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Role of CG and Non-CG Methylation in Immobilization of Transposons in *Arabidopsis*

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Summary

Methylation of cytosine residues in eukaryotic genomes is often associated with repeated sequences including transposons and their derivatives [1, 2]. Methylation has been implicated in control of two potential deleterious effects of these repeats: (1) uncontrolled transcription [2–4], which often disturbs proper expression of nearby host genes [5, 6], and (2) changes in genome structure by transposition and ectopic recombination [2, 7]. *Arabidopsis thaliana* provides a genetically tractable system to examine these possibilities, since viable mutants in DNA methyltransferases are available. *Arabidopsis* MET1 (METHYLTRANSFERASE 1, ortholog of mammalian DNA methyltransferase Dnmt1) is necessary for maintaining genomic cytosine methylation at 5′-CG-3′ sites [8, 9]. *Arabidopsis* additionally methylates non-CG sites using CHROMOMETHYLASE3 (CMT3) [10, 11]. We examined the mobility of endogenous *CACTA* transposons in *met1*, *cmt3*, and *cmt3-met1* mutants. High-frequency transposition of *CACTA* elements was detected in *cmt3-met1* double mutants. Single mutants in either *met1* or *cmt3* were much less effective in mobilization, despite significant induction of *CACTA* transcript accumulation. These results lead us to conclude that CG and non-CG methylation systems redundantly function for immobilization of transposons. Non-CG methylation in plants may have evolved as an additional epigenetic tag dedicated to transposon control. This view is consistent with the recent finding that CMT3 preferentially methylates transposon-related sequences [12].

Results and Discussion

Changes in transposon activity correlated with cytosine methylation were first described in maize [13–17]. Despite extensive investigation thereafter on both plants and animals (reviewed in [2, 18]), the role of DNA methylation in transposon immobilization remains controversial [2, 19–22].

Arabidopsis thaliana provides an ideal system to examine the role of DNA methylation genetically, since many viable DNA methylation mutants are available. *Arabidopsis* MET1 and CMT3 DNA methyltransferases are involved in methylation of CG and non-CG sites, respectively [8–11]. A third *Arabidopsis* gene necessary for DNA methylation is *DDM1* (*DECREASE IN DNA METHYLATION*), which encodes a putative chromatin remodeling factor and which is thought to affect DNA methylation indirectly through changes in chromatin structure [23–26]. The *ddm1* mutation affects both CG and non-CG methylation, although some sequences are not affected. A striking feature of *ddm1* and *met1* mutations is that they induce a variety of developmental abnormalities [9, 27–29]. One of the bases for the developmental abnormalities is transcriptional activation of normally silent genes (such as a homeobox gene *FWA* and disease-resistance genes) [30, 31]. More extensive genome-wide transcript analysis revealed that the *ddm1* mutation induces transcription from many heterochromatic sequences, substantial parts of which are transposon related [32–35]. Similarly, *met1* or *cmt3* mutations also induce transcription from several sequences including retroelement-related repeats [22, 30, 34, 35]. These results indicate that one role of DNA methylation is to suppress such background transcription [3, 4]. However, the effects of mutations in DNA methyltransferase genes on transposon mobilization (transposition) have remained unexplored.

Through genetic characterization of a *ddm1*-induced developmental abnormality, we previously identified the mobile endogenous *Arabidopsis CACTA* family transposons [27, 36]. *CACTA* and other transposons lose methylation and transpose at high frequency specifically in the *ddm1* background [36, 37], consistent with the interpretation that DNA methylation is necessary for transposon immobilization. However, because the primary function of DDM1 is likely to be chromatin remodeling rather than DNA methylation [25, 26], it remains unclear whether *ddm1*-induced transposition is mediated by loss of DNA methylation or by underlying changes in chromatin structure. Indeed, it has recently been shown that the *ddm1* mutation has additional effects to that of DNA methyltransferase mutants; it affects methylation of histone H3 lysine 9 in all loci examined [32, 35] as well as acetylation of histone H4 lysine 16 [38]. These observations suggest that the *ddm1* mutation affects histone modifications in pathway(s) independent of DNA methylation [35].

