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1 **DNA demethylation and hypermethylation are both required for late nodule development in**
2 **Medicago**

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10

11 Introductory paragraph

12

13 Plant epigenetic regulations are involved in transposable element (TE) silencing, developmental
14 processes and responses to the environment¹⁻⁷. They often involve modifications of DNA
15 methylation, particularly through the DEMETER (DME) demethylase family and RNA-dependent DNA
16 methylation (RdDM)⁸. Root nodules host rhizobia that can fix atmospheric nitrogen for the plant
17 benefit in nitrogen-poor soils. The development of indeterminate nodules, as in *Medicago*
18 *truncatula*, involve successive waves of gene activation⁹⁻¹², the control of which raises interesting
19 questions. Using laser capture microdissection (LCM) coupled to RNAseq (SYMBiMICS data¹¹), we
20 previously identified 4,309 genes (termed NDD) activated in the nodule differentiation and nitrogen
21 fixation zones, 36% of which belong to co-regulated genomic regions dubbed symbiotic islands¹³. We
22 found *MtDME* to be upregulated in the differentiation zone and required for nodule development,
23 and identified 474 differentially methylated regions (DMRs) hypomethylated in the nodule, by
24 analyzing ~2% of the genome⁴. Here, we coupled LCM and whole-genome bisulfite-sequencing
25 (WGBS) for a comprehensive view of DNA methylation, integrated with gene expression at the tissue
26 level. Furthermore, by CRISPR-Cas9 mutagenesis of *MtDRM2*, we showed the importance of RdDM
27 for CHH hypermethylation and nodule development. We thus proposed a model for DNA
28 methylation dynamics during nodule development.

29 Main text

30

31 We first performed WGBS using *M. truncatula* nodules at 6 days post inoculation with *Sinorhizobium*
32 *meliloti* (stage of maximal *MtDME* expression⁴) and nitrogen-starved non-inoculated root tips (RT)
33 (two replicates; sequencing coverage of ~16 to 25x, supplemental Table 1). Indeterminate nodules
34 consist of an apical meristem (or zone I), an infection and early differentiation zone (distal and
35 proximal zone II), a late differentiation zone (interzone II-III), and a nitrogen fixation zone (zone III).
36 To distinguish different developmental stages, we analyzed three laser-dissected nodule zones,

37 namely the meristematic (M), differentiation (Diff), and nitrogen-fixation (Fix) zones (Fig. 1a; three
38 replicates; average BsSeq coverage of 5.7, 10.5 and 15.8x respectively; supplemental Table 1). BsSeq
39 data showed a good sample reproducibility, and allowed two groups to be clearly distinguished, one
40 corresponding to the differentiated nodule tissues (Diff and Fix) and the other to the whole organs
41 and M zone (Fig. 1b). While little difference was observed between whole organs, an increase of CHH
42 methylation was found in the Diff and Fix zones (Fig. 1c, 1d), on all chromosome regions (Fig. 1e),
43 with an average methylation level of 12.0% and 15.1% respectively, vs. 6.8% in RT. Changes in DNA
44 methylation in all three contexts CG, CHG, and CHH were then revealed by DMR analyses, when
45 comparing Diff and Fix vs RT (Fig. 1f). The CHH DMRs (Fix vs RT) encompassed 22.8 Mb vs. only 0.82
46 Mb for the CG-CHG DMRs (5.3% and 0.2% of the nuclear genome, with an average size of 355 and
47 233 nt, respectively). The DMRs (shown with methylome data in the M.t 5.0 genome browser¹³
48 <https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>) were consistent with previously identified
49 DMRs⁴ and highly reproducible, whether comparing biological replicates, whole organ vs LCM
50 samples, or Diff vs Fix zones. In conclusion, a strong dynamics of DNA methylation during the late
51 stages of nodule development was uncovered.

52 At the gene level, in the CG context, average methylation profiles showed gene body methylation
53 with a strong decrease at the transcription start (TSS) and termination (TTS) sites (Fig. 2a), as
54 commonly observed in angiosperms¹⁴. While all samples were very similar when considering the
55 average of all genes, gene body hypomethylation was observed in nodule samples when focusing on
56 the 4,309 NDD genes. Such hypomethylation was not seen when considering the 4,309 genes most
57 expressed in roots, or TE-related repeats (hereafter called TEs for simplification) (Fig. 2a). By
58 contrast, in the CHH context, and the CHG context to a lower extent, a strong hypermethylation was
59 observed for all genes and TEs in the Diff and even more the Fix samples, with a peak of
60 hypermethylation in the 1kb region upstream the gene TSS (Fig. 2b and 2c).

61 CHG DMRs appeared more similar to CG DMRs than to CHH DMRs, both in terms of localization and
62 pattern. Thus, 98.8% of CG DMRs, 85.5% of CHG DMRs and 99.5% CG-CHG DMRs were hypo-
63 methylated in Fix vs RT, whereas all CHH DMRs were hyper-methylated (>99.9% in Diff and Fix vs RT),
64 in all cases with a large difference of methylation level (mean absolute difference of 0.53 and 0.42 in
65 the CG-CHG and CHH context, respectively) (supplemental Table 2). Many DMRs were close to genes,
66 with 71.9% of CG-CHG DMRs and 47.9% of CHH DMRs overlapping with genes extended by 1 kb on
67 each side. Moreover, whereas only 14% of CG-CHG DMRs overlapped with CHH DMRs, 75.9% of CG-
68 CHG DMRs overlapped with 1kb-extended CHH DMRs, indicating their proximity (see a
69 representative example in Extended Data Fig. 1). However, the CG-CHG DMRs preferentially targeted
70 genes (Fig. 2d) whereas the CHH DMRs mostly targeted TEs (66.1%, 88.5% and 96.6% overlap with
71 Tephra-annotated¹³, EDTA¹⁵-annotated and TASR¹⁶-annotated TEs, respectively). Finally, CG-CHG
72 DMRs were strongly enriched in NDD islands compared to their flanking regions, unlike in apical
73 (NDA) and non-spatially regulated (NDN) islands (Fig. 2e). Thus, in summary, late nodule
74 development is accompanied by hypomethylated CG-CHG DMRs centered on a limited number of
75 genes, and hypermethylated CHH DMRs targeting TEs, with nearly half of them close to CG-CHG
76 DMRs.

77 To further integrate gene expression (LCM-RNAseq) and methylation (LCM-BsSeq), we analyzed the
78 DMR distribution amongst the 16 expression patterns previously defined within the nodule¹³ (Fig. 3a-
79 b). CG-CHG DMRs tightly positively correlated with genes expressed in the Diff and Fix zones
80 (Patterns 5-11 and 14-16), maximally in Pattern 6 to 9 (47.5% of 1,218 genes with DMRs) (Fig. 3c;
81 Extended Data Fig. 2). These hypo-DMRs are associated with key or potential actors in nodule
82 development and activity, notably genes encoding 380 NCR peptides⁹, leghemoglobins¹⁷,
83 transporters, calmodulin-like proteins, symbiotic immune response regulators (MtDNF2¹⁸,
84 MtSymCRK¹⁹, MtNAD1²⁰, MtRSD²¹), redox control proteins and 508 long non-coding RNAs
85 (supplemental Table 3). When comparing the regulation of NDD genes with and without CG-CHG
86 DMRs (1,723 and 2,586 genes respectively), we found that gene induction in nodules was much

87 stronger for NDD genes with CG-CHG DMRs [median fold change (FC) vs root expression of 280.6 and
88 12.7, respectively; supplemental Table 4]. This was mostly due to a lower expression in roots (median
89 root expression with and without DMR of 0.01 and 0.09 counts per million, respectively), correlating
90 with a higher DNA methylation in roots (Extended Data Fig. 3). Finally, as previously observed¹³,
91 many NDD genes (particularly in expression patterns 5 to 10) presented a highly differential
92 distribution of histone marks, with repressive H3K27me3 marks in roots and active H3K9ac marks in
93 nodules (Fig. 3d), supporting complex epigenetic regulations in symbiotic islands.

94 In contrast to the CG-CHG DMRs, the abundance and distribution of CHH DMRs strongly differed
95 between the Diff and Fix zones (Fig. 3c). In the Diff zone, CHH DMRs were located next to 11.7% of
96 genes, preferentially those showing CG-CHG DMRs (Pearson correlation of 0.81; P-value < 0.001),
97 whereas, in the Fix zone, they were next to 45.9% of all genes, whatever their expression profile. An
98 attractive hypothesis is therefore that hyper CHH DMRs are generated first next to hypo CG-CHG
99 DMRs in the Diff zone and then spread genome-wide. Two pathways have been reported in
100 Arabidopsis for CHH methylation, involving RdDM and the CMT2 chromomethylase (in a DDM1-
101 dependent process), respectively⁸. *CMT2* and *DDM1* genes are weakly expressed in the Diff and Fix
102 zones, in contrast to RdDM genes (supplemental Table 4), making RdDM the best candidate pathway
103 for CHH hypermethylation. We tested this assumption using mutants of DRM2, the main DNA
104 methylase in RdDM. We identified three *M. truncatula* homologues of AtDRM2 with a good
105 conservation of its reported active sites²², termed MtDRM2, MtDRM2L1 (DRM2-like1) and
106 MtDRM2L2 (Extended Data Fig. 4). We then performed multi-guide CRISPR-Cas9 mutagenesis of both
107 *MtDRM2* and *MtDRM2L2* (the second best expressed family member in nodules), using hairy root
108 transformation. WGBS was carried out with pooled DNA from nodules showing various mutations in
109 *Mtdrm2/Mtdrm2l2* (hereafter termed *drm*; Extended Data Fig. 5), using nodules poorly affected in
110 size, thereby decreasing confounding effects due to developmental problems. Control samples
111 consisted of nodules transformed with a CRISPR construct targeting the *GUS* gene. CG methylation
112 was mostly unaffected in *Mtdrm* mutant nodules (17 DMRs hyper methylated in CRISPR-*GUS* vs *drm*

113 nodules). By contrast, CHH (Fig. 3e) and to a lower extent CHG hypermethylation were strongly
114 decreased (respectively 9,951 and 391 DMRs hypermethylated in both replicates of CRISPR-*GUS* vs
115 *Mtdrm* nodules with a difference in absolute methylation level >0.3 , vs. no hypermethylated DMR in
116 *Mtdrm* nodules) (supplemental Table 5). This implied that RdDM is involved in CHH and part of CHG
117 hypermethylation in nodules, which was supported by 87.4% of CHH DMRs overlapping with 24nt
118 siRNA clusters (71.9%, 91.4% and 97.7% of which co-localize with Tephra-, EDTA- and TASR-
119 annotated TEs, respectively). We set up a complementation experiment where the CRISPR-*DRM*
120 construct was co-transformed with a non-editable synthetic *MtDRM2* cDNA (termed *DRM2-R*)
121 expressed from a *pAtUBI* promoter. WGBS showed that *DRM2-R* allowed CHH hypermethylation of
122 genes and TEs to be recovered in nodules (Fig. 3e; only 186 DMRs hypermethylated in CRISPR-*GUS* vs
123 *Mtdrm/DRM2-R*).

