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Role of the *Arabidopsis DRM* Methyltransferases in De Novo DNA Methylation and Gene Silencing

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Summary

Proper DNA methylation patterning requires the complementary processes of de novo methylation (the initial methylation of unmethylated DNA sequences) and maintenance methylation (the faithful replication of preexisting methylation). *Arabidopsis* has two types of methyltransferases with demonstrated maintenance activity: MET1, which maintains CpG methylation [1–3] and is homologous to mammalian DNMT1, and CHROMOMETHYLASE 3 (CMT3), which maintains CpNpG (N = A, T, C, or G) methylation [3, 4] and is unique to the plant kingdom. Here we describe loss-of-function mutations in the *Arabidopsis DOMAINS REARRANGED METHYLASE* (*DRM*) genes [5] and provide evidence that they encode de novo methyltransferases. *drm1 drm2* double mutants retained preexisting CpG methylation at the endogenous *FWA* locus but blocked de novo CpG methylation that is normally associated with *FWA* transgene silencing. Furthermore, *drm1 drm2* double mutants blocked de novo CpNpG and asymmetric methylation and gene silencing of the endogenous *SUPERMAN* (*SUP*) gene, which is normally triggered by an inverted *SUP* repeat. However, *drm1 drm2* double mutants did not show reactivation of previously established *SUPERMAN* epigenetic silenced alleles. Thus, *drm* mutants prevent the establishment but not the maintenance of gene silencing at *FWA* and *SUP*, suggesting that the *DRM*s encode the major de novo methylation enzymes affecting these genes.

Results and Discussion

De novo cytosine methylation is important in many processes, including genomic imprinting and the silencing of transposons and newly introduced transgene DNAs [6–9]. Aberrant de novo methylation is associated with the silencing of tumor suppressor genes in human cancers [10]. Enzymes responsible for de novo methylation have been identified in mammals (Dnmt3a and b) [11, 12], but de novo enzymes from plants are unknown. The *Arabidopsis thaliana* genome contains two related cytosine methyltransferase genes, *DRM1* and *DRM2* (Figure 1A), whose catalytic domains show sequence similarity to those of the Dnmt3 methyltransferases [5, 11]. However, unlike Dnmt3s, the DRMs have unique N termini containing ubiquitin associated (UBA) domains. Furthermore, relative to all known eukaryotic methyltransferases, the DRMs show a rearranged structure within their methyltransferase catalytic domains such that motifs VI through X are N-terminal to motifs I through V [5]. The *DRM1* and *DRM2* genes seem to have arisen from a recent gene duplication, since they show close sequence similarity (Figure 1A) and physical linkage (approximately 1 centimorgan apart on chromosome V).

Characterization of the *drm* Mutants

To study the function of the *DRM* genes, we isolated T-DNA insertion mutations in both *DRM1* and *DRM2* (Figure 1B) and crossed these together to create *drm1 drm2* double homozygous plants. RT-PCR using primers on either side of the T-DNA insertions detected expression of both *DRM1* and *DRM2* in wild-type plants but not in *drm1 drm2* double mutants, confirming that the T-DNA insertions are likely to disrupt gene function (Figure 1C). *drm1 drm2* double homozygotes showed a morphology similar to the wild-type WS strain (Figures 1D and 1E), even after five generations of inbreeding. Using the methylation-sensitive restriction enzymes HpaII and MspI, which are inhibited by either CpG and/or CpNpG methylation in their recognition sites, we did not observe a detectable loss of methylation at the repetitive centromeric repeat sequences (Figure 1F) [13], suggesting that the *drm* mutations do not affect maintenance methylation of these repeats.

DRM2 Is Required for *FWA* Transgene Silencing

To test whether the *DRM* loci affect de novo methylation associated with transgene silencing, we used the *FWA* gene [14]. The promoter of *FWA* is normally methylated within two direct repeats, causing *FWA* expression to be silenced. In epigenetic *fwa* mutants in which this methylation has been lost, *FWA* expression is ectopically activated in vegetative tissue causing a dominant late flowering phenotype. These epigenetic *fwa* alleles are stable; the *FWA* direct repeats do not become spontaneously remethylated even after several generations of inbreeding [14]. However, when an extra copy of the *FWA* gene is transformed into wild-type plants, the direct repeats become de novo methylated at a very high frequency, and transgene expression is silenced [14].

Using *FWA* transformation as a de novo methylation assay, we transformed both the parental WS strain and the *drm* mutant strains. In wild-type WS, the resulting transgenic plants displayed an early flowering phenotype similar to that of wild-type (Figures 2A and 2B), showing that the *FWA* transgene was efficiently silenced. Southern blot analysis showed that the *FWA* transgene was de novo methylated at the CpG dinucleotides present within CfoI restriction sites (Figure 2C). However, *FWA* transformed into *drm1 drm2* double homozygotes produced plants with a late flowering phenotype, and the de novo methylation of the transgenes was blocked (Figure 2). Untransformed *drm* mutant plants do not show a late flowering phenotype (Figure 1D), and *drm* mutations do not affect preexisting methylation at

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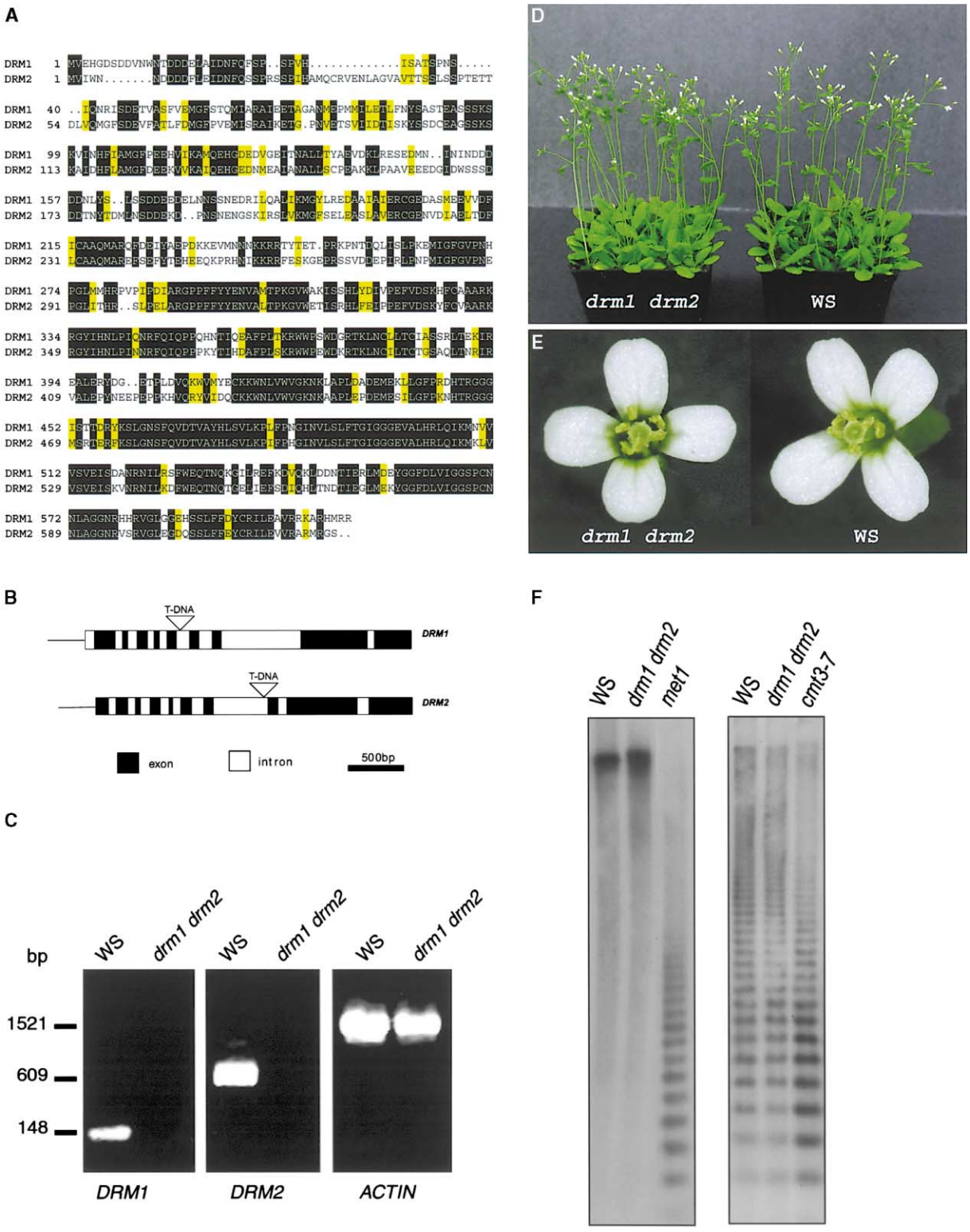

Figure 1. *DRM* Genes and Mutations

(A) ClustalX alignment of the inferred amino acid sequence of DRM1 and DRM2 (accession AF240695). The DRM1 sequence is inferred from the Columbia genomic sequence (accession ATF8M21), with the intron/exon borders determined by RT-PCR. Black shading shows identical residues, and yellow shading shows similar residues.

(B) Diagrams of the *DRM1* and *DRM2* genes, showing exons, introns, and positions of the T-DNA insertion mutations.

(C) RT-PCR expression of *DRM1*, *DRM2*, and *ACTIN*, in either wild-type WS (left lane of each panel) or *drm1 drm2* double mutant plants (right lane of each panel).

(D and E) Four-week-old *drm1 drm2* double mutant plants showing vegetative morphology (D) and floral structure (E) similar to wild-type WS plants.

(F) Southern blot analysis of centromeric repeat sequences. Genomic DNAs of the indicated genotype were digested with HpaII (left panel) or MspI (right panel).

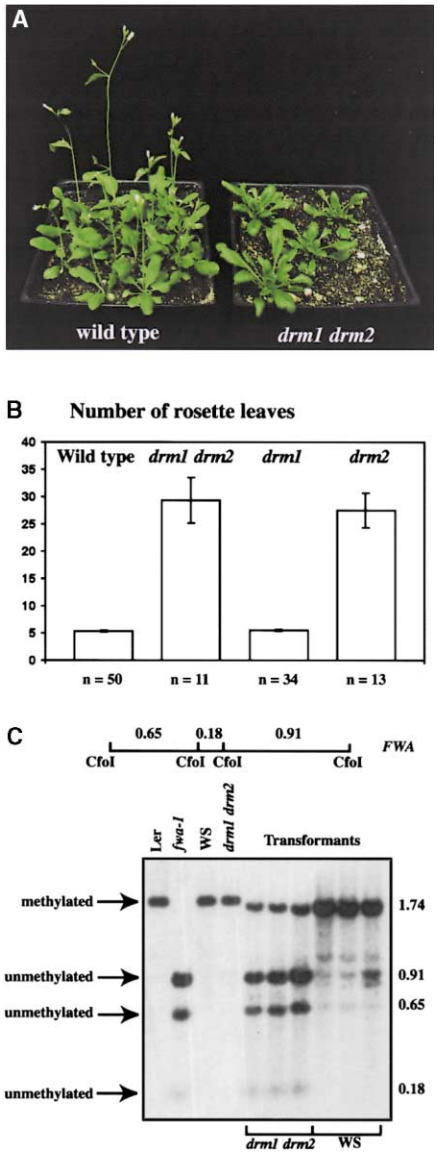

Figure 2. Effect of the *drm* Mutations on De Novo Methylation and Silencing of *FWA*

(A) Photograph of wild-type WS T1 plants (left) or *drm1 drm2* double mutant T1 plants (right) transformed with an *FWA* transgene.

(B) Quantitation of flowering time in plants of the indicated genotype transformed with an *FWA* transgene. Delayed flowering is associated with the production of additional rosette leaves. Thus, flowering time is expressed as number of rosette leaves produced per plant. Bars represent standard error. n = the number of independent transgenic plants analyzed.

(C) Top shows a diagram of the CfoI restriction fragments (in kilobases) present within the *FWA* promoter. The inner two CfoI sites are within the methylated direct repeats. Bottom shows a DNA blot of CfoI-digested genomic DNAs probed with a 1.74 kilobase fragment corresponding to the DNA shown at the top. The positions of the methylated and unmethylated bands are shown, as well as the size of the bands in kilobases. DNA from individual T2 plants of three independent transformants in WS or *drm1 drm2* plants are shown. The completely unmethylated *fwa-1* epigenetic allele is included as a control.

CfoI sites (Figure 2C). Therefore *DRM* is required for de novo methylation of *FWA* transgenes but is not required for maintenance of CpG methylation and silencing of the endogenous *FWA* gene.

The late flowering phenotype in *drm1 drm2 FWA* transformants was heritable in both the T2 and T3 generations. Furthermore, when we crossed late flowering *drm1 drm2 FWA* transformants with wild-type plants, the F1 plants retained a late flowering phenotype. Therefore, once *FWA* transgenes are hypomethylated (due to the presence of *drm* mutations), they retain the hypomethylated and active state even when exposed to wild-type *DRM* alleles in later generations. This suggests that *FWA* transgenes are most susceptible to *DRM*-dependent de novo methylation either during the transformation process itself or during the first generation after transformation. These results are consistent with the observation that the originally isolated *fwa* hypomethylated epigenetic alleles are stable in wild-type *DRM* backgrounds [14].

Using the *FWA* transformation assay, we also tested the *drm1* and *drm2* single mutants and found that *drm2* but not *drm1* blocked transgene-associated de novo methylation and silencing (Figure 2B). This is consistent with previous observations that *DRM2* RNA is expressed at much higher levels than *DRM1* RNA [5] and suggests that DRM2 is the predominant de novo methylase in *Arabidopsis*. Since we could not rule out a minor role for *DRM1*, we performed the remainder of our experiments using *drm1 drm2* double mutants.

The *DRM* Genes Are Not Required for Maintenance of *SUP* Gene Silencing

To study the role of the *DRM* genes in the maintenance of preexisting methylation and silencing at the *SUP* locus, we crossed the *drm1 drm2* double mutant to two different epigenetic hypermethylated *sup* alleles (*clark kent* alleles), *clk-3* and *clk-st*. *clk-3* is an allele in which the *SUP* gene has become densely hypermethylated and silenced but which spontaneously reverts to a wild-type unmethylated allele 3% of the time [15]. *clk-st* is a transgenic strain containing a 24 kilobase *SUP* inverted repeat transgene locus on chromosome III (see detailed description in the Supplementary Material available with this article online). In *clk-st*, both the inverted repeat *SUP* genes and the endogenous *SUP* gene are heavily methylated and silenced, causing a stable (nonreverting) epigenetic *clark kent* phenotype (Figure 3A) [3]. *drm1 drm2 clk-3* triple mutant plants and *drm1 drm2 clk-st* triple mutant plants retained a strong and heritable *clark kent* phenotype (Figure 3A), showing that *drm* mutations do not suppress preexisting gene silencing at the *SUP* locus.

We used bisulfite genomic sequencing of the 5′ end of the *SUP* locus to compare these triple mutant strains with control strains containing wild-type *DRM* genes (Table 1). *drm1 drm2* mutants retained a high level of CpNpG methylation in both the *clk-3* and *clk-st* backgrounds. Further, *drm1 drm2* mutants significantly reduced but did not eliminate *SUP* asymmetric methylation (a detailed study of the effect of the *DRM* genes on asymmetric methylation will be published elsewhere). CpG methylation is not adequately assayed in this re-

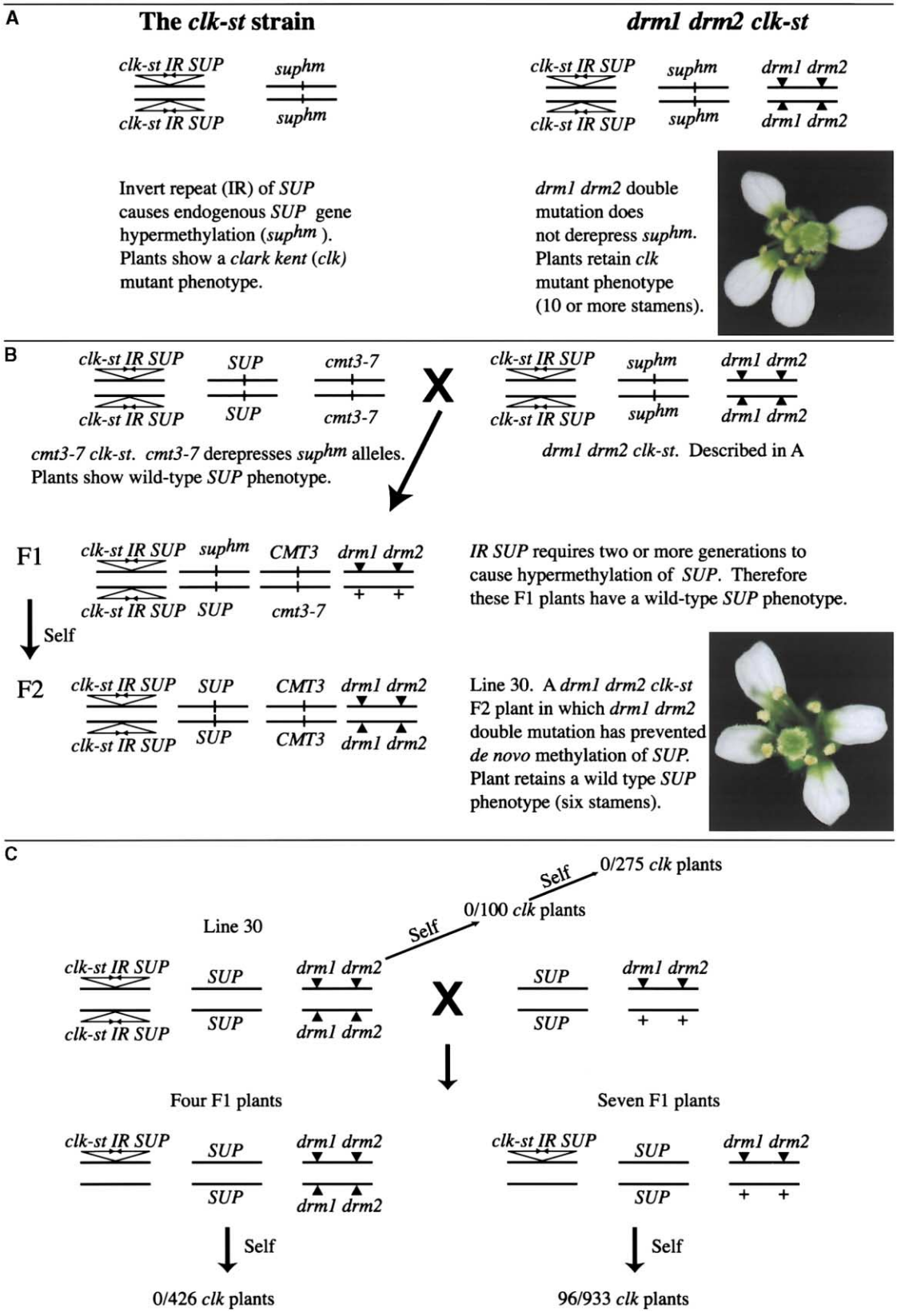

Figure 3. Role of *DRM* in Establishment of *SUP* Silencing

(A) Description of *clk-st* and *drm1 drm2 clk-st* plants. Photograph shows a *drm1 drm2 clk-st* flower with a *sup* floral phenotype (ten stamens and a defective gynoecium), demonstrating that *drm1 drm2* does not reactivate silenced *clk* alleles.

(B) Genetic scheme used to create line 30. Photograph shows a flower from a *drm1 drm2 clk-st* plant (line 30) with a wild-type *SUP* phenotype (six stamens and a normal gynoecium), demonstrating that *drm1 drm2* prevents reestablishment of gene silencing if *SUP* alleles have previously been exposed to *cmt3-7*.

(C) Genetic scheme used to further demonstrate that *drm1 drm2* double mutations block the establishment of *SUP* silencing. See text for explanation.

Table 1. Number of Cytosines Methylated in Different Sequence Contexts within Eight Cloned PCR Products of Bisulfite-Treated DNA from a 362 Nucleotide Region Near the 5′ End of the *SUPERMAN* Locus

	CpNpG	CpG	Asymmetric[a]
Total number of sites	72	8	576
Number methylated			
DRM1 DRM2 clk-st	36 (50.0%)	3 (37.5%)	87 (15.1%)
drm1 drm2 clk-st	34 (47.2%)	3 (37.5%)	33 (5.7%)
DRM1 DRM2 clk-3	27 (37.5%)	0 (0%)	28 (4.9%)
drm1 drm2 clk-3	24 (33.3%)	0 (0%)	15 (2.6%)
drm1 drm2 clk-st (Line 30)	0 (0%)	2 (25.0%)	0 (0%)
Line 30 × *drm1 drm2*	0 (0%)	0 (0%)	3 (0.5%)
Line 30 × *DRM1 DRM2*	36 (50.0%)	0 (0%)	56 (9.7%)

[a]Asymmetric is defined as CpHpH, where H = A, T, or C. Bisulfite genomic sequencing [3] utilized DNA from shoots of 3- to 4-week-old plants. The region of *SUP* corresponds to positions 992 to 1353 in GenBank accession AB025608.

gion, as there is only one CpG site, which shows low and spurious levels of methylation. In summary, *drm1 drm2* double mutants retained the majority of preestablished DNA methylation at *SUP*.

drm1 drm2 Mutations Block Inverted Repeat-Induced De Novo Methylation of *SUP*

To test whether the *drm* mutations block de novo methylation of *SUP*, we utilized the silencing properties of the *clk-st* strain. We found that the *SUP* inverted repeat transgene locus present in *clk-st* induces de novo methylation and gene silencing of a previously unmethylated and active *SUP* endogene. This silencing phenomenon occurs after two or more generations of exposure of the *SUP* endogene to the *SUP* inverted repeat (see Supplementary Material section for details). In order to test for de novo methylation, we first needed to erase the preexisting methylation present in *clk-st*. For this, we used the *cmt3-7* mutation (a null *CMT3* allele), which eliminates the majority of CpNpG and asymmetric methylation of *SUP* toward the 5′ end of the gene, causing reactivation of *SUP* expression [3]. Our genetic strategy (outlined in Figures 3B and 3C) was to use the *cmt3-7* mutation to erase *SUP* methylation and then simultaneously cross in a wild-type allele of *CMT3* and mutant alleles of *drm1* and *drm2*. In this way, we could determine whether *drm* mutations would block reestablishment of *SUP* methylation and silencing. As diagrammed in Figure 3B, we crossed a *cmt3-7 clk-st* plant to a *drm1 drm2 clk-st* plant. The F1 plants from this cross displayed a wild-type *SUP* phenotype. In the F2 progeny, we identified a plant that retained a wild-type *SUP* phenotype and that was homozygous for the wild-type *CMT3* allele, homozygous for the *clk-st* inverted repeat *SUP* locus, and homozygous for both *drm1* and *drm2*. We named this plant line 30 (Figure 3B). Bisulfite sequencing of line 30 near the 5′ end of the *SUP* gene showed that it had a very low level of cytosine methylation in CpNpG and asymmetric contexts (Table 1), confirming that the *SUP* genes in this line had not yet undergone de novo methylation. We then analyzed 100 self-pollinated F3 progeny and then 275 self-pollinated F4 progeny of line 30, and all displayed a wild-type *SUP* phenotype (Figure 3C). The stable wild-type phenotype of line 30 suggested that *drm1 drm2* double mutation blocked the de novo methylation and silencing of *SUP* that is normally induced by the inverted *SUP* repeat. To confirm this finding, we crossed line 30 with a plant doubly heterozygous for *drm1* and *drm2* (Figure 3C), to test whether reintroduction of wild-type *DRM* alleles would cause de novo methylation. Eleven F1 plants from this cross were genotyped for the *drm* mutations and then allowed to self-pollinate. Four F1 plants were *drm1 drm2* double homozygotes, and the F2 progeny from these plants all retained a wild-type *SUP* floral phenotype (a total of 426 plants analyzed). Bisulfite sequencing confirmed that these plants showed a very low level of cytosine methylation (Table 1; labeled Line 30 × *drm1 drm2*). The remaining seven F1 plants were *drm1 drm2* double heterozygotes, and the F2 progeny from all seven segregated plants with a *clk* phenotype (96 *clk* plants out of 993 total). Bisulfite sequencing of several of these *clk* plants confirmed that CpNpG and asymmetric methylation were reestablished (Table 1; labeled Line 30 × *DRM1 DRM2*). The results of these experiments show that the de novo methylation and silencing of *SUP* that is caused by the *clk-st* inverted repeat is dependent on the presence of wild-type *DRM* alleles.

Conclusion

Our results suggest that the *DRM* genes are important for the establishment but not the maintenance of gene silencing at *FWA* and *SUP* and are required for de novo methylation of cytosines in all known sequence contexts, CpG, CpNpG, and asymmetric. While the direct repeat containing *FWA* gene was only susceptible to *DRM*-dependent de novo methylation in the first generation after transformation, the *SUP* inverted repeat containing transgene locus was affected by *DRM* genes many generations after integration. One interpretation of this finding is that DRMs may methylate direct repeats and inverted repeats by different mechanisms.

The observation that *drm* mutants block de novo methylation of *FWA* and *SUP* but do not cause a major loss of preexisting methylation of these genes after inbreeding suggests that *FWA*, *SUP*, and other sequences such as centromeric repeats, do not normally lose their methylation during the plant life cycle. These data are consistent with results showing a lack of genome remethylation after exposure to demethylating mutants [16] and support a long-standing notion that a fundamental distinction between plant and animal DNA methylation is a lack of genome-wide resetting (demethylation and de novo methylation) during plant development [17]. Our

results also have implications for the mechanisms of genomic imprinting in plants. We have not observed imprinting-related seed development defects in the *drm* mutants, like those found in other methylation mutants, such as *ddm1* and antisense-*MET1* [18–21]. Furthermore, in contrast to *ddm1* and antisense-*MET1* mutants [18–21], *drm1 drm2* double mutants did not rescue seeds with a maternal mutant allele of the imprinted *MEDEA* locus (X.C., T. Kinoshita, R. Fischer, and S.E.J, unpublished data). Thus, as opposed to genomic imprinting in mammals [8, 17], de novo methylation may not play a significant role in plant imprinting. Instead, plants may reserve de novo methylation for genome defense processes, such as transposable element management and the RNA-directed de novo methylation associated with posttranscriptional gene silencing [6, 9, 22].

Experimental Procedures

Genetic Analysis of the *drm1* and *drm2* Mutations

The *DRM1* and *DRM2* T-DNA insertion mutations were isolated as described by the NSF Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis/). The *DRM1* T-DNA insert corresponds to position 96275 of BAC clone F8M21 (GenBank accession number AL353993), and the *DRM2* T-DNA insert corresponds to position 53805 of BAC clone T15N1 (GenBank accession number AL163792). Each mutant was backcrossed to the wild-type line WS, to eliminate unrelated T-DNAs. *DRM1* and *DRM2* are approximately 1 cM apart on chromosome V. To isolate a recombinant containing both the *drm1* and *drm2* mutations, we first crossed a *drm1* homozygote to a *drm2* homozygote and then crossed the resulting *trans* double heterozygote to a wild-type WS plant. F1 plants from this second cross were PCR genotyped to identify *cis* double heterozygotes, which were then self-pollinated to segregate *drm1 drm2* double mutants. The *drm1 drm2* doubly heterozygous plant used in the cross diagrammed in Figure 3C was first backcrossed five additional times to the wild-type Landsberg *erecta*, to further reduce the chance of this line containing unrelated mutations. The molecular markers used to genotype the *drm1* and *drm2* mutations were composed of the following combinations of three oligonucleotide primers: *DRM1* (JP 617, 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′; JP 807, 5′-TGCGATTGACAATTTCCAATTTTCTCCAT-3′; and JP 956, 5′-TTCTTGGTGTCTCAGTGTATGTTCGCCTT-3′) and *DRM2* (JP 617, 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′; JP 686, 5′-CCTCC TCCAGTAAACTGACGACGATACAA-3′; and JP 621, 5′-CAAAAAG CAAAAGAGAGTTAGGTTGACTT-3′).

FWA Transformation Experiments

A 6.1 kilobase XbaI to HindIII fragment of the *FWA* gene derived from cosmid clone WS20 (from the *fwa-1* mutant in the Ler ecotype) [14] was cloned into the Cambia 1300 vector, mobilized into *Agrobacterium* strain AGL-0 and selected on LB plates containing 50 μg/ml rifampicin and 50 μg/ml kanamycin. Plants were transformed using vacuum infiltration [23], and transformed seedlings were selected on MS plates containing 25 μg/ml Hygromycin. Plants were then transferred to soil and scored for flowering time. Genomic DNA was extracted from whole plants of T2 individuals and digested with the restriction enzyme CfoI, which cuts twice in the repeats and is sensitive for methylation. Southern blots were probed with a 1.7 kilobase PCR-generated DNA fragment corresponding to positions 498 to 2281 in GenBank accession AF178688.

Construction of *drm1 drm2 clk-3* and *drm1 drm2 clk-st* Triple Mutants

drm1 drm2 plants were crossed two successive times to *clk-3 gl1-1* plants [15] (*gl1-1* is a linked mutation that eliminates the epidermal hairs). F1 plants from the second cross were selected that were homozygous for *gl1-1* and that showed a strong *clark kent* phenotype. F2 progeny plants were then screened for the *drm1* and *drm2* mutations by PCR genotyping. Several *drm1 drm2* double homozygotes were selfed for two generations to confirm the stability of the *clark kent* phenotype. Several *clk-3 gl1-1 DRM1 DRM2* control plants were also isolated for the bisulfite sequencing reported in Table 1. To construct the *drm1 drm2 clk-st* triple mutant plants, *drm1 drm2* plants were crossed two successive times to *clk-st*. F1 plants from the second cross were selected that were homozygous for the inverted repeat *SUP* transgene and that showed a strong *clark kent* phenotype. Several *drm1 drm2* double homozygote F2 progeny plants were selfed for three generations to confirm the stability of the *clark kent* phenotype. Several *clk-st DRM1 DRM2* plants were also selected as controls for bisulfite sequencing (Table 1).

Supplementary Material

Supplementary Material including Supplementary Results and Discussion is available at http://images.cellpress.com/supmat/supmatin.htm.

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