of genes.
702 The black bar shows the proportion of all genes with a mCHH island while the other bars show
703 the proportion of genes with altered expression in specific mutant backgrounds that have mCHH
704 islands, and * denotes significantly higher proportion than expected relative to all genes (p-val <
705 0.01, chi squared test).
706
707

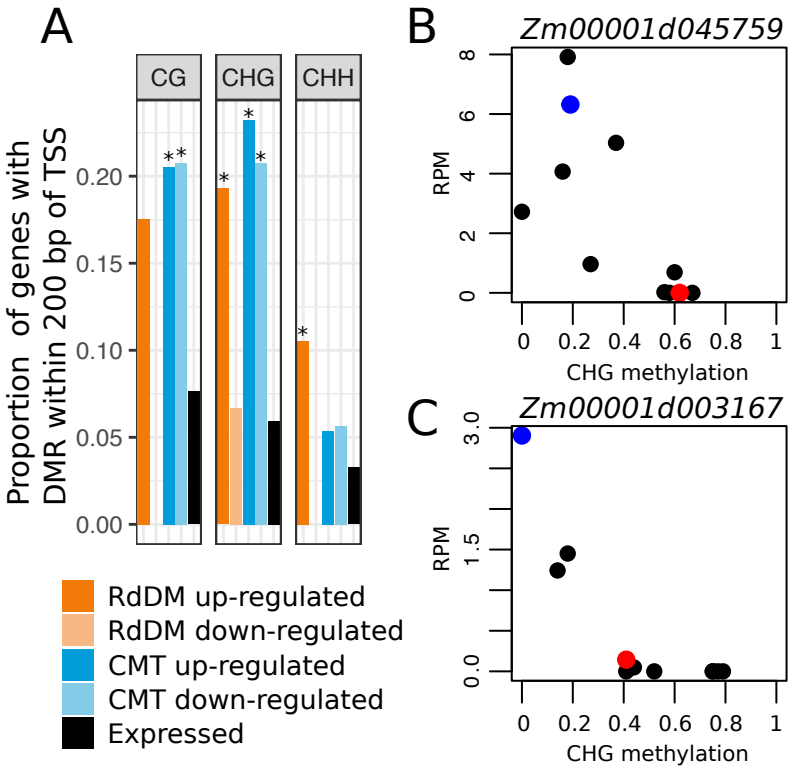


Figure 3. Natural variation for methylation level. A. DMRs in diverse genotypes located within 200 bp the TSS for all genes and for genes with differential expression in either CMT or RdDM mutants. Significant enrichment compared with expressed set is denoted with * (p-value < 0.01, chi squared test). B-C. Examples of genes up-regulated in CMT that have negatively correlated expression and CHG methylation at the bin overlapping the TSS. Data points show values for B73 (red), *zmet2-m1* mutants (blue), and 9 diverse genotypes (B97, CML322, HP301, IL14, Mo17, Oh43, P39, Tx303, and W22, black).

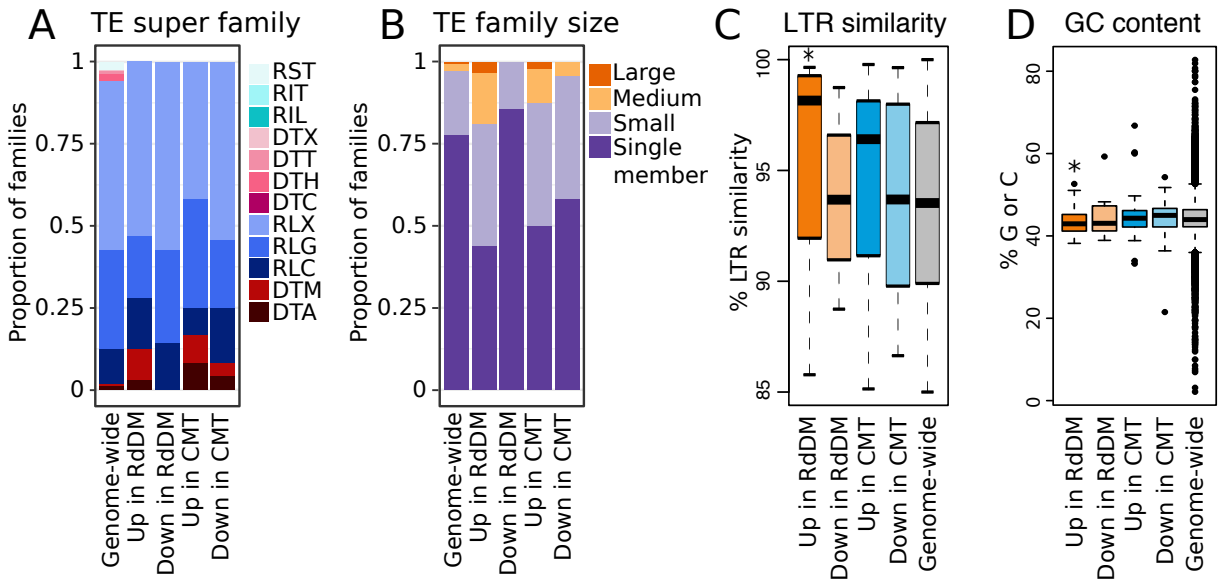
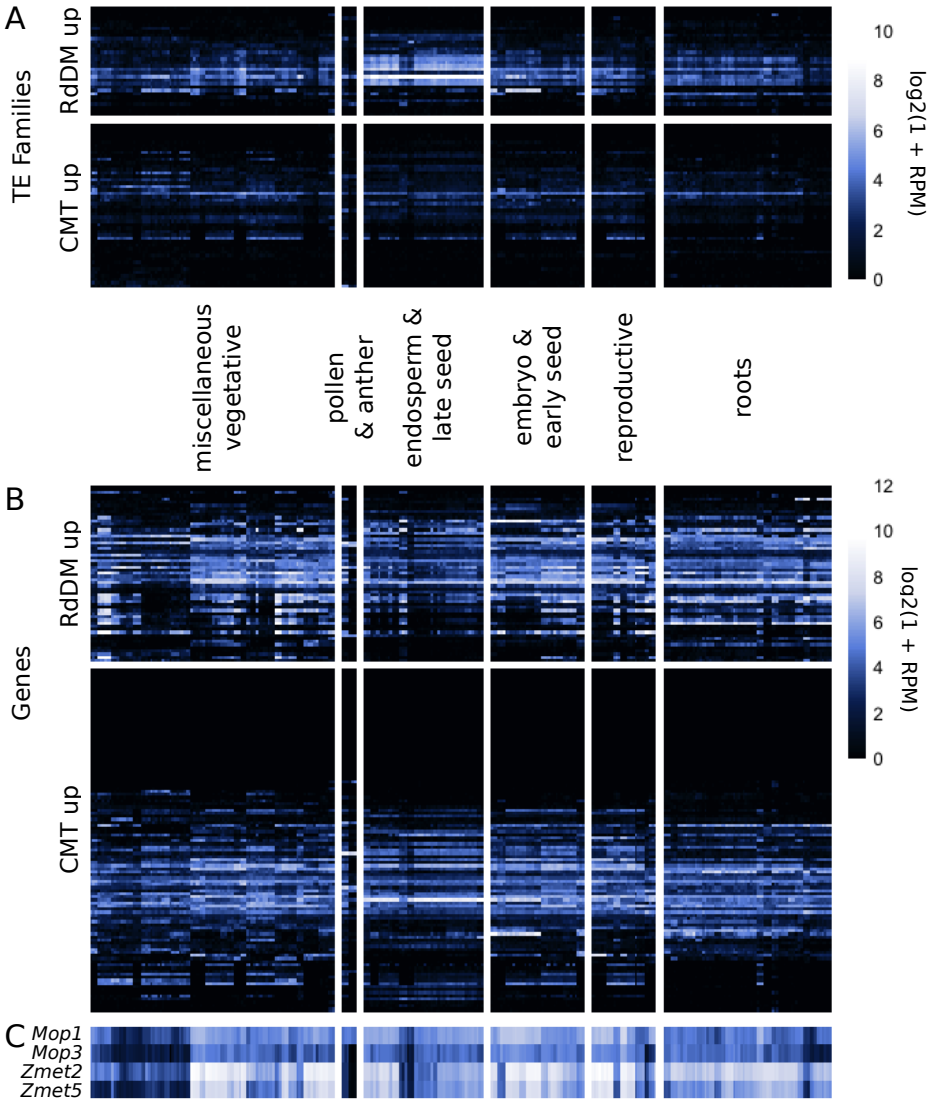


Figure 4. Attributes of TE families with altered expression in methylation mutants. A. TE super family membership for TE families genome-wide and with varied expression in mutants, where RST = SINE, RIT = LINE, RIL = LINE-L1-like, DTX = TIR-unclassified, DTT = TIR-Tc1/Mariner, DTH = TIR-PIF/Harbinger, DTC = CACTA, RLX = LTR-unclassified, RLG = LTR-Gypsy, RLC = LTR-Copia, DTM = TIR-Mu, and DTA = TIR-hAT. B. Size distribution for TE families genome-wide and with varied expression in mutants, where small: 2-9 members, medium: 10-99 members, and large: >= 100 members. C. Boxplot of the average LTR similarity per-family for LTR TE families genome-wide, along with those LTR families with differential expression in methylation mutants. D. Boxplot of the average GC content per-family for TE families genome-wide and with expression changes in the mutant. * denotes significant deviation from the mean for all TE families (p-value < 0.01, t-test).



730

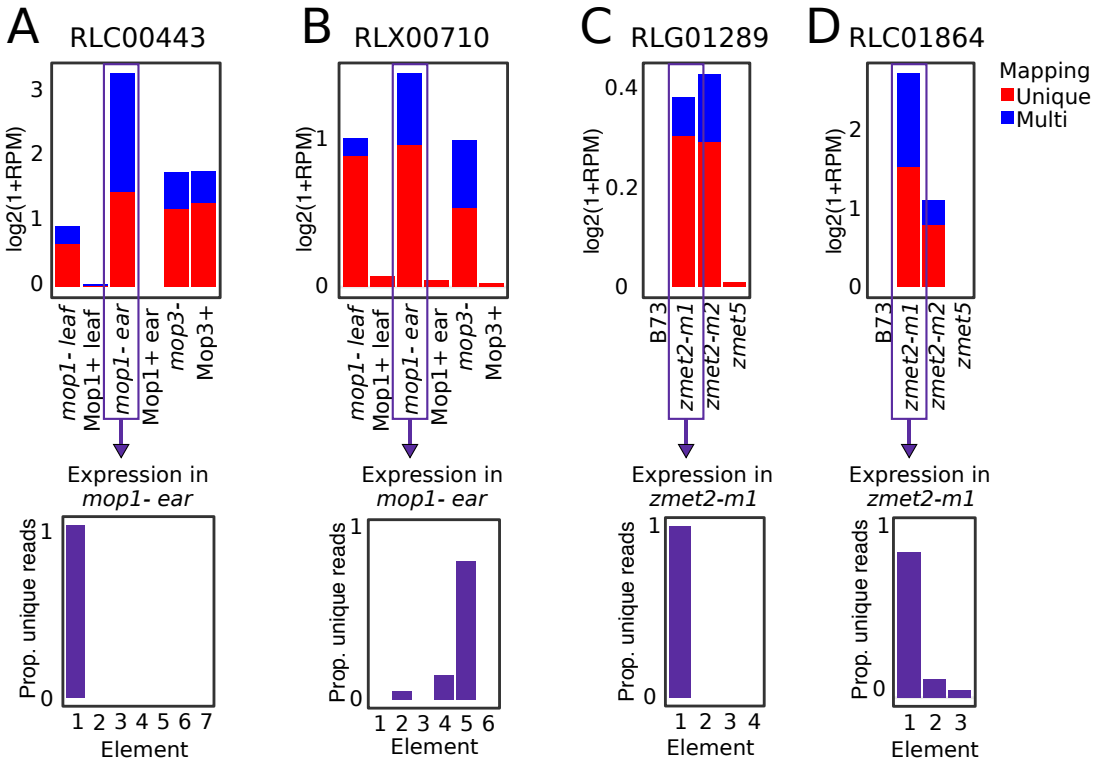
731 Figure 5. Developmental expression of TE families (A) and genes (B) up-regulated in RdDM and
732 CMT mutants, along with the typical expression of genes mutated in this study (C), where rows
733 show TE families or genes and columns show RNA-seq libraries. Developmental samples are
734 grouped by tissue type, with seed samples split into two clusters based on relative contribution
735 of endosperm: endosperm & late seed (12+ days after pollination) and embryo & early seed (up
736 to 10 days after pollination). For full list of tissue assignments and RNA-seq library accession
737 numbers, see Table S1. Approximately one third of TE families up-regulated in RdDM mutants
738 have higher expression in endosperm than other tissues across development, a pattern not

739 observed for genes. In contrast, many more TE families and genes up-regulated in CMT

740 mutants are never or lowly expressed during typical development.

741

742



743

744 Figure 6: TE families up-regulated in methylation mutants can have expression of a single
745 element or multiple elements. A-B Show two LTR families up-regulated in RdDM mutants, and
746 C-D show two LTR families up-regulated in CMT mutants. All families have both unique (red)
747 and multi-mapped (blue) reads. The proportion of unique-mapping reads assigned to each
748 element is shown for a representative library. Unique-mapping reads showed confident
749 expression of only a single member of a family (A and C) or coordinated expression of more
750 than one member of a family (B and D).

SUPPLEMENTAL MATERIALS

Supplemental Figures S1-S4: attached as separate document

Table S1: Complete list of datasets used in this study.

Table S2: Expression values, differential expression calls, list assignments, and DMR calls for all genes.

Table S3: Expression values, differential expression calls, list assignments, and descriptions for all TE families.

Table S4: Unique mapping read counts and descriptors for each unique transposable element.