124 To assess the impact of RdDM on nodule development, we examined the phenotype of nodules
125 induced by *S.meliloti nifH:GFP* (a marker of late rhizobium differentiation) (Fig. 3f-g). While the
126 nodule number was not significantly modified (Extended Data Fig. 6a), *nifH:GFP* expression was
127 affected in 50% of CRISPR-*DRM* nodules (n=114), vs 4.5% of CRISPR-*GUS* (n=89) and 3.2% of CRISPR-
128 *DRM pUBI:DRM2-R* (n=31) nodules. In the most severe cases (about 25% of independently
129 transformed roots), small, round-shaped and white nodules were formed with very low *nifH:GFP*
130 expression and nitrogenase activity (based on an acetylene reduction assay) (Fig. 3g; Extended Data
131 Fig. 6b; Extended Data Fig. 7). As for WGBS, we analyzed the transcriptome of *Mtdrm* nodules slightly
132 affected in size. Similar number of genes were up- and down-regulated vs CRISPR-*GUS* nodules (1,011
133 and 913 genes respectively, $FC>2$, $FDR<0.05$), while more TEs were up- than downregulated in *drm*
134 nodules (282 and 91 TEs respectively, $FC>2$, $FDR<0.05$), consistent with an increased transcription of
135 less methylated TEs. Down-regulated genes, including 83 *NCR* genes, were predominantly found in
136 patterns 8 to 11, as strongly upregulated genes ($FC>4$) (supplemental Table 6). This indicated a
137 particular impact of non-CG methylation on transcriptional regulation in the late differentiation zone.
138 Of note, *MtROS1* and *MtCMT2* (DNA demethylase and CHH methylase genes, respectively) were

139 down- and up-regulated in the mutant respectively, possibly as compensation for the loss of
140 DRM2^{8,23,24}. Most Pattern 8 to 11 genes that were down and upregulated in *drm* nodules had a CHH
141 DMR (Fix vs RT) within 1kb (85.1% and 67.6% respectively, supplemental Table 7), suggesting that the
142 methylation status of TEs may either positively or negatively impact the expression of certain Diff/Fix
143 genes.

144 Two non-exclusive hypotheses could explain an increase in RdDM during late nodule development:
145 (i) an increased expression of TEs, leading to increased siRNA production; (ii) a more conducive
146 environment due to chromatin relaxation²⁵. A re-analysis of SYMBiMICS data (see Methods) revealed
147 1,923 nodule-expressed TEs, with 1,321 of them expressed in the Diff/Fix zones, enriched in type I
148 TEs (43.2% vs 34% for all chromosomal TEs) (supplemental Table 7). These Diff/Fix TEs showed a high
149 induction level (up to a median 47-fold increase vs zone 1 in Pattern 9) and were often close to NDD
150 genes (48.1% within 1 kb). Visual inspection of RNAseq data on the Mt5.0 genome browser
151 suggested that TEs could be expressed either autonomously or by read-through transcription from
152 strongly expressed genes (see an example in Extended Data Fig. 8). A higher fraction of Diff/Fix TEs
153 overlapped with CHH DMRs compared to the total TE population (44.7% vs. 23.3%, respectively, with
154 a mean overlap length of 761 vs. 228 nt). Thus, an increased TE expression is a likely cause of
155 genome-wide hypermethylation, but probably not the only one. To assess our second hypothesis, i.e.
156 a possible decondensation of heterochromatin, we examined DAPI-stained nodule sections by
157 confocal microscopy. We observed enlarged nuclei in the Diff/Fix zones, but their chromocenters
158 remained well visible (Extended Data Fig. 9), which did not support an extensive decondensation of
159 pericentromeric heterochromatin. Decondensation of facultative heterochromatin in chromosome
160 arms remains however conceivable, all the more since we found that all eight *M. truncatula* *H2A.W*
161 genes encoding a histone 2 variant involved in heterochromatin compaction²⁶ are strongly down-
162 regulated in the nodule interzone and zone III (supplemental Table 4).

163 In conclusion, while we previously uncovered (i) the importance of DNA demethylation for Medicago
164 nodule development⁴ and (ii) symbiotic islands enriched with differential mCHH and histone marks in
165 nodules versus roots¹³, here we showed the extent and dynamics of DNA methylation in the nodule,
166 by coupling LCM and WGBS. We established that CG-CHG demethylation is restricted to genes in a
167 few genomic regions, including symbiotic islands, in contrast to CHH hypermethylation, that affects
168 TEs first next to CG-CHG DMRs and then throughout the genome. This hypermethylation is RdDM-
169 mediated and important for nodule development. In Arabidopsis, RdDM-dependent hyper-CHH
170 DMRs have been described in male sexual-lineage cells²⁷, the embryo²⁸ and root meristematic cells²⁵
171 but are unusual in differentiated somatic cells. The CHH hypermethylation reported here is more
172 reminiscent of that reported for miRNA genes in soybean nodules²⁹ and the so-called mCHH islands
173 found in maize and other plant species next to highly expressed genes, and proposed to act as
174 barriers between euchromatin and heterochromatin to prevent TE expression^{14,30}.

175 In our model for DNA methylation dynamics in *M. truncatula* nodules (Fig. 4), a subset of late
176 symbiotic genes are found in genomic regions that are strongly repressed in the root by DNA
177 methylation and repressive histones. A combination of CG-CHG demethylation and histone
178 modifications would make these regions accessible to the transcriptional machinery in the nodule
179 differentiation zone. This would lead to transcriptional induction of Diff/Fix genes and nearby TEs,
180 either from their own promoters or by read-through transcription from strongly expressed upstream
181 genes. This would trigger an increased siRNA production and RdDM, as a plant protection against TE
182 activation, progressively targeting all related TEs. In our model, TE hypermethylation would thus be
183 primarily a consequence of TE transcription, as well as possibly relaxation of facultative
184 heterochromatin. Since the methylomes of the nodule meristem and Diff/Fix zones are very different
185 (Fig. 1b-c), the previously discussed hypothesis⁴ that siRNAs produced in the Diff/Fix zone might
186 migrate to the meristem to protect it against TE activation seems unlikely. In any case, RdDM is
187 important for optimal expression of late symbiotic genes, possibly to decrease interference between

188 TE and gene expression. Taken together, our data provide new insights on the complex epigenetic
189 landscape regulating gene expression during nodule development and nitrogen fixation.

190 Methods

191

192 **Plant growth and inoculation**

193 Non-transformed and *Agrobacterium rhizogenes*-transformed *M. truncatula* cv Jemalong A17 plants
194 were grown in aeroponic caissons as described³¹, with the following chamber conditions:

195 temperature: 22°C; 75% hygrometry; light intensity: 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; light-dark photoperiod: 16h-8h.

196 Plants were grown for about seven days in caisson growth medium supplemented with 5 mM

197 NH_4NO_3 , then nitrogen-starved for three days, just before rhizobium inoculation. Plants were

198 inoculated with 10 mL of a suspension ($\text{OD}_{600\text{nm}} = 1$) of *Sinorhizobium meliloti* 2011 (for 10 L of plant

199 growth medium), containing the pXLGD4 *hemA:lacZ* plasmid (GMI6526)³², grown at 28°C on TY solid

200 medium (Bacto Tryptone 5 g.L⁻¹ (Becton Dickinson), Yeast Extract 3 g.L⁻¹ (Duchefa Biochemie), Agar-

201 Agar 15 g.L⁻¹, pH 7) supplemented with 10 $\mu\text{g}\cdot\text{mL}^{-1}$ of tetracycline and 6 mmol.L⁻¹ calcium chloride.

202 Following *S. meliloti* inoculation, plants were grown in nitrogen-free medium. Under these

203 conditions, nodules became pink and nitrogen-fixing at about 7 dpi (non-transformed plants) or 10

204 dpi (composite plants transformed by *A. rhizogenes*).

205 **Root transformation**

206 Root transformation was carried out using *Agrobacterium rhizogenes* ARqua1 as described³³, except

207 that plants were kept at 20°C for three weeks after transformation. Transformed roots were selected

208 by kanamycin (25 mg/L) and systematically checked for the expression of a *DsRed* reporter gene

209 present on the T-DNA construct. The nodulation phenotype of composite plants was analyzed

210 following aeroponic growth in caissons.

211 **RNA and DNA extraction**

212 For BsSeq or RNAseq analyses of transformed roots/nodules, at least six independently transformed

213 root systems were used per replicate, and at least 15 nodules per root system. For RNAseq analyses

214 (three biological replicates per sample) RNA was extracted using the Qiagen RNeasy Plant Mini Kit
215 (Qiagen) with RNase-free DNaseI (Qiagen) treatment following the manufacturer's procedure. RNA
216 was quantified using a Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington,
217 DE, USA) and analyzed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).
218 For bisulfite sequencing, DNA was prepared using the Qiagen DNA easy kit or as described for high
219 molecular weight plant genomic DNA³⁴. It was quantified using a Qubit fluorometer (Invitrogen) and
220 analyzed with a Fragment Analyzer (Agilent).

221 **Laser micro-dissection of nodule zones**

222 Laser microdissection of 15 dpi nodules was carried out as described^{11,35}, using 24 µm nodule
223 sections and four biological replicates. Rep 1 was only used to set up conditions for the whole
224 procedure. DNA was extracted with the QIAmp micro DNA kit (QIAGEN), following the recommended
225 procedure (« Isolation of Genomic DNA from laser-microdissected tissues »). DNA was quantified
226 using a Qubit with the Qubit dsDNA HS Assay, and then pooled from 20 (rep 2 and 4) or 21 (rep 3)
227 nodules before library production. The amount of pooled DNA was: 81, 87, 99 ng for the dissected
228 meristematic zone (rep 2, 3, 4 respectively); 203, 218, 171 ng for the differentiation zone; 342; 361,
229 360 ng for the fixation zone (including *S. meliloti* DNA).

230 **Bisulfite sequencing and methylome data analyses**

231 WGBS of RT, N6, M, Diff and Fix samples was performed at the GeT-PlaGe core facility, INRAE
232 Toulouse (<http://www.get.genotoul.fr>). WGBS libraries were prepared according to Swift
233 Biosciences's protocol using the Accel-NGS Methyl-Seq DNA Library Kit for Illumina Sequencing.
234 Briefly, DNA was fragmented by sonication and bisulfite conversion was performed using the EZ DNA
235 Methylation-Lightning Kit (ZYMO Research) following manufacturer's recommendations. Sample
236 purifications were performed using SPRI select magnetic beads. Then, adaptors were ligated and
237 sequencing tags were added by PCR (10 PCR cycles). Library quality was assessed using a Fragment
238 Analyzer (Advanced Analytical Technologies, Inc.) and libraries were quantified by Q-PCR using the
239 Kapa Library Quantification Kit. Sequencing was performed using an Illumina HiSeq3000 (paired end

240 150 bp) and the Illumina HiSeq3000 Reagent Kits. WGBS of transformed root tips, CRISPR-*GUS*,
241 CRISPR-*DRM* and *DRM2-R* samples was performed by BGI Hong-Kong, using the EZ DNA Methylation-
242 Gold kit (ZYMO Research) and a HiSeq Xten (Illumina) platform (paired end 150 bp).