To directly test if loss of DNA methylation is sufficient for mobilization of transposons, we examined mobility of *CACTA* transposons in mutants of DNA methyltransferase genes. We first tested the effects of a mutation in the major DNA methyltransferase gene *MET1*. The *met1-1* mutation in the Columbia (Col) background in-

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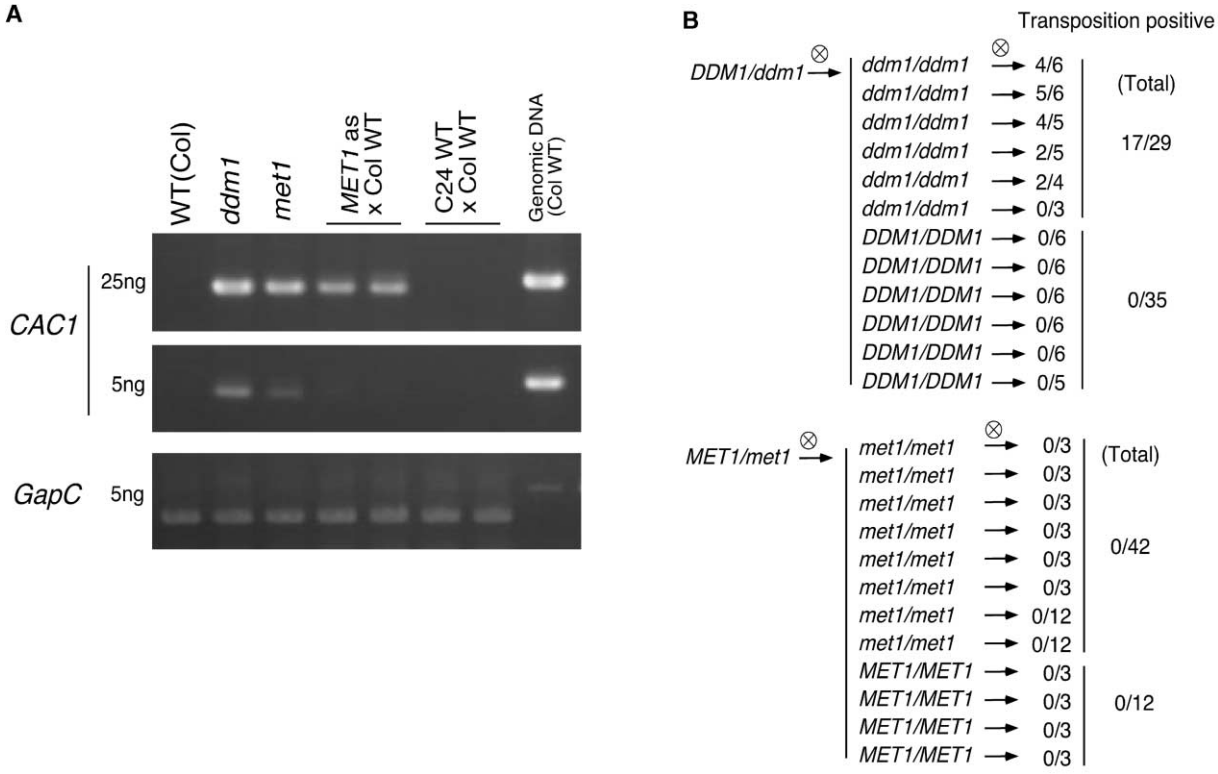

Figure 1. Transcription and Transposition of *CACTA* Elements in *met1* and *ddm1* Mutants

(A) The *CACTA* transcript detected by RT-PCR. The *MET1* antisense line is in the C24 background, which does not have any *CACTA* elements (unpublished). Therefore, the *MET1* antisense transgenic line was crossed to wild-type Col to examine effects of the transgene on *CACTA* activity. Total RNA from each genotype was reverse transcribed and amplified as described in Experimental Procedures. PCR reactions from two different dilutions of the cDNA, corresponding to 25 ng and 5 ng of input RNA, are shown for each plant. Length of predicted PCR products: *CACTA1*, 0.64 kb for cDNA and 0.72 kb for genomic DNA; *GapC*, 0.54 kb for cDNA and 0.82 kb for genomic DNA.

(B) Scoring of *CACTA* transposition. In order to detect independent immobilization events, we examined transposition in self-pollinated progeny from several independently segregating *ddm1* or *met1* homozygotes. The parental *DDM1/ddm1* and *MET1/met1* heterozygotes were backcrossed six times to wild-type Col in the heterozygous state. This procedure replaced hypomethylated *CACTA* elements with normally methylated copies [47]. No transposition was observed in 38 self-pollinated progeny from the (*MET1* antisense × Col) plant, 22 of which had the *MET1* antisense transgene as well as a *CACTA1* copy (not shown). Details of the Southern analysis are described in Experimental Procedures. The circled × indicates self-pollination.

duced accumulation of *CACTA* transcripts (Figure 1A). Similarly, *CACTA* transcripts were detected in transgenic plants expressing the *MET1* gene in antisense orientation (Figure 1A). Despite the transcript accumulation, no transposition was detectable either in the *met1* mutants or in *MET1* antisense plants (Figure 1B and legend). These results contrast with the high-frequency transposition observed in the *ddm1* mutant (Figure 1B) [36].

The *ddm1* mutation affects both CG and non-CG methylation, but *met1* mutants retain methylation at non-CG sites. We therefore examined the effect of combining mutations in the CG methylase *MET1* and the non-CG methylase *CMT3* genes. The double heterozygotes (*MET1/met1*, *CMT3/cmt3*) were selected from a cross between a *cmt3-i11* homozygous mutant in the Wassilewskija (WS) background and a *MET1/met1* heterozygote backcrossed to the Col background, which has potentially active *CACTA* members [36]. By genotyping self-pollinated progeny from the double heterozygotes, we found several *met1/met1-cmt3/cmt3*, *met1/met1-CMT3/CMT3*, *MET1/MET1-cmt3/cmt3*, and *MET1/MET1-CMT3/CMT3* homozygotes. Southern analysis of *CACTA* elements using the methylation-sensitive restriction enzymes *Msp*I and *Hpa*II revealed that the *met1* mutation affected CG methylation, whereas the *cmt3* mutation affected non-CG methylation (Figure 2A) as is the case at other loci [10, 11]. The *cmt3-met1* double mutation and the *ddm1* mutation affected both CG and non-CG methylation (Figure 2A and Figure S1 in Supplementary Material available online).

In each genotype class in the segregating families, we examined *CACTA* transcript levels by RT-PCR. We detected accumulation of *CACTA* transcripts in *cmt3* as well as *met1* mutants (Figure 2B). We next examined transposition of *CACTA* elements in the segregating F_2 generation by Southern analysis using *Eco*RV, which is insensitive to cytosine methylation. Several faint bands in new positions were detectable in six out of ten *cmt3-met1* plants in the F_2 families, while no additional bands were detectable in either single mutants or in wild-type in the same segregating families (Figure 2C). The transpositions in the double mutants were confirmed by genomic amplification and determination of the nucleotide

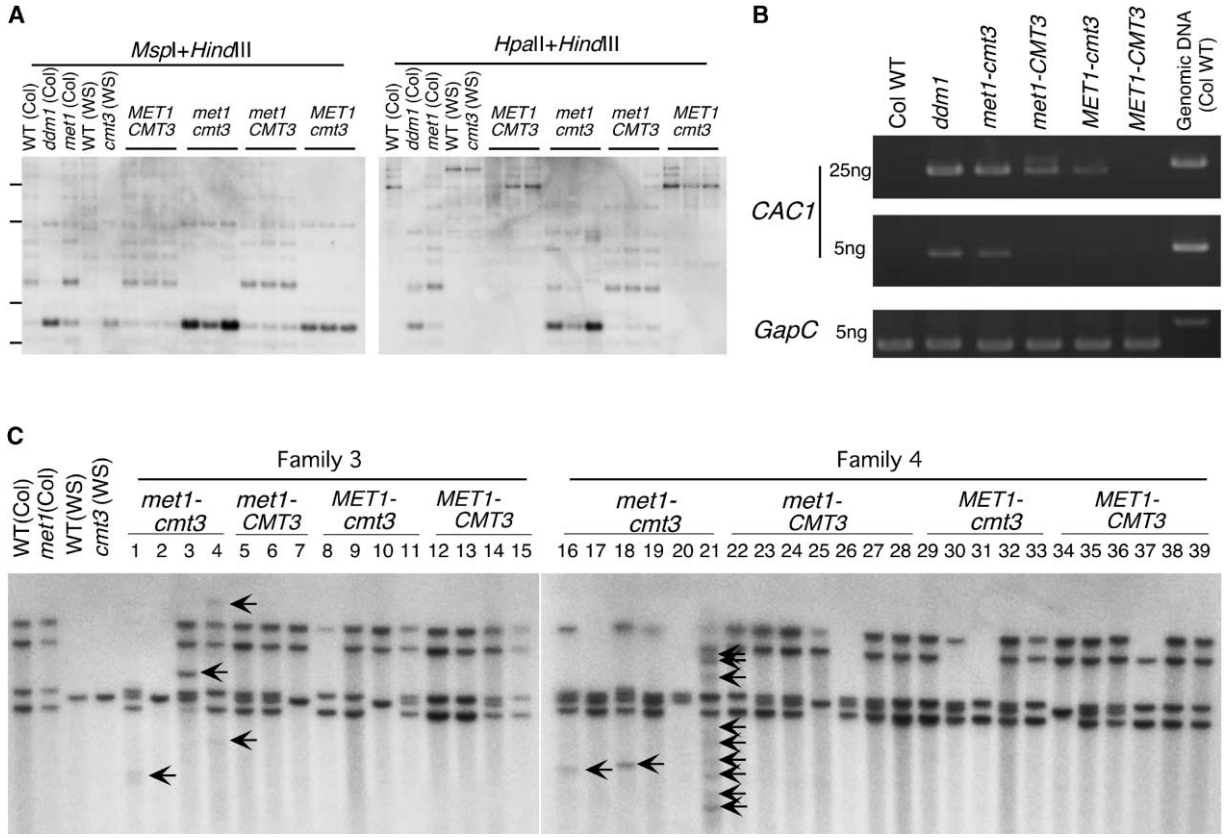

Figure 2. Methylation Status and Transposition of *CACTA* Elements in DNA Methyltransferase Mutants

Genomic DNA was prepared from mature leaves of plants with the indicated genotypes for Southern analysis.

(A) CG and non-CG methylation status examined by the methylation-sensitive restriction enzymes *Msp*I or *Hpa*II in addition to *Hind*III. Filters were probed with a *CACTA* sequence (probe D in [36]). Both *Hpa*II and *Msp*I recognize CCGG sites. *Msp*I does not cleave CCGG when the first C is methylated. Therefore, in *Msp*I cleavage the shift of bands downward by the *cmt3* mutation indicates loss of methylation in the first C (non-CG sites). *Hpa*II does not cleave CCGG when the second C is methylated. Therefore, in *Hpa*II cleavage the shift of bands downward by the *met1* mutation indicates loss of methylation in the second C (CG sites). The *cmt3-met1* double mutation and *ddm1* mutation affect both CG and non-CG methylation. DNA length markers are 7.74, 4.26, 1.49, and 0.93 kb.

(B) *CACTA1* transcripts accumulate in *met1*, *cmt3*, and *met1-cmt3* double mutants. The examined plants correspond to those shown in Figure 2C; plant 4 (*met1-cmt3*), 6 (*met1-CMT3*), 9 (*MET1-cmt3*), and 15 (*MET1-CMT3*).

(C) Wild-type, *cmt3*, *met1*, and the double mutants segregating in the F_2 generation of families 3 and 4. Details are described in Experimental Procedures. Segregation of *CACTA* copies was observed because families 3 and 4 are derived from a cross between Col (*MET1/met1*) and WS (*cmt3/cmt3*).

sequence of the region flanking *CACTA1* (see Supplementary Material). To estimate the transposition frequency on a larger scale, we examined the self-pollinated progeny from each genotype class. Almost all of the examined double mutant F_3 plants showed new bands, most of which were not shared by siblings, reflecting independent transposition events (Figure 3A, Table 1). No transposition was observed in wild-type or *met1* single mutants (Figure 3B, Table 1, Supplementary Material), consistent with the results described above. Interestingly, in the *cmt3* mutants, bands in new positions were observed in about 10% of the examined F_3 plants (Figure 3C, Table 1, Supplementary Material), suggesting that *CACTA* elements transpose at a low frequency in the *cmt3* mutant background. The transpositions in the *cmt3* mutants were confirmed by sequencing the region flanking *CACTA1* (Supplementary Material). The transposition frequency, however, was much increased in the double mutants (Figure 3A and Table 1). In addition to the increase in the proportion of plants showing transposition, many of the double mutant plants showed multiple new bands reflecting multiple independent transpositions and increase in the transposon copy number (Figure 3A).

The transposition frequency in the double methyltransferase mutant was even higher than that induced by the *ddm1* mutation (Table 1), perhaps because the primary function of the DDM1 gene product is chromatin remodeling [25, 26] and the effect of the *ddm1* mutation on DNA methylation is secondary and incomplete. Furthermore, the results in the F_2 generation (Figure 2C) indicate that the high frequency of transposition in the double mutants was induced somatically, even before passing through the gametophyte stage. