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Supplemental Data

Multimegabase Silencing in Nucleolar Dominance Involves siRNA-Directed DNA Methylation and Specific Methylcytosine-Binding Proteins

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S1 nuclease protection assay conditions

For S1 nuclease protection assays, RNA was mixed with 5 'end-labeled probe DNA and incubated at 37˚C overnight in hybridization buffer (40 mM PIPES pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% deionized formamide). 150 units of S1 nuclease (Invitrogen) in digestion buffer (5% glycerol, 1 mM $ZnSO_4$, 30 mM sodium acetate, 50 mM NaCl) was then added and incubated at 37˚C for 45 minutes. Reactions were stopped with 0.3% SDS, 8 mM EDTA. Nucleic acids were ethanol precipitated and subjected to electrophoresis on 10% polyacrylamide/7M Urea sequencing gels. Dried gels were subjected to phosphorimaging.

Small RNA library details. Small RNA sequences analyzed in Figure 2 were determined by 454 sequencing. The library used to generate Figure 2C was generated using 15-30 nt RNAs isolated from floral tissue. Its construction, sequencing and analysis is described in Mosher et al, 2008. The libraries used to generate Figure 2D, comparing *A. thaliana* wild-type (ecotype Col-0) and *dcl3-1* mutants are described in Kasschau et al., 2007. These siRNA sequence data are available at the Arabidopsis small RNA project

(ASRP) website (http://asrp.cgrb.oregonstate.edu/db/). For wild type Col-0, a total of 78,583 small RNAs were sequenced. For *dcl3-1*, 30,318 small RNAs were sequenced. Both libraries were generated using RNA from inflorescence tissue. The entire 454 sequencing data set has the GEO (Gene Expression Omnibus) series number GSE6682. The GEO sample numbers for the data sets used in Figure 2D are GSM154336 (Col-0, inflorescence tissue; stage 1-12 flowers) and GSM154363 (*dcl3-1*, inflorescence tissue, stage 1-12 flowers).

Table S1. Members of the Arabidopsis DNA methyltransferase gene family, their expression status and the specific genes targeted for knockdown using RNAi

Plant homology group[1]	Gene	Gene locus identifier	Expressed	Mammalian homolog	RNAi target
I	MET1	At5g49160	Yes	DNMT1	Yes
I	MET2	At4g14140	Yes	DNMT1	Yes
I	MET2b	At4g08990	No	DNMT1	No
I	MET3	At4g13610	Yes	DNMT1	Yes
II	CMT1	At1g80740	Yes	Plant specific	Yes
II	CMT2	At4g19020	Yes	Plant specific	Yes
II	CMT3	At1g69770	Yes	Plant specific	Yes
III	DRM1	At5g15380	No	DNMT3	Yes
III	DRM2	At5g14620	Yes	DNMT3	Yes
III	DMT10	At3g17310	No	DNMT3	No
IV	DMT11	At5g25480	No	DNMT2	No

Table S2. Genes of the Arabidopsis methyl-CpG-binding domain protein family, their expression status and specific genes targeted for knockdown using RNAi

Plant Homology Group[1]	Gene	Accession number	Expressed	RNAi target
I	MBD10	At1g15340	Yes	Yes
I	MBD11	At3g15790	Yes	Yes
II	MBD1	At4g22745	Yes	Yes
II	MBD3	At4g00416	No	No
II	MBD4	At3g63030	Yes	Yes
III	MBD2	At5g35330	Yes	Yes
III	MBD12	At5g35338	No	No
IV	MBD5	At3g46580	Yes	Yes
IV	MBD6	At5g59380	Yes	Yes
V	MBD9	At3g01460	Yes	Yes
VI	MBD7	At5g59800	Yes	Yes
VII	MBD8	At1g22310	Yes	Yes
VIII	MBD13	At5g52230	No	No

[1]Homology groups are based on: Nathan M. Springer and Shawn M. Kaeppler (2005) Evolutionary Divergence of Monocot and Dicot Methyl-CpG-Binding Domain Proteins. Plant Physiol. 138: 92–104.

Table S3: Oligonucleotides used in Fig. 2A as probes to detect promoter siRNAs in *A. thaliana*. Numbers refer to positions relative to the 45S rRNA transcription start site (+1). Sequences shown were all followed by the sequence: CCTGTCTC, which serves as an adapter for probe generation using the mirVana probe kit.