243 For each library, the raw BSseq reads were processed with Methylypy v1.4.1 using bowtie2 (v.2.3.4.3)
244 as aligner with Methylypy embedded default parameters "-X 1000 -k 2 --no-mixed --no-discordant --
245 sensitive", and MtrunA17 R5.0¹³ genome for mapping. PCR duplicates were removed with
246 MarkDuplicates (Picard suite v2.18.1). The methylation level of each cytosine was then computed
247 with Methylypy v1.4.1³⁶. The non-conversion rate of each sample was obtained by analyzing the rate
248 of methyl cytosines in the chloroplast genome (MtrunA17CP). The clustering analysis of Fig. 1b was
249 performed with methylKit³⁷ (R package, version 1.10), with a minimal coverage of ten for cytosines,
250 correlation-based distances and Ward's method. Identification of differentially methylated sites
251 (DMS) and differentially methylated region (DMR) calling were performed with Methylypy,
252 independently for CG, CHG, CG-CHG and CHH contexts, only considering cytosines covered by at least
253 four reads. DMS at a distance of less than 250 nt from each other were collapsed into DMRs. We also
254 tested distances of 50 nt, 150 nt and 350 nt, with qualitatively similar results. Were retained the
255 DMRs with FDR<0.05, containing at least 4 DMS, found in at least two replicates of each sample, and
256 with a minimal difference of absolute methylation level of 0.1 for CHH, 0.2 for CHG, 0.3 for CG-CHG
257 and 0.4 for CG³⁸.

258 The relative position of DMRs with other DMRs, genes or TEs were computed using Bedtools v2.30.0
259 (command "intersect" and "closest"). The relative proportion of methylated cytosines (Fig. 1c) was
260 determined using a betabinomial test and considering cytosines with a minimal sequencing coverage
261 of four. Representations of cytosine methylation level and DMR density were obtained using Circos³⁹
262 (<http://circos.ca>) (Fig. 1e), ViewBS⁴⁰ (v 0.1.9, using default parameters; Fig. 2a-c, Fig. 3e), and
263 DANPOS⁴¹ (bin size set to 200 bp; Fig. 2d-e, Fig. 3c-d, Extended Data Fig. 3).

264 Despite the fact that very little LCM DNA remained and that whole organs dilute the signals, five
265 CHH-DMRs could be independently validated by Chop-PCR⁴², using DNA from LCM-BsSeq analyses for

266 one of them, and newly prepared genomic DNA from root tips and 15 dpi nodules for the others
267 (Supplemental Table 8). For those, DNA was prepared by CTAB extraction from three biological
268 replicates, consisting of pools of 50 nitrogen-starved root tips from six plants and pools of 303, 434
269 and 357 nodules from five, ten and eight plants, respectively. One μg of DNA was digested with
270 either *Nla*III, *Alu*I or *Dde*I for one hour at 37°C according to manufacturer's procedure
271 (ThermoFisher). The control DNA consisted of one μg of DNA treated under the same conditions
272 without restriction enzyme. Quantitative PCR reactions were performed on a Roche Light Cycler 480
273 using the Light Cycler Fast Start DNA Master SYBR Green I kit according to manufacturer's
274 instructions (Roche). Cycling conditions were as follows: 95°C for 10 min, 50 cycles at 95°C for 5 sec,
275 60°C for 5 sec, and 72°C for 15 sec.

276 **Transposable element analyses**

277 A two-step protocol based on the mapping of siRNA reads was used to complement the published
278 structural annotation of TEs (Tephra pipeline¹³). In a first step the TASR pipeline¹⁶ was run on the
279 genome using 24nt siRNA reads extracted from sRNA datasets previously listed¹³ (excluding A17
280 mutant datasets) and from a Stem and Leaves dataset downloaded from NCBI (GEO Accession
281 GSE13761). siRNAs of 24nt with at least 10 reads among all datasets were selected.
282 The filtered siRNA dataset used as input of TASR contains 1,652,379 unique siRNA sequences with a
283 median number of sequenced reads of 18 (mean 38). TASR 1.1 pipeline was run with parameters: -
284 nsirna 4 -win 100 -minlen 80 -maxlen 20000 -idclust 0.9 -overlapclust 0.8 -cnumber 2 -idenblast 90 -
285 evaluate 1e-200 -usearchv usearch8.0.1623_i86linux64. The perl code was marginally modified to set -
286 K bowtie parameter to 10000 and to disable centroid selection (execution failure in some families).
287 Then, bedtools v2.27.1 was run to merge close repeat regions (merge -d 100). The TASR-based
288 annotation identified 13,309 regions spanning 69,918,880 nucleotides (minimum, maximum and
289 mean region lengths are 397, 204337, 5253 nucleotides, respectively).
290 The second step of the protocol aimed at increasing the sensitivity of the TE annotation. The bank
291 previously built by TASR was used as input repeat library of RepeatMasker 4.1.0, run with

292 parameters: -gff -s -no_is -norna -nolow -e rmbblast. Then, bedtools v2.27.1 (merge -d 100) was run
293 to merge close regions. The final dataset (labeled TASR10-round2-RepeatMarker in the genome
294 browser) identified 257,443 regions spanning a total of 218,292,089 nucleotides (minimum,
295 maximum and mean region lengths are 25, 318,396, 847 nucleotides, respectively).
296 The two annotations substantially overlapped, with 93.2% Tephra-annotated repeats intersecting
297 with TASR -annotated repeats. 86.9% of Tephra-annotated repeats were also found to overlap with
298 TEs annotated with the EDTA pipeline¹⁵. All bioinformatic studies were performed with the published
299 Tephra-annotated TEs¹³ but TASR- and EDTA-annotated TEs can be found in the Mt5.0 genome portal
300 (downloads section) and browser (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>).

301 **RNAseq data analysis**

302 To evaluate TE expression in nodules, SYMBiMICS data¹¹ were analyzed with the pipeline used for the
303 MtExpress gene expression atlas⁴³ and *M. truncatula* annotation version 5.1.8. nf-core/rnaseq
304 pipeline version 3.0 (doi:10.5281/zenodo.4323183) was used with the following parameters " --
305 skip_alignment --pseudo_aligner salmon ", followed by transcript assignment and quantification with
306 salmon (version 1.4.0). The chosen expression threshold for retained genes and TEs was one CPM
307 (count per million reads) for the sum of 15 libraries counts (i.e. three replicates of five nodule zones).
308 Normalization was performed using trimmed mean of *M* values method⁴⁴. Differentially expressed
309 genes and TEs were detected with EdgeR Bioconductor package⁴⁵ version 3.34.0, using the GLM
310 (Fitted generalized linear models) likelihood ratio test, with an FDR adjusted p-value⁴⁶. Analyses of
311 GO term enrichment were performed using the topGO package version 2.44.0⁴⁷.
312 Expression patterns (termed RG-Patterns for repeat and gene patterns), including 17,406 and 1,923
313 nodule-expressed genes and TEs, were defined for genes and TEs differentially regulated between at
314 least two nodule zones (FDR<0.01 and LFC >1; supplemental Table 7), similarly to the 16 expression
315 patterns previously defined from genes only¹³. The relative position of expressed TEs and genes was
316 analyzed using Bedtools (v2.30.0).

317 siRNA distribution was analyzed using Shortstack v. 3.8.5⁴⁸, with siRNA clusters independently
318 defined for different siRNA sizes (namely, 21, 22, 21-22 and 24 nt), using siRNAs from N0, N4, N6,
319 N10 siRNA libraries previously generated¹³. The position of siRNA clusters vs DMRs, genes and TEs
320 was analyzed using Bedtools v2.30.0.

321 **CRISPR-Cas9 mutagenesis, genotyping and complementation assay**

322 The guide RNAs (gRNA; listed in supplemental Table 9) were designed with CRISPOR (version 4.8,
323 <http://crispor.tefor.net/>) program, with the INRA A17r5.0 r1.6 *M. truncatula* genome release¹³ and
324 the "20bp-NGG-Sp Cas9, SpCas9-HF1, eSpCas9 1.1" option (appropriate for the use of *Streptococcus*
325 *pyogenes* Cas9). T-DNAs contained the *S. pyogenes Cas9* coding DNA sequence, to which a SV40 NLS
326 sequence was added at the C-terminus, as well as three gRNAs, interspaced by tRNAs as described⁴⁹,
327 and preassembled as a synthetic polycistronic gene. The guides were expressed under the control of
328 *M. truncatula U6.1* (MtrunA17_Chr3g0136831) and *U6.6* (MtrunA17_Chr7g0251721) RNA Pol III-
329 controlled promoters. Retained guides did not contain a TTTT stretch and did not show a perfect
330 match with potential off target genes in the 12 nt following the NGG Protospacer Adjacent Motif
331 (PAM). The backbone plasmids required for the assembly of the binary vectors were provided by the
332 ENSA project (Engineering Nitrogen Symbiosis for Africa; <https://www.ensa.ac.uk>) and the cloning
333 strategy was based on the Golden Gate cloning technology⁵⁰. T-DNAs included a kanamycin
334 resistance module (*p35S:KanR:TNos*) and a DsRed fluorescent reporter module
335 (*pAtUbi10:DsRed:TOcs*), located respectively close to the right and left borders in order to counter-
336 select partial insertions of T-DNAs.

337 Genotyping was done on individual transformed root systems, by extracting DNA from nodules or a
338 root segment adjacent to nodules. Nested PCR was performed, followed by agarose gel
339 electrophoresis analysis and systematic sequencing of PCR products.

340 For the *drm* complementation assay, a synthetic *MtDRM2* cDNA (ProteoGenix, Schiltigheim, France)
341 was designed, in which all guide recognition sequences were mutated (with conservation of encoded
342 amino acids). The synthetic construct (*MtDRM2-R*, 3533 nt; supplemental Note 1) included the *AtUBI*

343 promoter and the 35S terminator, flanking the cDNA and sequences required for Golden Gate
344 cloning. The whole module was inserted into the plasmid used for CRISPR-Cas9 *DRM2/DRM2L2*
345 mutagenesis. Samples used for phenotyping and WGBS were checked to have bi-allelic edits of
346 *MtDRM2* and *MtDRM2L2*, and no edits of the *MtDRM2-R* cDNA.

347 **Gene and protein sequence analyses**

348 The phylogenetic tree was generated using Phylogeny.fr⁵¹ (PhyML/OneClick). Multiple alignment
349 analyses were performed using Multalin⁵² version 5.4.1. Correspondences between gene names and
350 Mt5.0 or Mt4.0 gene identifiers were obtained using Legoo⁵³ and the Downloads section of Mt5.0
351 genome browser, as of 20211025.

352 **Root nodule analysis and phenotyping**

353 To analyze wild type nodule nuclei and chromocenters, 15 dpi nodules were fixed in 0.2%
354 paraformaldehyde, then embedded in 8% low-melting agarose (NuSieve™ GTG™ Agarose Lonza); 80
355 µm sections (microtome LEICA VT 1000S) were stained with 0.5µg/ml DAPI (4', 6-diamidino-2-
356 phenylindole) and observed using a confocal microscope Leica SP8 (excitation 415 nm; emission 411-
357 478 nm).

358 For mutant vs control nodule phenotyping, root segments nodulated with *S.meliloti* 2011 *nifH::GFP*
359 were harvested at 15-18 dpi from four independent caissons (in two independent experiments).

360 Nodules were visually scored for their size, shape and color. Acetylene reduction assays were then
361 performed, with a 4 hour incubation followed by quantification of ethylene production using an
362 Agilent 7820A gas chromatograph. All nodulated roots were then observed with bright field and
363 fluorescence imaging, using a stereo microscope Leica DFC7000T. They were given a GFP signal score
364 from 0 to 4, corresponding to 0%, ≤25%, 25 to 50%, 50 to 75% and >75% GFP+ nodules, respectively.

