Supplemental Data

DEMETER DNA Glycosylase Establishes MEDEA Polycomb Gene Self-Imprinting by Allele-Specific Demethylation

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Supplemental Experimental Procedures

Primers for Bisulfite Sequencing
Primers for the – 4 kb region were MEA3904 (5’-AACCTTATTACATRTAATRRCACACT-3’) or MEA3979 and MEA4510. The –3 kb region was amplified with MEA5187BFc (5’-CAAAATACTCTATTCTACATTCCACATCTAT-3’) and MEA5810BRc (5’-TAAATAATTTAAATGAGTGGGTTAGTAAATAG-3’), followed by a nested amplification with MEA5212 and MEA5810BRc. The –500 bp region was amplified with MEA7671 (5’-TAACCATTAACATTTAAATCTCTT-3’) or MEA7529 and MEA7935. MEA-ISR was amplified from Ler and Col-gl backgrounds using JP1026 and JP1027 (Cao and Jacobsen, 2002). A large deletion and extensive polymorphisms prevented the use of these primers in RLD. Instead, the first repeat was amplified with RLDBi (5’-TAATTTAAAAAATAATGATGTTGGTTAAGTTC-3’) and RLDBi4 (5’-AAAAARRTTTTATTAAATATTAAATATRA-3’). For MEA coding region bisulfite sequencing, Col-gl rosette leaf DNA was bisulfite treated as previously (Xiao et al., 2003) and methylation on the bottom strand determined. We sequenced 7 clones from MEA8355F (5’-TTTCTCTCCAAACATATAAAATAC-3’) to MEA8755R (5’-GAYTAATGTATATATTAGTGATAT-3’), 5 clones each from MEA8646F (5’-CTCTTCTRTATTTTTTTTCTRAAAATTAARRA-3’) to MEA9066R (5’-TGATYAYTYTGGYTTTTTTGGYTAATGAT-3’) and from MEA9294F (5’-CAGTTTTTTCRARAARCTCAAAAACCACTT-3’) to MEA9801R (5’-TAAATGAAAAAYTAAYYTATAAATYTGGY-3’), 8 clones from MEA9810F (5’-CTTRATTATTTATATCTTCCATATTTAAACAC-3’) to MEA10221R (5’-GTGGYTAATATTAAAAGAAAAATTTATAYATG-3’), 10 clones from MEA10310F (5’-CCTTTCRACARCTTCTAACACCTAAACATC-3’) to MEA10650R (5’-GGATYTGAGAYYAYAATTTGTTGATATAG-3’), 8 clones each from MEA10528F (5’-CTATTCCTTTAATTACRTTTATTTACTTAC-3’) to MEA10905R (5’-GGTTTTGTTAAGGYYTATATAYATGATATG-3’) and MEA10761F (5’-TGTTTTAATCTTATTTTTTACT-3’) to MEA11285R (5’-TAYAAAYTYATGTGAATTAATATTATYTATG-3’), 6 clones from MEA11131F (5’-ATAARCACACACACCAACTCTTCAART-3) to MEA11460R (5’-CAAATTCTATAATCAAATATTCAAACC), 7 clones from MEA11571F (5’-
CATACAATTCCTCCTAAACCAATAA-3') to MEA11987R (5'-
GATYATTYAAGGTAAAGGTAGGAAGAAYA-3'), 8 clones each from MEA11906F
(5'-CTRATCACTCATRAARCTAATRARCRT-3') to MEA12300R (5'-
GAGTTTGAGTTTYTTGGAATATYTTYAATATG-3') and MEA12234F (5'-
TCRTRATCACTTTACTRCTTTTRATTTR-3') to MEA12647R (5'-
GTTTTGGTTTAGTAAYAYAAAATAGYATTA-3'), and 9 clones from MEA12740F (5'-
CAATRTTTATRTTRTTARTTTRCATARACC-3') to MEA13093R (5'-
GTTTAGATAYTAAATGTTAGATGYATYAAAT-3'). This covers 91 of the 99 CG sites
present from the MEA transcription start site to the beginning of the 3’ repeats.

Amplification and Cloning of the MEA Allele in dme-2 Mutant Endosperm
The –500bp region and MEA-ISR were amplified with Pfu Turbo DNA polymerase (Stratagene)
from the same dme-2 DNA used for the experiment in Figure 2. PCR products were cloned into
the pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The primers for amplifying the –500
bp region were MEA8323Xba (5'-
ATATTCTAGACTTTTTTTCTCGTCTTCTCTGATGTTGGT-3') and UCB3SR12R-sac1 (5'-
GGGAGCTCGTTAAGCCTGTGGTTGACAAC-3'). The primers for amplifying the MEA-ISR
were B5-7RR (5'- TTAGGTATTAGCTCGTTTGGTTTTA-3') and MEA 3 REP (5'-
CTTAAAAGATTTTCAACTCATTTTTTAAAAGG-3').

Cloning, Expression, and Purification of DME in E. coli
A full-length DME cDNA (Choi et al., 2002) was used as template in a PCR reaction with
oligonucleotides JH021 (5'-TTAATCTAGAATGCAGAGCATTATGGACTCG-3') and JH017
(5'-CGGTCGACTTAGGTTTTGTTTCAATTTGC-3'), which add XbaI and SalI
restriction sites (underlined), respectively. The 5.2 kb PCR product was digested with XbaI and
SalI and cloned into the pMAL-c2x vector (NEB) to create c2x-DME. To generate a N-terminal
537 amino acid deletion, c2x-DME was digested with XbaI and Bsu36I. The 3’ overhangs were
filled in with T4 DNA polymerase and self-ligated, creating the c2x-DME∆N537 clone. The
construct with an D1304N point mutation was generated using the full-length DME(D1304N)
cDNA clone (Choi et al., 2004), following the same procedure as above. This fuses DME in
frame downstream of maltose-binding protein (MBP). The c2x-DME∆N537 or c2x-
DME∆N537(D1304N) clones were transformed into E. coli Rosetta cells (Novagen).
Transformed cells were grown at 28°C in LB supplemented with 0.2% glucose, 100 µg/mL of
ampicillin, and 50 µg/mL of chloramphenicol until the OD600 reached 0.4. Protein expression
was induced with 10 µM of IPTG at 18°C for 1 hr. The culture was centrifuged at 6,500 rpm for
15 min at 4°C and the pellet was resuspended in 30 mL of 4°C column buffer (20 mM Tris-HCl,
pH 7.4, 200 mM NaCl, 1mM EDTA). Cells were sonicated for 2 min on ice (output power 4;
duty cycle 50%; Branson Sonifer 250). The lysate was centrifuged at 9,000 rpm for 25 min at
4°C and the supernatant was collected and subjected to gravity column purification. The MBP-
DME∆N537 and MBP-DME∆N537(D1304N) fusion proteins were purified following the
manufacturer’s protocol through amylose resin (New England Biolabs). Eluted protein was
dialedyzed in the Slide-A-Lyzer dialysis cassette (10,000 MWCO; Pierce) against 50% glycerol at
4 °C overnight. Protein concentration was determined by the Bradford method using the Protein
Assay kit (Bio-Rad Laboratories) and stored at –20 °C until use.
Substrate Preparation for DNA Glycosylase Activity Assays

Synthetic oligonucleotides were purchased either from Operon or Midland Certified. All oligonucleotides were 35-nucleotides in length with modifications denoted within parentheses as shown below:

- MEA-1.6F, 5'-CTATACCTCCTCAACTCCGGTCACCGTCTCCGGCG
- MEA-1.6F18meC, 5'-CTATACCTCCTCAACT(5-meC)GGTCACCGTCTCCGGCG
- MEA-1.6F17meC, 5'-CTATACCTCCTCAACT(5-meC)CGGTCACCGTCTCCGGCG
- MEA-1.6F22meC, 5'-CTATACCTCCTCAACTCCGGT(5-meC)ACCGTCTCCGGCG
- MEA-1.6F18AP, 5'-CTATACCTCCTCAACTC(abasic)GGTCACCGTCTCCGGCG
- MEA-1.6F17AP, 5'-CTATACCTCCTCAACT(abasic)CGGTCACCGTCTCCGGCG
- MEA-1.6F15AP, 5'-CTATACCTCCTCAAT(abasic)TGTCACCGTCTCCGGCG
- MEA-1.6F12AP, 5'-CTATACCTCCTCAACT(abasic)GTCACCGTCTCCGGCG
- MEA-1.6F18T, 5'-CTATACCTCCTCAACTCTGGTCACCGTCTCCGGCG
- MEA-1.6R, 5'-CGCCGGAGACGGTGACCGGAGTTGAGGAGGTATAG
- MEA-1.6R17meC, 5'-CGCCGGAGACGGTGAC(5-meC)GGAGTTGAGGAGGTATAG
- MEA-1.6F12AP, 5’-CTATACCTCCTCAACT(abasic)GTCACCGTCTCCGGCG
- MEA-1.6F18T, 5’-CTATACCTCCTCAACTCTGGTCACCGTCTCCGGCG
- MEA-1.6R, 5’-CGCCGGAGACGGTGACCGGAGTTGAGGAGGTATAG
- MEA-1.6R17meC, 5’-CGCCGGAGACGGTGAC(5-meC)GGAGTTGAGGAGGTATAG

Twenty pmol of oligonucleotide were end-labeled in a 50 µL reaction using 20 units of T4 polynucleotide kinase in the presence of 30 µCi of (γ-32P)ATP (6000 Ci/mmol, Perkin Elmer Life Sciences) at 37°C for 1 hr. The labeled oligonucleotide was purified using a Qiaquick Nucleotide Removal Kit (Qiagen) as described by the manufacturer.

Labeled oligonucleotides were annealed to the appropriate complementary oligonucleotides in 10 mM Tris-HCl (pH 8.0), 1mM EDTA and 0.1 M NaCl. The mixture was boiled in water for 10 min and then slowly cooled to room temperature overnight. MspI or HpaII restriction endonuclease digestion followed by gel electrophoresis was used to determine the efficiency of annealing. Only substrates that were greater than 90% double-stranded were used in glycosylase activity assays.

NaBH4 Trapping Assays

5’-labeled oligonucleotide substrates (13.3 nM) were incubated with DME protein (250 nM) in a 15 µl reaction with 40 mM HEPES-KOH (pH 8.0), 0.1 M KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 200 µg/mL BSA at 37°C. After 1 hr of incubation, 1 M NaBH4 was added to a final concentration of 100 mM and the reaction tubes were placed at 37°C for an additional 10 min. An equal volume of 2x SDS-PAGE loading buffer (90 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.02% bromophenol blue, 100 mM dithiothreitol) was added to terminate the trapping reaction. Products were boiled for 10 min before loading onto a 10% SDS-PAGE gel. The wet gel was exposed to Kodak Biomax MS film for 12-18 h at –80°C.

Bacterial Cell Toxicity Assays

Bacterial strains AB1157 (F−thr-1 ara-14 leuB6(Am) lacY1 (gpt-proA2)62 tsx-33 supE44(Am) galK2 rac hisG4(Oc) rfbD1 mgl-51 rpsL31 kgkK51 xyl-5 mtl-1 argE3(Oc) thi-1) and its isogenic AP endonuclease mutant RPC501 (xth nfo) were kindly provided by R. P. Cunningham (Cunningham et al., 1986). Strains GM30 (F−thr-1 ara-14 leuB6 tonA31 lacY1 tsx-78 supE44 galK2 galT22 hisG4 rpsL136 xyl-5 mtl-1 thi-1) and its isogenic dcm-6 derivative, GM31, were kindly provided by Martin G. Marinus (Palmer and Marinus, 1994).

The c2x-DMEAN537 and c2x-DME∆N537(D1304N) plasmids were individually transformed into the strains above by electroporation and cells were grown on LB/Glu/Amp plates (LB supplemented with 0.2% glucose and 100 µg/mL of ampicillin) at 37°C overnight.
Fresh colonies were picked and resuspended in 5 mL of LB/Glu/Amp liquid medium. After 12-14 h incubation at 37°, the culture was diluted 100,000-fold in LB medium and 100 µL was plated on the LB/Glu/Amp plates with 0, 2, 5, 10, 25, 50, and 100 µM of IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma). The plates were incubated at 28° for 20 to 28 hr and the number of colonies was counted.

**Chromatin Immunoprecipitation (ChIP) Procedures**
LNA nucleotide analogues (Promega) contain a 2'-O, 4'-C methylene bridge that locks the ribose moiety into a C3'-endo conformation (Koshkin et al., 1998; Obika et al., 1998; Singh et al., 1989). Region one (-4 to +440) of MEA was amplified with MEA-LNA006 (5'-CACCAACATCAGAGAAGACGAGAAAAG-3') and MEA-LNA004 (5'-GATTATGACTAATGTATAACTGTTTAC-3'). Region 2 (-947 to –547) of MEA was amplified with MEA-LNA002 (5'-GGGTCTCAATTTTGTGAACTGGTGTG-3') and MEA-LNA003 (5'-CCGATATTTTTTACTATTTATAACGTTAATTAC-3'). LNA nucleotides are underlined and are complementary to the RLD template sequence but have a mismatch with the Ler template due to a polymorphism. To demonstrate the specificity of LNA-containing primers, approximately 50 pg of Ler and RLD genomic DNA were used as a control. To increase the sensitivity of the LNA PCR reaction, 1 µCi of α-dATP-P32 was added to each PCR reaction. A polymorphism within region 1 (+60, T in RLD, C in Ler) was used to check the parental origin of PCR products by sequencing. PCR products from region 1 from wild type (Ler crossed to RLD) and mea (Ler mea/mea crossed to RLD) were cloned into TOPO TA-cloning vector (Invitrogen, CA). 22 clones each were sequenced to determine the origin of amplification templates. Primer sequences and reaction conditions for Actin gene amplification were as described (Johnson et al., 2002).
Supplemental References


Table S1. Percent Methylation in Rosette Leaves

<table>
<thead>
<tr>
<th>Accession</th>
<th>-4 kb</th>
<th>-3 kb</th>
<th>-0.5 kb</th>
<th>MEA-ISR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N(^a)</td>
<td>% CG</td>
<td>% CNG</td>
<td>% CNN</td>
</tr>
<tr>
<td>Col-gl</td>
<td>23</td>
<td>91</td>
<td>76</td>
<td>39</td>
</tr>
<tr>
<td>RLD</td>
<td>29</td>
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<td>Ler</td>
<td>5</td>
<td>96</td>
<td>80</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) = number of clones sequenced.

Table S2. CG Methylation of MEA in the –500 bp and MEA-ISR Regions of Dissected Seeds

<table>
<thead>
<tr>
<th>Cross(^a)</th>
<th>Allele</th>
<th>–500 bp</th>
<th>MEA-ISR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N(^b)</td>
<td>% CG</td>
</tr>
<tr>
<td>RLD x Col-gl</td>
<td>Maternal Endosperm</td>
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<td>24</td>
</tr>
<tr>
<td></td>
<td>Maternal Embryo</td>
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<td>77</td>
</tr>
<tr>
<td></td>
<td>Paternal Endosperm</td>
<td>39</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Paternal Embryo</td>
<td>20</td>
<td>85</td>
</tr>
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<td>RLD x Ler</td>
<td>Maternal Endosperm</td>
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<td>Maternal Embryo</td>
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<td></td>
<td>Paternal Embryo</td>
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</tr>
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<td>Maternal Embryo</td>
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<tr>
<td></td>
<td>Paternal Endosperm</td>
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<tr>
<td></td>
<td>Paternal Embryo</td>
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<td>2</td>
</tr>
</tbody>
</table>

\(^a\) = female parent is written on the left.
\(^b\) = numbers of clones sequenced.
Figure S1. Purification of Wild-Type and Mutant DME Proteins

(A) Wild type (MBP-Δ537DME) DME was produced in bacteria and purified as described in the Supplementary Experimental Procedures Section. Samples at different stages of the purification process (CL, cleared lysate; FT, flow through of the amylose column, EL, eluted fractions from the amylose column) were subjected to electrophoresis on an SDS-polyacrylamide gels. The gels were either stained (left) or blotted (right) and reacted with anti-MBP antibody (MBP-AB) with a chemiluminescent detection system.

(B) Wild type, and mutant DME, D1304N (MBP-Δ537DME(D1304N)) and K1286Q (MBP-Δ537DME(K1286Q)) were purified. Eluted fractions from amylose columns were subjected to electrophoresis on an SDS-polyacrylamide gels. The gels were either stained (left) or blotted (right) and reacted with anti-MBP antibody (MBP-Ab) using a colorimetric detection system.
Figure S2. Sequence of Maternal MEA Allele in Endosperm of dme-2 Col-gl Pollinated by RLD

Crosses, seed dissection, and DNA isolation are as described for Figure 2A. Primers and cloning are described in the Supplemental Experimental Procedures section. Reference sequence is Col-gl and ‘N’ is any base. CpG sites in red are hypomethylated in a DME-dependent manner in the endosperm. Twelve sequenced clones of the −500 bp region of the MEA promoter (A) and the MEA-ISR (B) had no C→T transition mutations at CpG sites.