Probe	**Sequence**
Atr(-150-100) sense	TACCAGAAAATAGGATTTAGTATCCTTATGATGCATGCCAAAAAGAATTT
Atr(-150-100) antisense	AAATTCTTTTTGGCATGCATCATAAGGATACTAAATCCTATTTTCTGGTA
Atr(-100-50) sense	TCAAATTCCAAGTATTTCTTTTTTCTTGGCACCGGTGTCTCCTCAGACAT
Atr(-100-50) antisense	ATGTCTGAGGAGACACCGGTGCCAAGAAAAAAGAAATACTTGGAATTTGA
Atr(-50+1) sense	TTCAATGTCTGTTGGTGCCAAGAGGGAAAAGGGCTATTAAGCTATATAGG
Atr(-50+1) antisense	CCTATATAGCTTAATAGCCCTTTTCCCTCTTGGCACCAACAGACATTGAA
Atr(+1+50) sense	GGGGTGGGTGTTGAGGGAGTCTGGGCAGTCCGTGGGGAACCCCCTTTTTC
Atr(+1+50) antisense	GAAAAAGGGGGTTCCCCACGGACTGCCCAGACTCCCTCAACACCCACCCC
Atr(+50+100) sense	GGTTCGGACTTGGGTAGCGATCGAGGGATGGTATCGGATATCGGCACGAG
Atr(+50+100) antisense	CTCGTGCCGATATCCGATACCATCCCTCGATCGCTACCCAAGTCCGAACC
Atr(+100+150) sense	GAATGACCGACCGTCCGGCCGCCGGGATTTTCGCCGGAAAACTTTTCCGG
Atr(+100+150) antisense	CCGGAAAAGTTTTCCGGCGAAAATCCCGGCGGCCGGACGGTCGGTCATTC

Table S4: Oligonucleotides used in Figures 3E and 3J as probes for siRNA blot hybridization in *A. suecica* WT and transgenic lines. Sequences shown were followed by the sequence: CCTGTCTC, which serves as an adapter for probe generation using the mirVana probe kit.

Probe	**Position**	**Sequence**
At 45S prom	-46 to -10	AATAGCCCTTTTCCCTCTTGGCACCAACAGACATTG
Aa 45S prom	-46 to -10	TTTGTCCATTTTTGGGTCTGGCACCAGTGGAGATGC
siR1003	(5S rRNA)	AGACCGTGAGGCCAAACTTGGCAT
pHell-DCL3	(*A.s. DCL3*-RNAi)	AGACAGAAGCCTTGACAAGCTTCCTGACCAAGTAAA GGTCCATCTCAACT
pHell-RDR2	(A.s. *RDR2*-RNAi)	ATCTGAGTCTTGACAAAGAAGACAGGATCATAACAT ATAGAACTTTGCTG

Table S5: PCR primers used in Figures 3A and 3F to evaluate *RDR2* and *DCL3* mRNA levels in *A.suecica* wild-type, *RDR2*-RNAi and *DCL3*-RNAi lines.

	Forward (5'→3')	**Reverse (5'→3')**
RDR2	GATTGATGCATTTCTTCTCAAGCTG	GCATTTGCGGGAAGCTTGCTCC
DCL3	TCATCTTCATCGGCTGCAGGTTC	TGGTTCTGTCTTCACAACCATCTC
PFK	CGCCGGAATTTCGATCAATCCT	CGCCACGAAAACCAAACAGAC

Figure S1. *DDM1* knockdown has no effect on nucleolar dominance.

A. Centromere repeat methylation assay using the methylation sensitive restriction endonucleases *Msp*I (M) or *Hpa*II (H) and Southern blot hybridization using a 180 bp centromere repeat probe. Genomic DNA isolated wild-type *A. suecica* (A.s.) or two independent *DDM1*-RNAi lines is compared. Note the increased HpaII digestion in the RNAi lines.

B. *DDM1* knockdown does not derpress the silenced *A. thaliana*-derived rRNA genes in *A. suecica*. RNA was isolated from *A. thaliana*, *A. arenosa*, wild-type *A. suecica*, *A. suecica* plants grown on media containing 10 ug/ mL of 5-aza-2' deoxycytsoine (aza-dC), or five independent *A. suecica DDM1*-RNAi lines. Equal aliquots of purified RNA were then subjected to S1 nuclease protection using probes specific for *A. thaliana* or *A. arenosa* rRNA gene transcripts initiated at the transcription start sites (+1) of their respective promoters. Note that aza-dC causes a dramatic increase in transcription from both the *A. thaliana* and *A. arenosa*-derived rRNA genes, unlike *DDM1* knockdown.

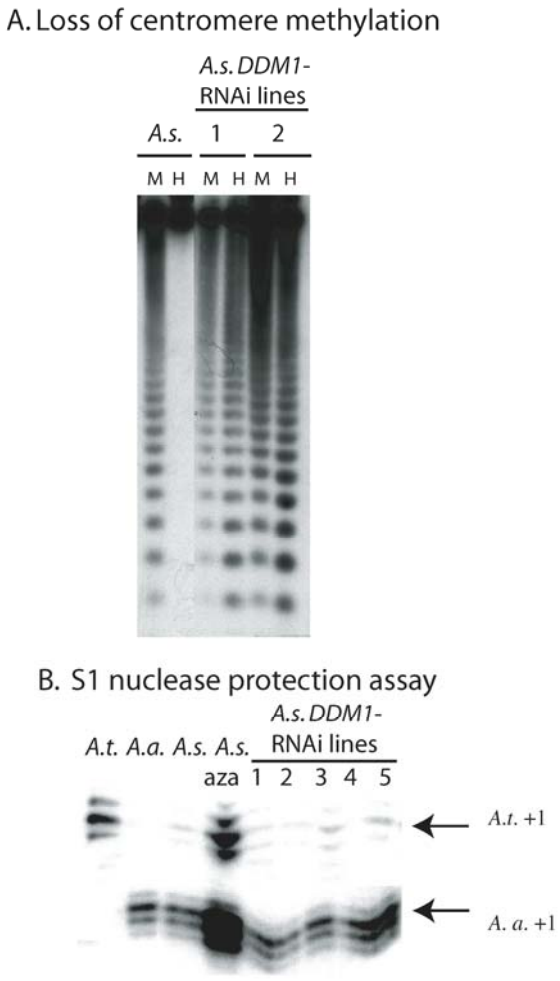

Figure S2. ChIP-chop assay showing that the subset of *A. thaliana*-derived rRNA genes promoters associated with the euchromatic mark, H3K4me3 are relatively hypomethylated compared to the subset of genes associated with the heterochromatic mark, H3K9me2. Chromatin was immunoprecipitated using antibodies specific for H3K4me3 or H3K9me2. Purified DNA was then subjected to cleavage by *Mcr*BC , which specifically digests DNA bearing two or more purine-methylcytosine dinucleotide motifs, followed by PCR using primers that amplify the specified regions. PCR primers were: -397F: ACCGGGTCCGAGGATT, -123F: CCTTATGATGCATGCCAAAAAGAATT, +65R: ACAGACATTGAAATGTCTGAGGAGAC,+5R:CCCCCTATATAGCTTAATAGCCCTTTT. Note that DNA associated with H3K9me2 is more *Mcr*BC-sensitive than is DNA associated with H3K4me3, as shown initially by Lawrence et al. (2004).

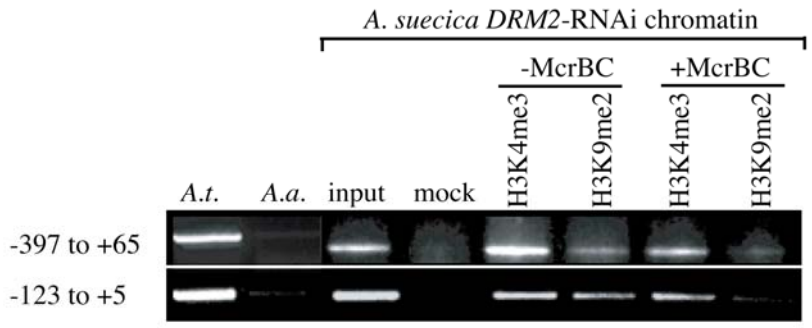

Figure S3. Size distributions and relative abundance of sequenced small RNAs matching the intergenic spacer (IGS), external transcribed spacer (ETS) or rRNA gene coding regions. IGS small RNAs correspond almost exclusively to the sizes of siRNAs produced by the four Dicer endonucleases: 21, 22, 23 and 24 nt. By contrast, small RNAs corresponding to regions encompassed by rRNA gene primary transcripts, namely the ETS and 18S-5.8S-25S rRNA coding regions, are heterogeneous in size. Pre-rRNA processing and rRNA degradation products are likely to constitute most of the ETS and coding region small RNAs.