365 A fraction of the nodules was then used for DNA and RNA extraction, while another fraction was
366 fixed in 1.5% glutaraldehyde, and embedded in 5% low-melting agarose. Nodule sections (60 µm
367 thick) were prepared from 41 transformed roots (in two independent experiments), with ~10 to 20

368 nodules per root, and observed using a Zeiss Axiophot light/fluorescence microscope, following
369 staining with 0.5µg/ml DAPI.

370 **Data availability:**

371 Raw reads from BsSeq and RNAseq experiments have been deposited at the Sequence Read Archive
372 (SRA) (project accession numbers: SRP355902 and SRP349933). Data related to gene annotation, TE
373 annotation (Tephra-based, TASR10_round2_RepeatMasker-based and EDTA-based), methylome and
374 DMR analyses are available at the *M. truncatula* genome portal and browser
375 <https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>

376 Correspondence and requests for material should be addressed to PG.

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385

386 Author contributions

387 Y.P., M-F.J. and P.G. conceived the research plans; Y.P., S.M., and M-F.J. performed most of the
388 experiments; O.B. performed bisulfite sequencing; E.S., S.C., and J.G. performed bioinformatics
389 analyses; Y.P., M-F.J. and P.G. analyzed the data; P.G. conceived the project and wrote the article
390 with contributions of J.G., Y.P. and M-F.J.

391 Competing financial interests

392 The authors declare no competing financial interests

393 Figure Legends

394

395 **Fig. 1.** Coupling laser capture microdissection (LCM) to whole-genome bisulphite sequencing (WGBS)
396 strongly increases the sensitivity of detection of differentially methylated DNA regions (DMRs).

397 **a**, representative image (out of 61 microdissected nodules, in three independent replicates) of a
 398 nodule following LCM of meristematic (M), differentiation (Diff) and nitrogen fixation (Fix) nodule
 399 zones; bar= 100 μ m. **b**, methylome-based clustering analysis of WGBS libraries, with three biological
 400 replicates of laser-dissected M, Diff and Fix zones, as well as two biological replicates of N-starved
 401 root tips (RT) and whole nodules six days-post-inoculation with *Sinorhizobium meliloti* (N6). **c**, mean
 402 methylation level in the CG, CHG and CHH contexts of genomic DNA from RT, N6, M, Diff and Fix
 403 samples, with a level of 1 corresponding to 100% of methylated cytosines. **d**, relative proportion of
 404 cytosine methylation levels in mCG, mCHG and mCHH contexts (minimal coverage of 10 reads) in RT
 405 and Fix. **e**, distribution on the eight *M. truncatula* chromosomes of methylated cytosines and DMRs
 406 (Fix vs RT), as well as genes, transposable elements (TEs) and symbiosis related islands (SRIs)
 407 expressed in the nodule differentiation zone. Centromeres and SRIs are depicted by thick black lines
 408 and grey lines, respectively. **f**, number of DMRs detected in the CG, CHG and CHH contexts, with
 409 pairwise comparisons of whole organs (N6 and RT) or laser-dissected samples (M, Diff, Fix); indicated
 410 DMRs were found in at least 2 biological replicates of each comparison, with a minimal difference of
 411 absolute methylation level of 0.4 for CG, 0.2 for CHG and 0.1 for CHH contexts. H= A, C or T
 412 nucleotide.

413

414 **Fig. 2.** Average methylation patterns of genes and transposable elements (TEs), in whole organs and
 415 laser-dissected nodule differentiation and nitrogen-fixation zones, and DMR localization.

416 **a, b, c**, CG, CHG and CHH contexts, respectively. From left to right, average methylated cytosine (mC)
 417 profile of all annotated genes (50,773 genes), of NDD genes (4,309 genes upregulated in the nodule
 418 differentiation and fixation zone), of the top 4,309 genes expressed in roots, and of small TEs
 419 (215,171 TEs <1 kb; 89% of total Tephra-annotated TEs). Two biological replicates of root tips (RT), 6
 420 dpi nodules (N6) and laser-dissected nodule differentiation (Diff) and fixation (Fix) zones. Flanking
 421 regions of 2 kb for genes and 1 kb for TEs. Profiles correspond to Cs shared between all samples, with
 422 a minimal coverage of four reads.

423 **d**, average distribution of differentially methylated regions (fixation zone vs root tips) (DMRs) on NDD
 424 genes.

425 **e**, average CG-CHG DMR density in the 211 symbiotic differentiation islands (NDD; 1,558 genes) vs.
 426 49 apical (NDA; 242 genes) and 57 non-spatially regulated (NDN; 275 genes) control islands¹³ (island
 427 underlined, with length normalized to 50 kb and 50 kb flanking regions)

428 TSS= transcription start site. TTS= transcription termination site.

429

430 **Fig. 3.** Localization of DMRs and histone marks in relation to gene expression profiles, and
 431 importance of *MtDRM2* for nodule CHH hypermethylation and late nodule development.

432 **a**, zonation of the *M. truncatula* nodule (ZI: zone I, meristematic region; ZII: distal zone II,
 433 (pre)infection; ZIIp: proximal zone II, early differentiation; IZ: interzone II-III, late differentiation; ZIII:
 434 zone III, nitrogen-fixation) and *pDME:GUS* expression (blue signal around IZ) (n=30 in three
 435 replicates). **b**, the 16 expression patterns previously defined³, with strong and gradual differences
 436 between nodule zones for patterns 1-11 and 12-16, respectively; differentiation zone: patterns 5-10
 437 and 14-15; fixation zone: patterns 11 and 16. **c**, distribution of CG-CHG and CHH DMRs at the
 438 transcription start site (TSS) and 2 kb flanking regions of all 16 pattern genes and non-spatially
 439 regulated (NDN) genes, observed for the laser-dissected differentiation (Diff) and nitrogen-fixation
 440 (Fix) zones. **d**, distribution of active H3K9ac and repressive H3K27me3 histone marks on gene bodies
 441 (normalized to 2kb) and 2 kb flanking regions, in root tip and whole nodule samples¹³. In c and d,

442 genes were ranked first by expression pattern and then by decreasing expression level. **e**, Average
443 CHH methylation of all genes and small transposable elements (215,171 TEs<1 kb), in *drm* mutant
444 nodules (CRISPR-Cas9 mutagenesis of *MtDRM2* and *MtDRM2L*) compared to control nodules
445 transformed with a *GUS*-targeting CRISPR-Cas9 construct and *drm* mutant nodules complemented
446 with a synthetic non-editable *MtDRM2* cDNA (*MtDRM2-R*). Two biological replicates from whole-
447 genome bisulfite sequencing are shown. **f, g, h**, sections of *S. meliloti nifH::GFP*-induced nodules,
448 transformed with a *GUS*-targeting CRISPR-Cas9 construct (**f**), or *MtDRM2* and *MtDRM2L* genes (**g, h**),
449 with in addition *MtDRM2-R* cDNA in **h**; observed using light and fluorescence microscopy (left and
450 right panels, respectively). **f, g, h**: 150, 200 and 40 sectioned nodules from 9, 9 and 4 independently
451 transformed roots, respectively. Note the strong GFP signal in the ZIII of the control nodule and the
452 mutant nodule complemented with *MtDRM2-R*, whereas in *drm* mutant nodules the GFP signal is
453 either absent (**g** top: round-shaped nodule) or present in only a few cells (**g** bottom: elongated
454 nodules). Bars = 100 μ m.

455

456 **Fig. 4.** Model for the dynamics of DNA methylation during nodule development.

457 **a**, In the root, genes expressed in the nodule differentiation zone and transposable elements (TEs)
458 are silenced, with cytosine methylation in all contexts (mCG, mCHG, mCHH: violet, blue and yellow
459 solid circles, respectively), and repressive histone marks (H3K9me2 and H3K27me1 for
460 heterochromatic TEs; H3K27me3 for genes)^{5,13}. The histone H2A.W variant contributes to
461 heterochromatin condensation²⁶.

462 **b**, In the nodule differentiation zone, mCs are removed in all contexts, notably by DME. Histone
463 repressive marks are replaced by activating histone marks (such as H3K9ac), particularly in symbiosis-
464 related islands involved in nodule differentiation (SRIs-NDD)¹³ while the eight *MtH2A.W* genes are
465 down-regulated. This leads to local chromatin opening and enables the transcription machinery
466 including transcription factors (TFs) to access the promoters and activate gene expression.
467 Transcriptional activation of some TEs also occurs, either from TE promoters or by transcriptional
468 read-through from upstream highly expressed genes.

469 **c**, In the nodule differentiation and nitrogen fixation zones, TE transcription leads to the production
470 of siRNAs and increased RNA-directed DNA methylation (RdDM), targeting siRNA-related TEs first *in*
471 *cis* and then *in trans*, genome-wide. Increased RdDM might also result from a better access of the
472 RdDM machinery to the relaxed facultative heterochromatin (e.g. due to *MtH2A.W.1-8*
473 downregulation). In the fixation zone, no DNA re-methylation takes place, and differentiation zone
474 genes remain expressed or not depending on the presence or absence of required TFs.

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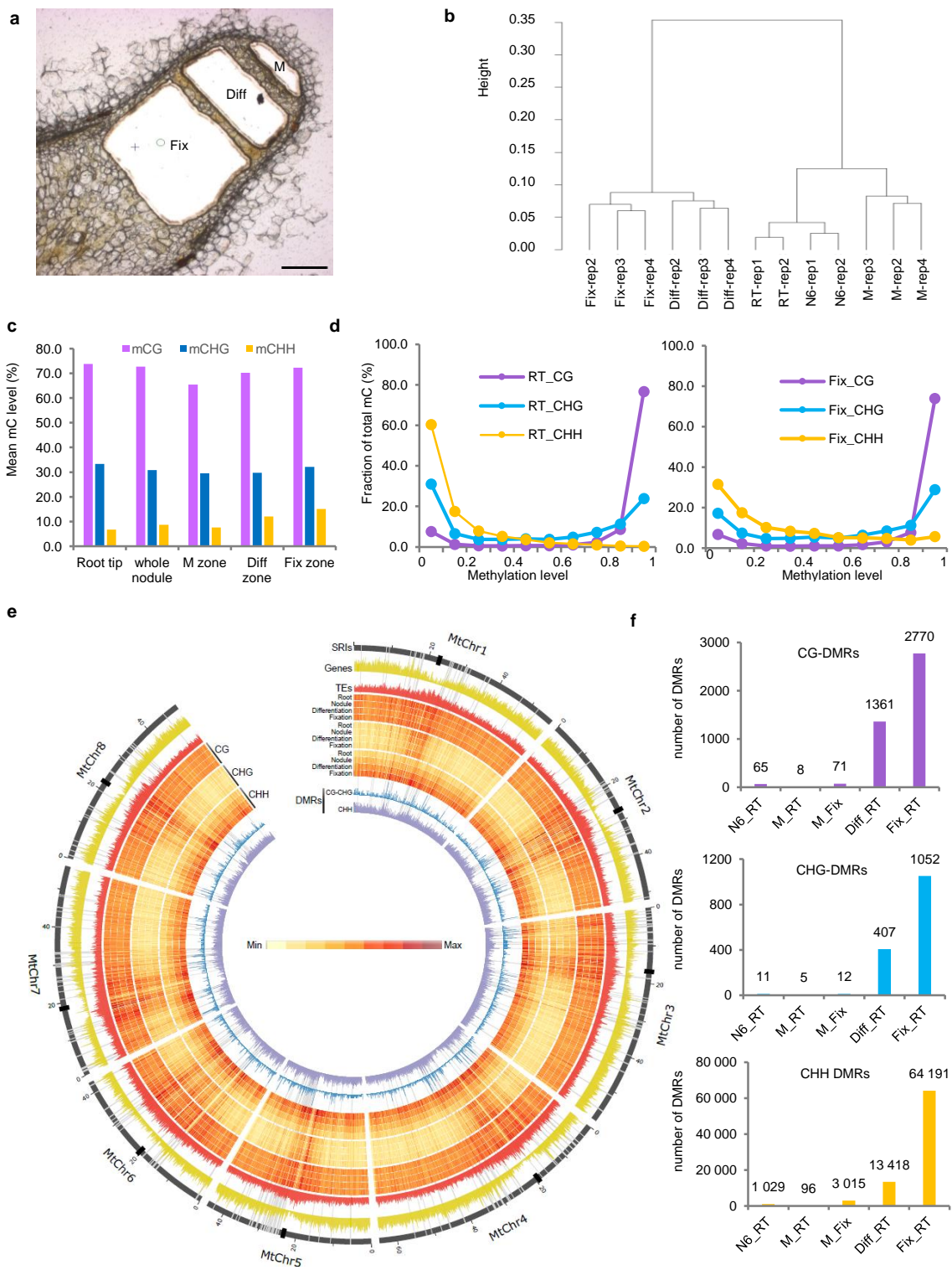


Figure 1

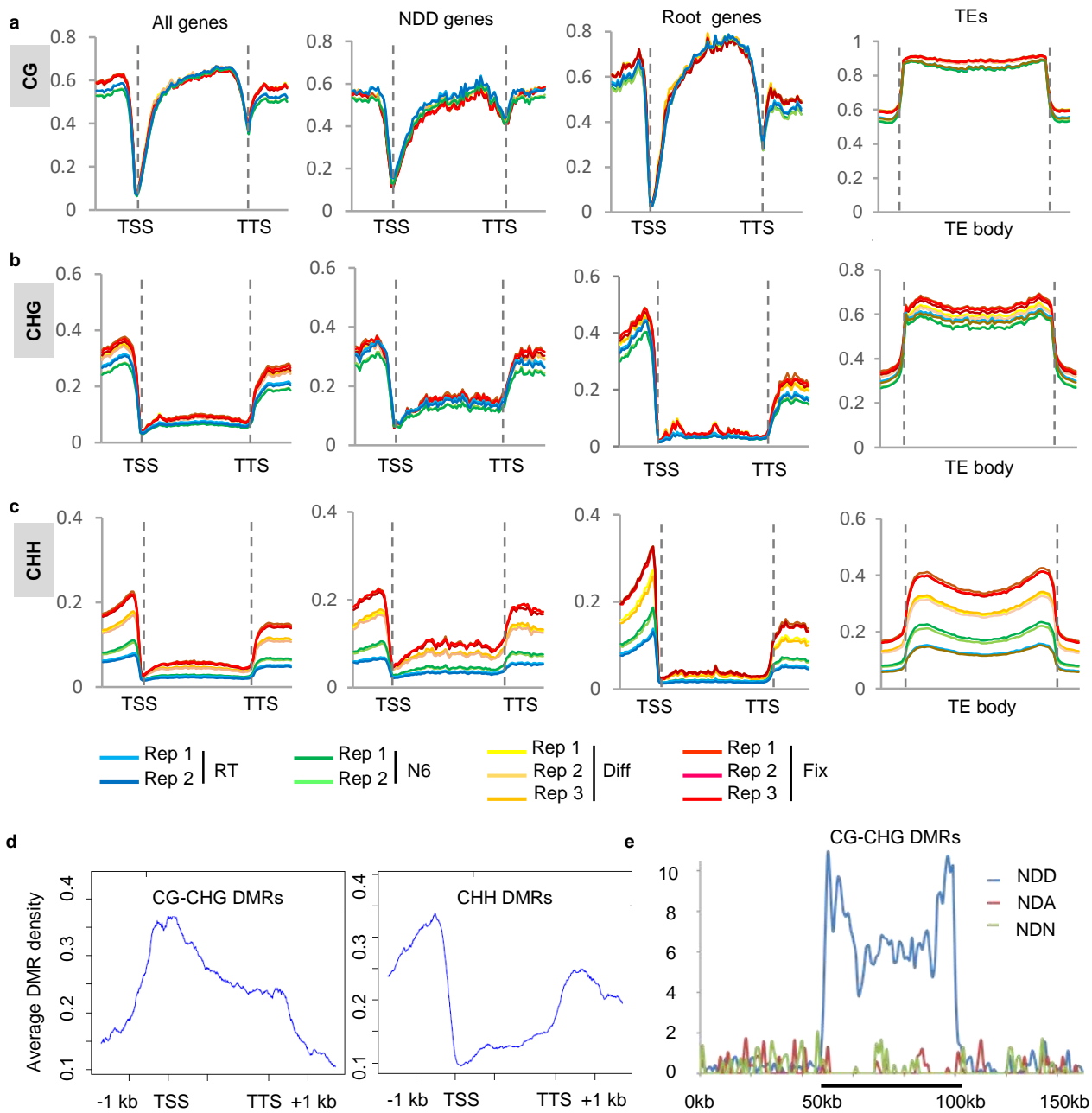


Figure 2

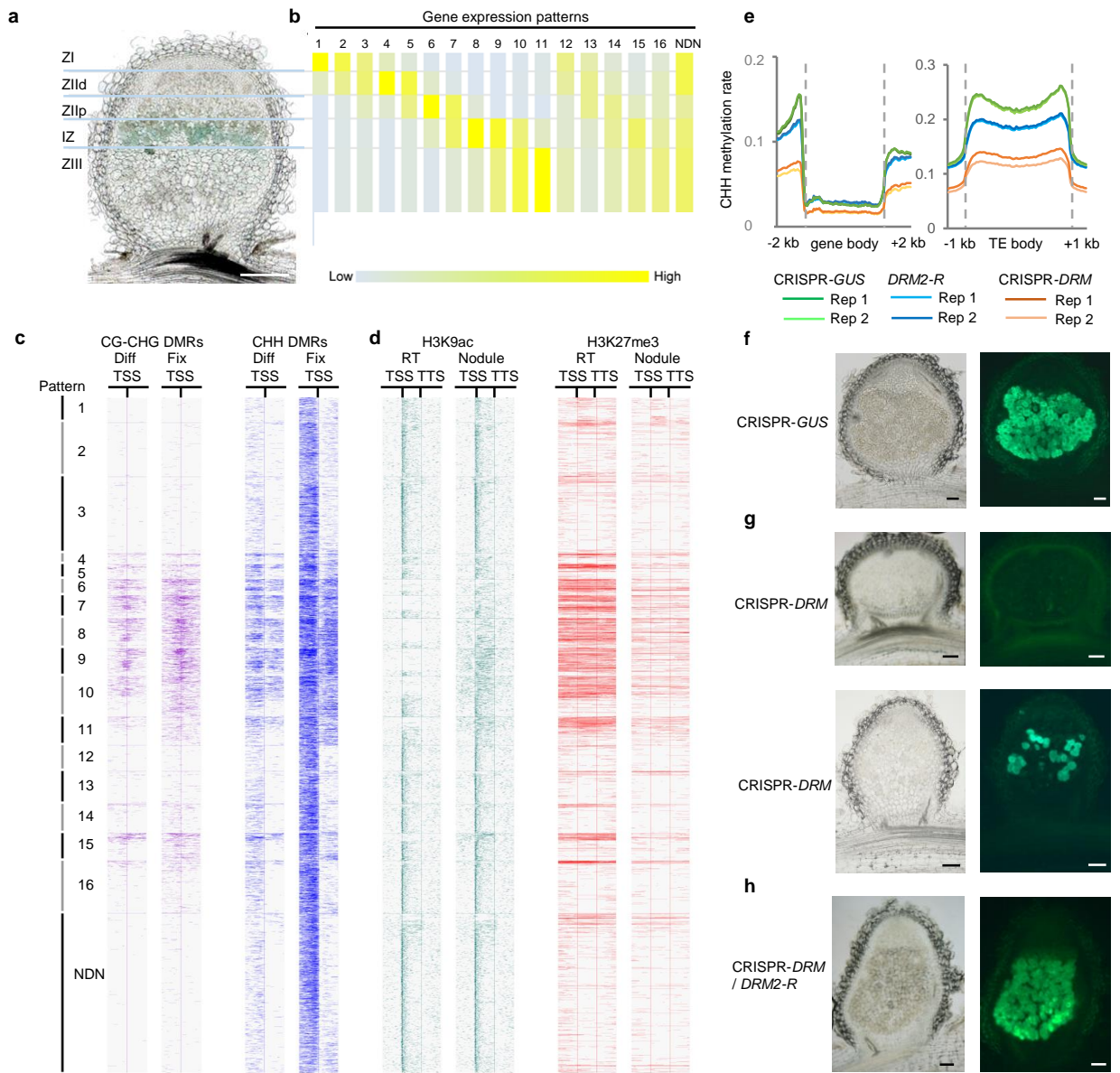


Figure 3

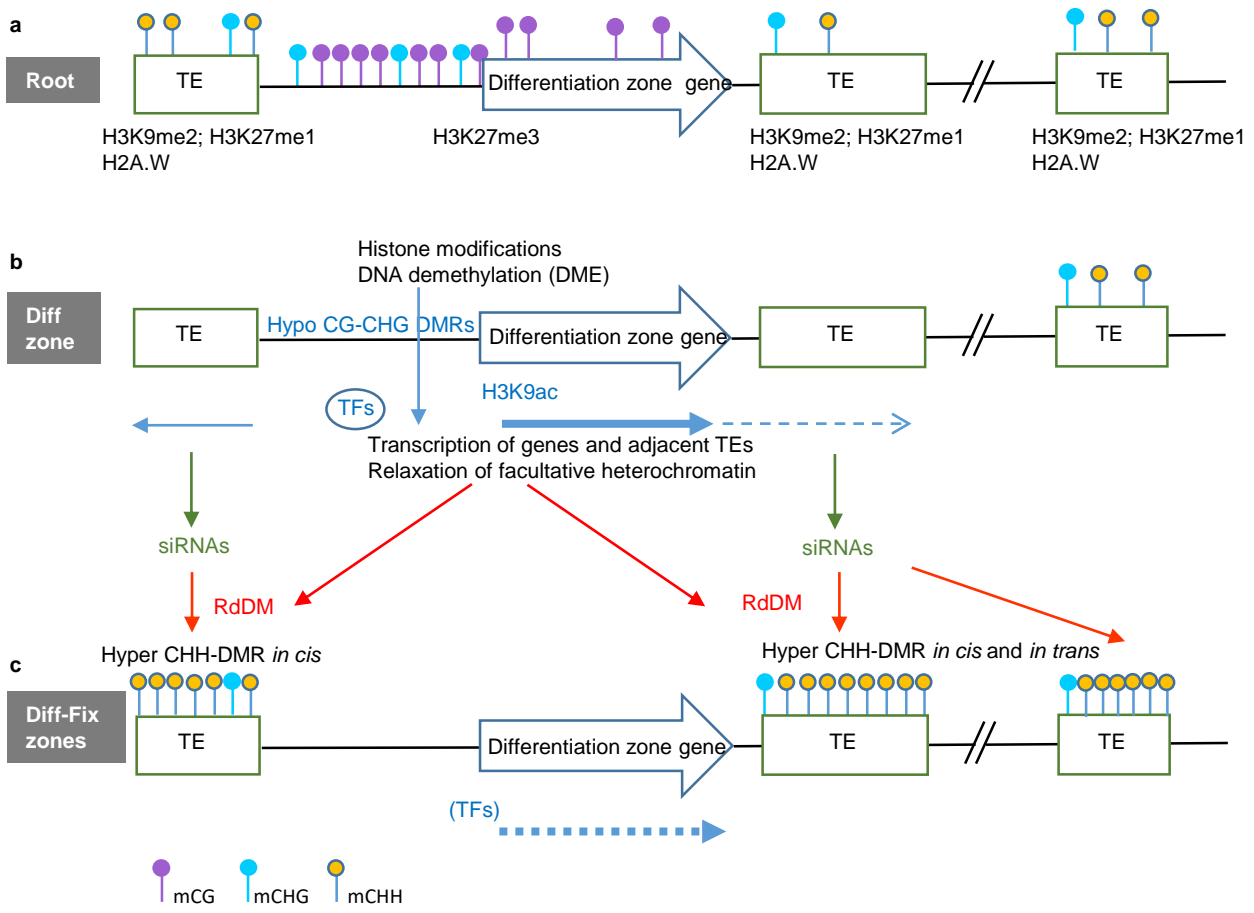
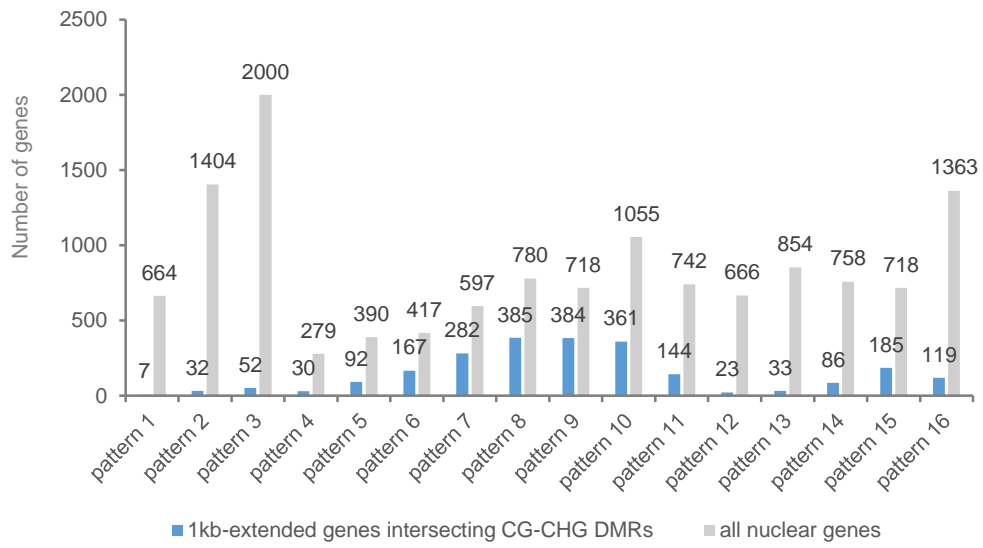
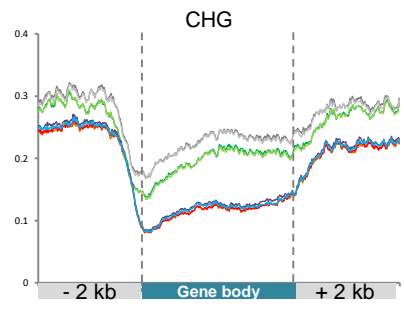
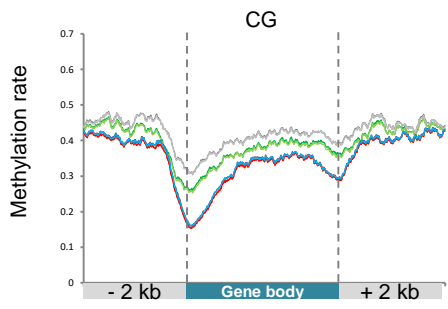


Figure 4

Extended Figures 1 to 9

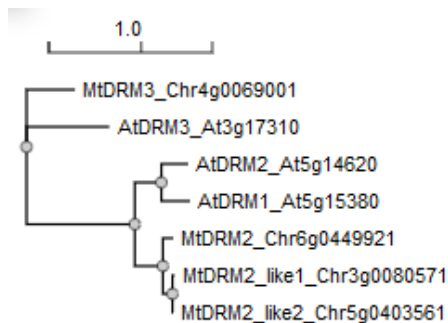




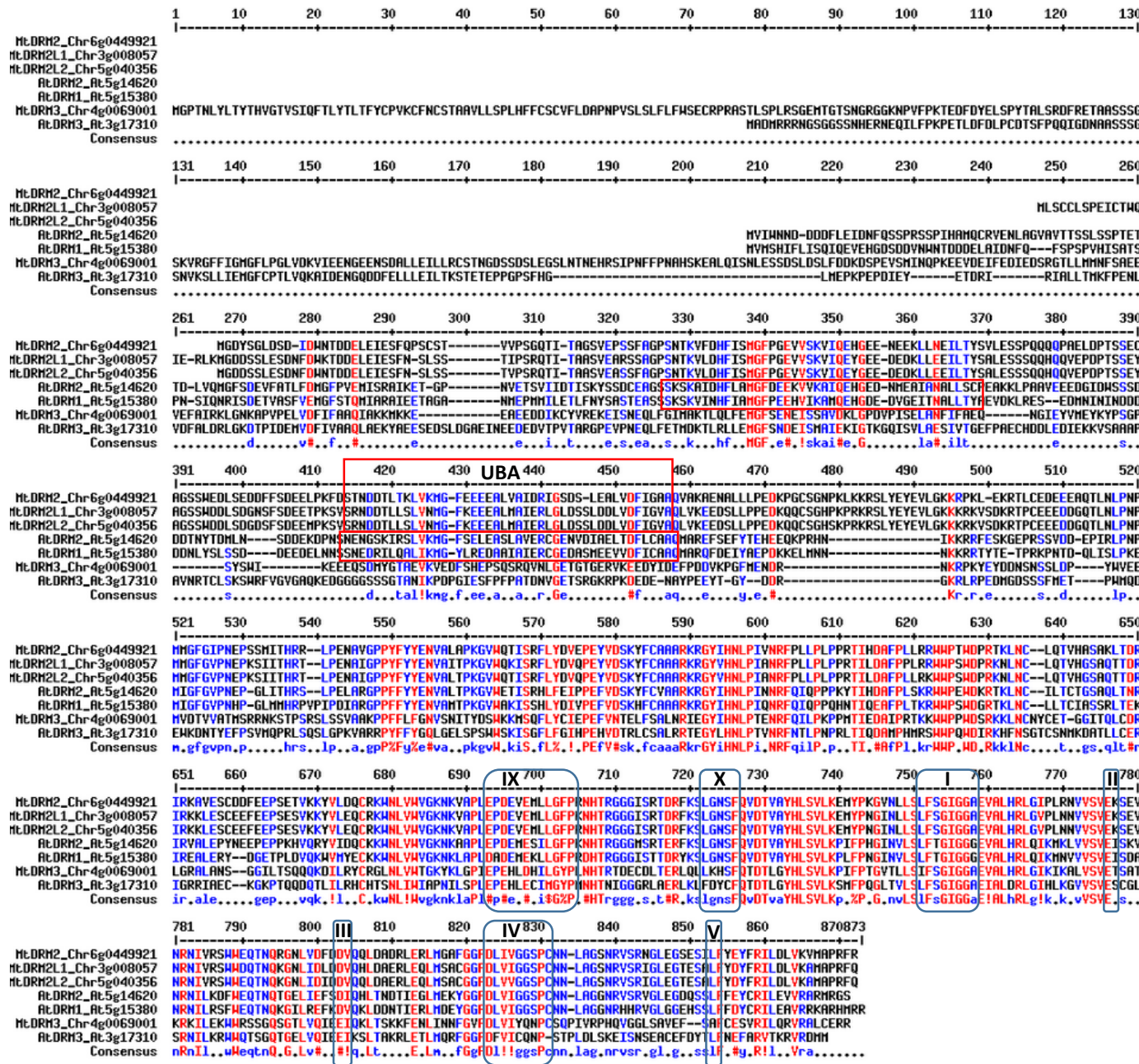


- DMR-plus, root tips
- DMR-plus, nodules
- DMR-minus, root tips
- DMR-minus, nodules

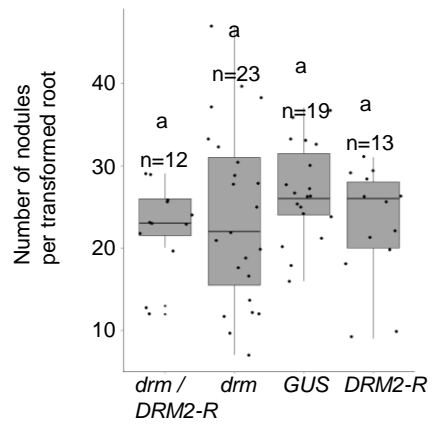
a



b

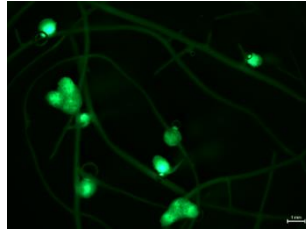


a



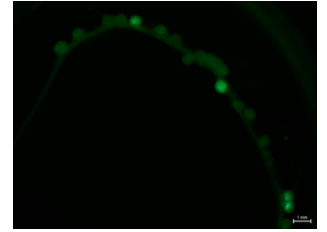
b

CRISPR-*GUS*



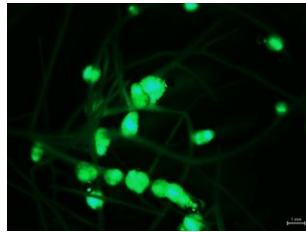
GFP score: 4; ethylene area / nodule: 8.4 pA*s

CRISPR-*DRM2/DRM2L2*



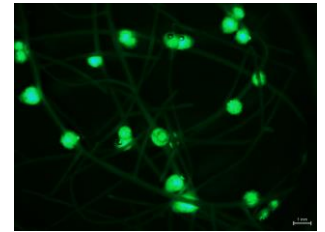
GFP score: 1; ethylene area / nodule: 0.8 pA*s

pAtUBI:DRM2-R

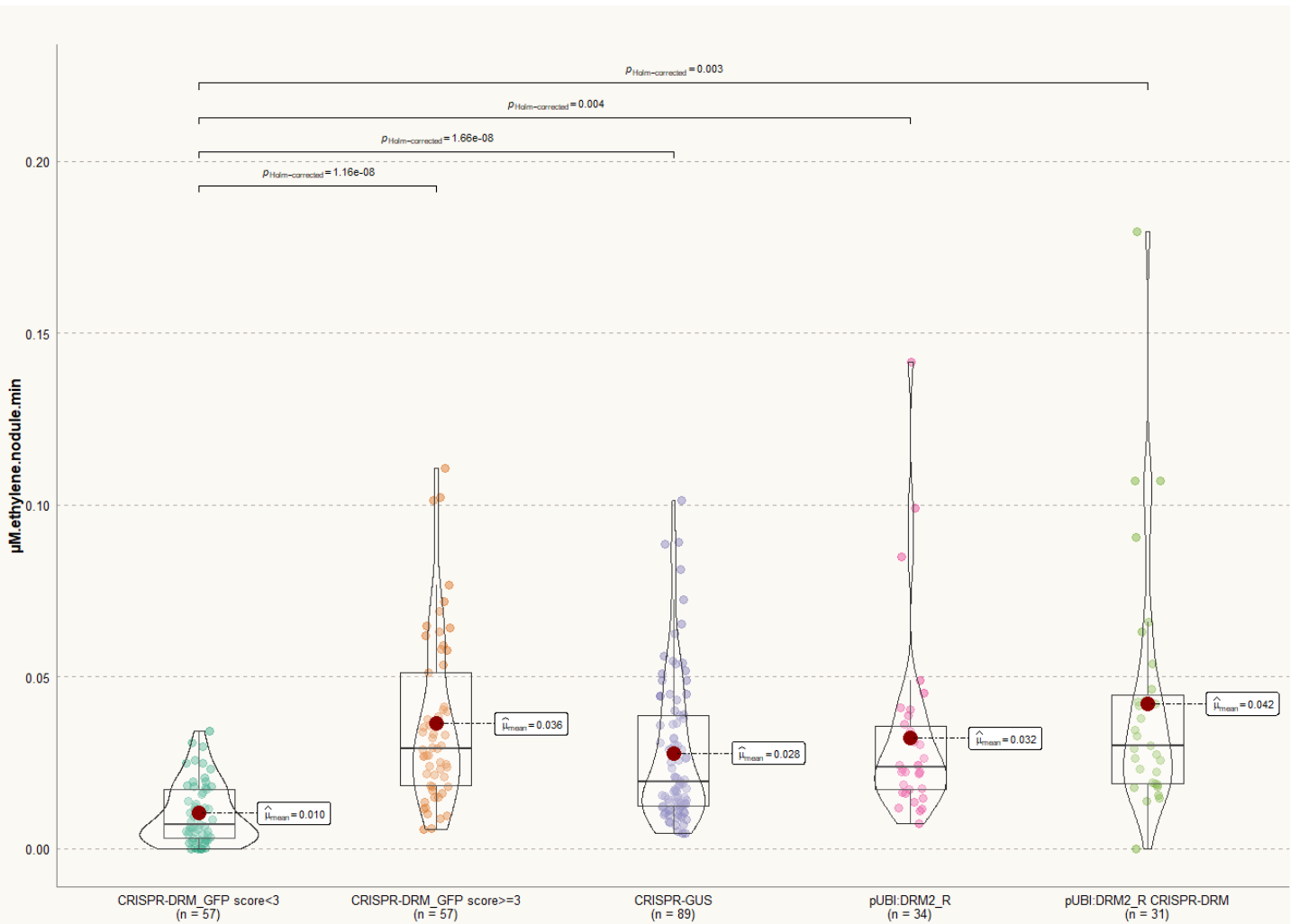


GFP score: 4; ethylene area / nodule: 3.5 pA*s

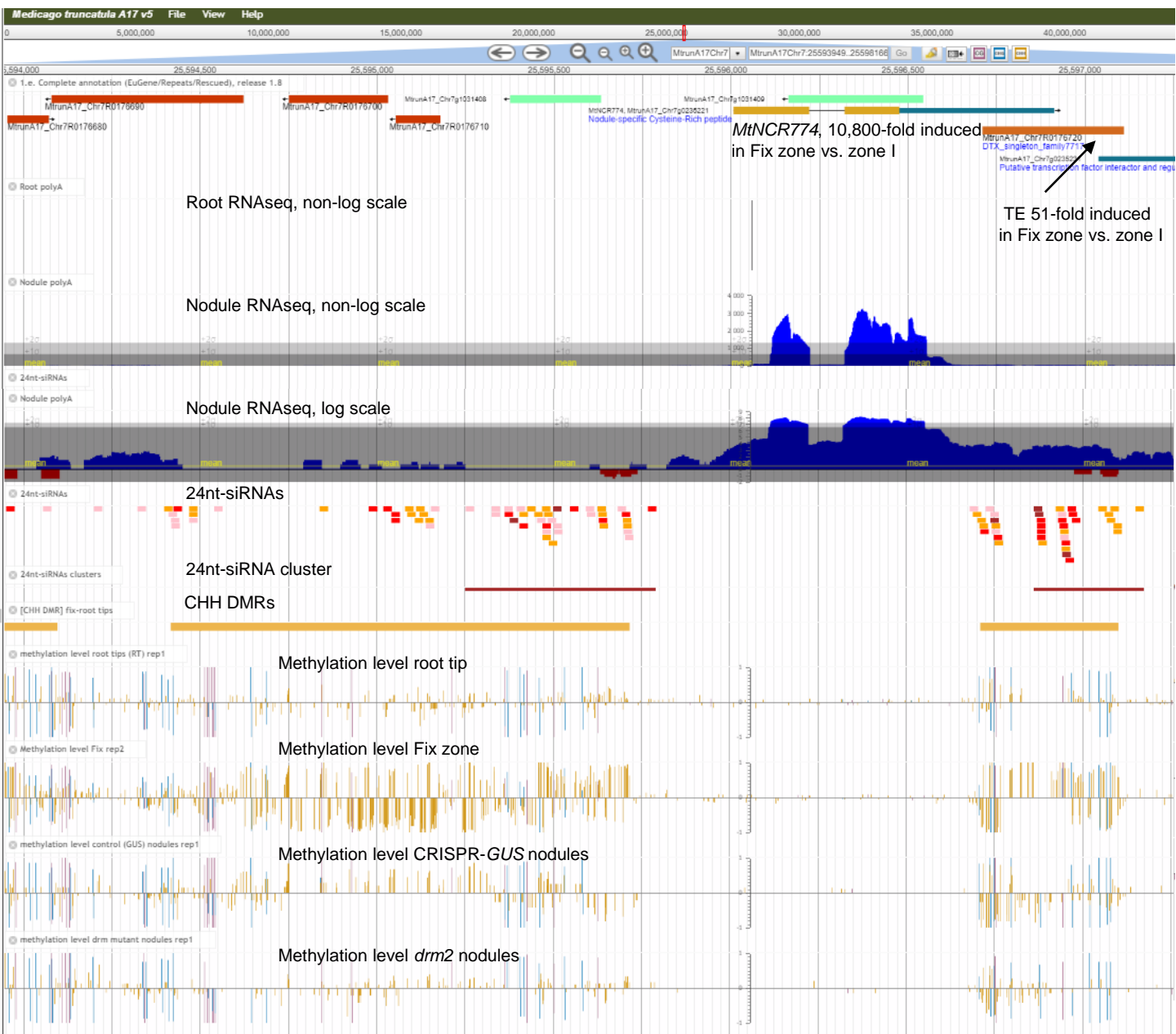
CRISPR-*DRM2/DRM2L2 pAtUBI:DRM2-R*



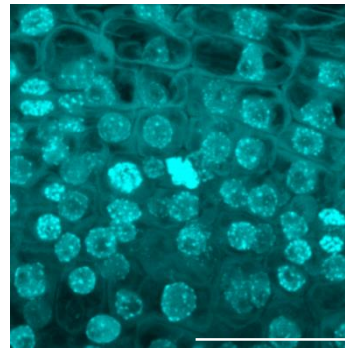
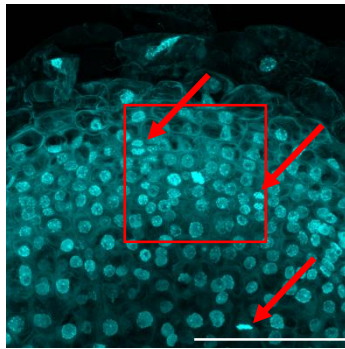
GFP score: 4; ethylene area / nodule: 6.6 pA*s



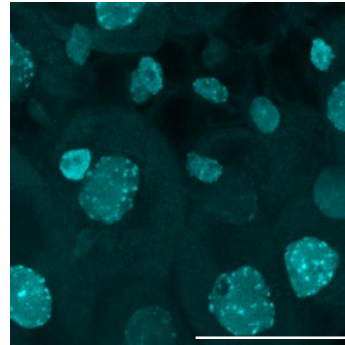
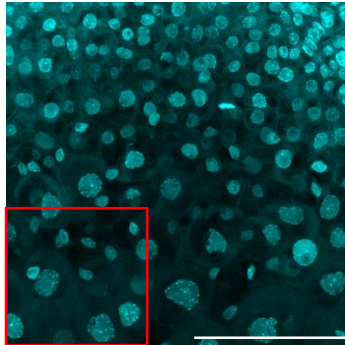
Pairwise test: Games-Howell test. Comparisons shown: only significant



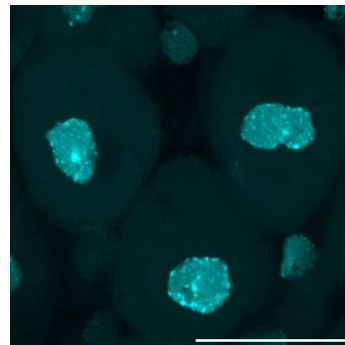
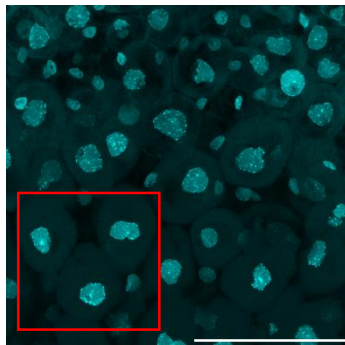
Zone I
Zone II d



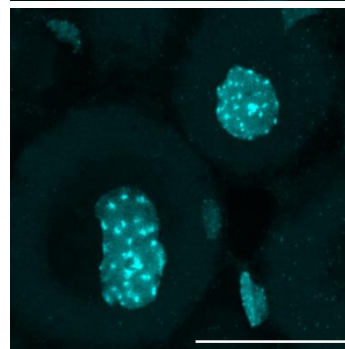
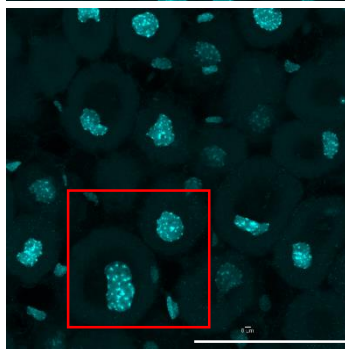
Zone II d
Zone II p



Interzone II-III



Zone III



Supplemental Tables 1, 8 and 9
Supplemental Note 1

Supplemental Table 1. Whole-genome bisulfite sequencing libraries and BsSeq data summary.
corresponding names in the *M.truncatula* genome browser are in parentheses; estimated genome size = 430Mb

sample	sequencing platform	read length	single-end (1) / paired-end (2)		uniquely mapping reads	uniquely mapping reads %	non-clonal reads	non-clonal reads %	useful reads %	sequencing depth	non-conversion rate %
			raw reads								
root tips (RT) rep1	GeT Plage	150	1	198 744 374	58 646 991	29.5%	46 501 259	79.3%	23.4%	16.2	1.26
root tips (RT) rep2	GeT Plage	150	1	188 125 142	94 395 254	50.2%	72 308 131	76.6%	38.4%	25.2	1.07
6 dpi nodules (N6) rep1	GeT Plage	150	1	298 305 105	68541505	23.0%	48 103 904	70.2%	16.1%	16.8	1.09
6 dpi nodules (N6) rep2	GeT Plage	150	1	298 440 135	74343704	24.9%	54 204 340	72.9%	18.2%	18.9	1.07
laser-dissected meristematic zone (Meristem rep2)	GeT Plage	150	1	399 966 743	69 966 258	17.5%	18 684 803	26.7%	4.7%	6.5	1.65
laser-dissected meristematic zone (Meristem rep3)	GeT Plage	150	1	290 385 535	79 699 717	27.4%	15 210 897	19.1%	5.2%	5.3	2.11
laser-dissected meristematic zone (Meristem rep4)	GeT Plage	150	1	352 885 728	60 100 310	17.0%	15 065 571	25.1%	4.3%	5.3	1.72
laser-dissected differentiation zone (Diff rep2)	GeT Plage	150	1	169 696 939	66 412 042	39.1%	29 992 961	45.2%	17.7%	10.5	1.63
laser-dissected differentiation zone (Diff rep3)	GeT Plage	150	1	174 374 489	66 159 837	37.9%	30 506 850	46.1%	17.5%	10.6	1.6
laser-dissected differentiation zone (Diff rep4)	GeT Plage	150	1	204 599 807	52 958 871	25.9%	29 552 672	55.8%	14.4%	10.3	1.56
laser-dissected fixation zone (Fix rep2)	GeT Plage	150	1	209 807 770	68 238 361	32.5%	47 547 459	69.7%	22.7%	16.6	2.18
laser-dissected fixation zone (Fix rep3)	GeT Plage	150	1	190 976 920	72 515 451	38.0%	45 607 620	62.9%	23.9%	15.9	1.88
laser-dissected fixation zone (Fix rep4)	GeT Plage	150	1	177 392 752	66 952 746	37.7%	42 646 733	63.7%	24.0%	14.9	2.26
CRISPR-GUS root tips (bRT rep1)	BGI	150	2	110 472 066	46 047 157	41.7%	33 572 922	72.9%	30.4%	23.4	1.19
CRISPR-GUS root tips (bRT rep2)	BGI	150	2	101 908 938	46 078 533	45.2%	33 548 766	72.8%	32.9%	23.4	1.26
CRISPR-GUS nodules (bGUS rep1)	BGI	150	2	119 566 679	28 799 615	24.1%	20 789 431	72.2%	17.4%	14.5	1.01
CRISPR-GUS nodules (bGUS rep2)	BGI	150	2	112 338 095	29 120 814	25.9%	21 146 217	72.6%	18.8%	14.8	1.15
CRISPR-DRM nodules (bDRM rep1)	BGI	150	2	85 503 038	32 533 045	38.0%	25 256 897	77.6%	29.5%	17.6	1.19
CRISPR-DRM nodules (bDRM rep2)	BGI	150	2	116 447 133	43 630 496	37.5%	33 644 685	77.1%	28.9%	23.5	1.28
CRISPR-DRM /pAtUbi:DRM2-R nodules (DRM2-R rep1)	BGI	150	2	128 638 179	40 325 617	31.3%	30 097 018	74.6%	23.4%	21.0	1.29
CRISPR-DRM /pAtUbi:DRM2-R nodules (DRM2-R rep2)	BGI	150	2	125 208 648	32 192 586	25.7%	24 050 933	74.7%	19.2%	16.8	1.11

Supplemental Table 8. Chop-PCR validation of CHH DMRs.

Whole organ analyses CHH DMR position	Methylation-sensitive restriction enzyme	mean delta CT nodule (n=3)	SE delta CT nodule	mean delta CT root tips (n=3)	SE delta CT nodule	Shapiro test*	Fisher's test*	one-sided t.test
Chr1_35839283-35840045	Alul	7.53	1.12	12.54	1.30	0.9730	0.8565	0.0324
	Ddel	9.76	3.28	14.93	3.16	0.4665	0.9270	0.1904
Chr2_32878129-32878605	Alul	9.00	1.58	12.67	3.27	0.0523	0.3803	0.2149
	Ddel	6.66	0.42	7.59	0.50	0.2860	0.3103	0.4908
Chr3_51183413-51184086	Alul	4.05	1.30	8.54	0.95	0.6230	0.6981	0.0455
	Ddel	8.13	1.56	10.99	0.79	0.4083	0.5227	0.1082
Chr4_30558968-30560144	Alul	2.03	0.96	5.38	2.13	0.6684	0.3397	0.1409
	Ddel	3.09	1.37	4.37	1.32	0.6019	0.9645	0.2960
Chr4_31795384-31796098	Alul	1.71	1.18	7.54	1.41	0.8531	0.8236	0.0256
	Ddel	4.14	1.93	9.64	1.50	0.8754	0.7566	0.0614
Chr4_31798378-31799002	Alul	5.94	1.24	14.82	0.86	0.6587	0.6447	0.0035
	Ddel	5.83	1.83	21.72	1.74	0.4678	0.9517	0.0028
Chr4_9710107-9710722	Alul	4.76	1.45	6.42	1.06	0.2362	0.7014	0.2339
	Ddel	4.94	1.81	6.96	0.92	0.2130	0.4092	0.2174
Laser dissected Fix zone vs root tip DNA	Methylation-sensitive restriction enzyme	delta CT Fix zone (n=2)**		delta CT root tips (n=2)**				
Chr1_26645685-26647163	NlaIII-1	-1.04		3.36				
	NlaIII-2	1.82		2.94				
	Ddel-1	1.47		5.74				
	Ddel-2	0.33		6.55				

delta CT: compared to mock non-digested samples

n= biological replicates, using pools of 303, 434 and 357 nodules (15 dpi) from 5, 10 and 8 plants, respectively.

*Shapiro test: normality test; Fisher's test: variance equality test

** DNA remaining from LCM-BsSeq experiments; the results for each replicate are shown since no statistical test was possible (only two replicates being available)

Supplemental Table 9. Multi-guide (mg) cloning, sequencing and Chop-PCR primers

cloning primers

mgDRM2a-F	5' to 3' sequence
mgDRM2a-R	TAGGTCTCCTCTATGAATATGGTTTTAGAGCTAGAA
mgDRM2b-F	CGGGTCTCATAGAGACTGCGCTGCACCAGCCGGG
mgDRM2b-R	TAGGTCTCAAACCCCATCATGTTTTAGAGCTAGAA
mgDRM2c-F	CGGGTCTCGGTTTGGTATTCTGCACCAGCCGGG
mgDRM2c-R	TAGGTCTCATGTATCCTAAAGTTTTAGAGCTAGAA
mgDRM2L2d-F	CGGGTCTCATACTCTCCTTCTGCACCAGCCGGG
mgDRM2L2d-R	TAGGTCTCCTCTCCATCGACTGTTTTAGAGCTAGAA
mgDRM2L2e-F	CGGGTCTCGGAGAGCTACTTCTGCACCAGCCGGG
mgDRM2L2e-R	TAGGTCTCGAAACAATATCTGTTTTAGAGCTAGAA
mgDRM2L2f-F	CGGGTCTCGTTTGCCAAACTGCACCAGCCGGG
mgDRM2L2f-R	TAGGTCTCTTTCCTCAATAGGGTTTTAGAGCTAGAA
	CGGGTCTCAGGAAATGGTGGCTGCACCAGCCGGG

sequencing primers

DRM2-F1	GGTTGTTTCATTGGCCTCACTGT
DRM2-F2	GAATGCCCTTCTGCTTCCTG
DRM2-F3	CGCTATCCGGGATTGACAGCTA
DRM2-F4	CTGTTTTTCCAATGAACTTTCCA
DRM2-F5	CCTTACTAAGGAGGTGGTGGCC
DRM2-R1	AGGATGCATTCTTACCGATGAACA
DRM2-R2	ACTGACTCAACCTAACCAACCA
DRM2-R3	CGCGGTGGAAGAGGTAAAAGAG
DRM2-R4	GATTCCGAGCCCTCAAGTCCAT
DRM2-R5	TCTAGGCGCCATAACCTTCACT
DRM2L2-F1	GGCCTAGACTCCTCACTTGACG
DRM2L2-F2	GCAGTGGGCATTCAAACCAAG
DRM2L2-R1	CACTCATGAGCTGCTCAAACG
DRM2L2-R2	CCAACAACGAGATCAAACCA
DRM2L2-R3	GGCAACTTTATTCTTGCCACCC

Chop-PCR primers

Chr1-26645685..26647163_F	AGCTGGTGGGGTCCATGACACA
Chr1-26645685..26647163_R	AGCAGGCTTACAACCTTCTTGGC
Chr1_35839283..35840045_F	ACCCACCGAAATCGACACTAGGGG
Chr1_35839283..35840045_R	GGTTGAGCTGAGTTGAGATTTGCA
Chr2_32878129..32878605_F	GTTGAGGATGTGGTCGTCTTAGCT
Chr2_32878129..32878605_R	TGCACAACCGCGACAAGAAGGA
Chr3_51183413..51184086_F	CCGAAAATGACGCGACATACCCCA
Chr3_51183413..51184086_R	TCGGTATTTTCAAATTCACCGCTGT
Chr4_24118045..24119280_F	TCCCTTGA CTGGGACCCACTT
Chr4_24118045..24119280_R	CGGCATTGGTGAAAACGTGTCACG
Chr4_30558968..30560144_F	AGACGAGCCACACAATCCAGTG
Chr4_30558968..30560144_R	TTCTTCGTCTTCCAAGGCCCA
Chr4_31798378..31799002_F	AGACACCAGGAGACAAGCCCTGA
Chr4_31798378..31799002_R	ACCGTGGAAAGCTATGTCTGAACGT
Chr4_9710107..9710722_F	TGCTCAAAAAGGTGCCACGTGT
Chr4_9710107..9710722_R	GGTTCAAACCTGGACCTGGCA

Supplemental note 1. Synthetic *DRM2-R* sequence used for cloning a non-editable Mt*DRM2* cDNA

ACGTGCAGAAGACAAGTAAGGAGgacgtcgttggttggtgcttccttacattctgagcctcttccttctaatacactcatctgcatcttcttgtcc
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TTCAAGAGCATGAGGAGAATGAAGAGAACTGCTTAATGAGATTCTACATACTCAGTTCTTGAAAGTTCTCCTCAGCAGC
AACAGCCAGCTGAAGTAGACCCACCTCATCCGAGTGTGCGGGGAGTTCGTGGGAGGATTTATCGGAAGATGATTTTTTTT
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GAGGCTTAGTGGCCATCGACAGAATATCAGACTCACTGAAGCTCTGGTGCATTTTATTGGTGTGCTCAAGTGGCGAAA
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CGAATTCTAGTTTGTCTTCACAGA

XXXX : sequences added for Golden Gate cloning using Bpil

xxxx : *AtUBI* promoter

xxxx : 35S terminator

XXXX : *DRM2* cDNA

XXXX : mutated *DRM2* sequences (targeted by *DRM2* guides)

x: mutated nucleotides