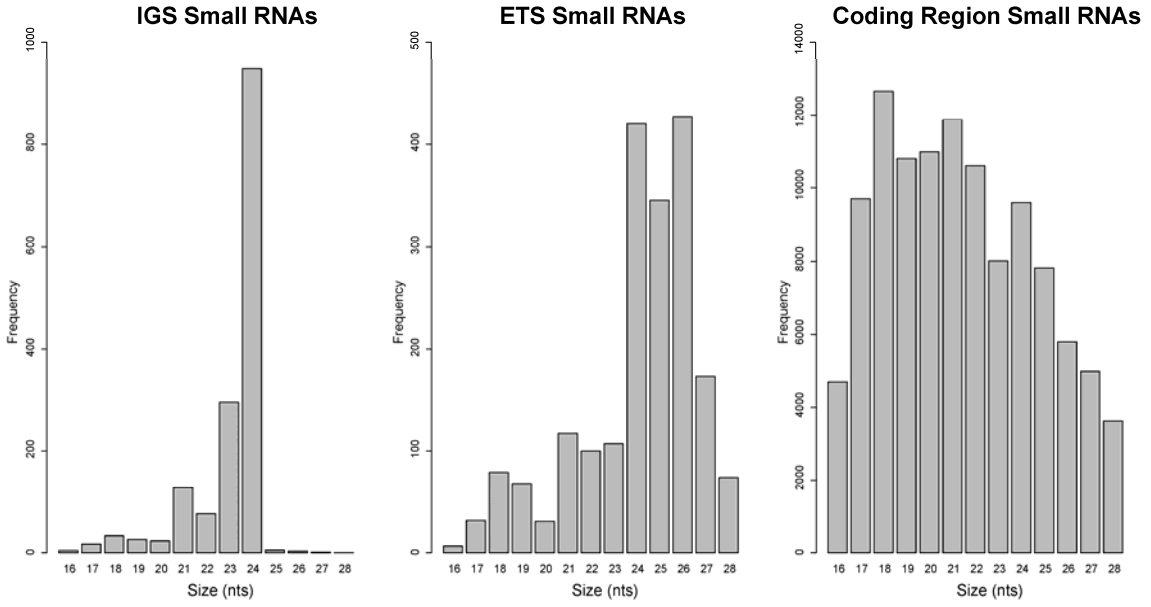

Figure S4. Localization of MBD6-YFP in *A. thaliana* nuclei.

MBD6-YFP recombinant protein expressed in transgenic plants was localized using anti-YFP antibody (green signals). NORs were localized using an rRNA gene probe (red signals). DNA was counterstained with DAPI (blue signals). The nucleolus, in which active rRNA genes are transcribed, appears as a black hole following DAPI staining. Inactive rRNA genes within NORs are highly condensed and localize at the outer edge of the nucleolos. Note that although there are four NORs in *A. thaliana*, they tend to coalesce, such that fewer than four NOR signals are typically observed by FISH. In the nuclei shown, either two or three NOR signals are apparent.

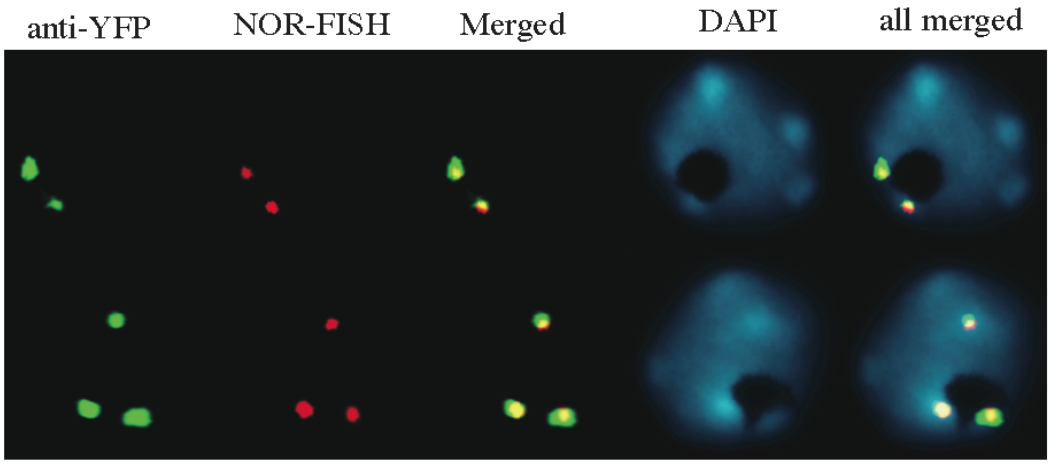

Figure S5. Comparison of siRNA levels in wild-type (WT), *DRM2*-RNAi and *MBD6*-RNAi lines of *A. suecica*. The RNA blots show siRNAs corresponding to the *A. thaliana* or *A. arenosa* 45S rRNA gene promoters or 5S rRNA gene spacers. The micro RNA, miR163 and ethidium-bromide stained 5S rRNA serve as loading controls.

Note that RNAi-mediated knockdown of *DRM2* in *A. suecica* causes decreased siRNA production, consistent with the decreases in DCL3-dependent siRNAs that have been reported previously in *A. thaliana drm1 drm2* mutants (e.g. see Pontes et al., 2006). The current interpretation is that *de novo* methylation influences the production of aberrant transcripts that serve as siRNAs. In MBD6-RNAi lines, siRNA levels are not decreased, but are slightly increased relative to wild-type (Fig. 6C), suggesting that in the absence of MBD6, production of aberrant transcripts from methylated DNA may be increased.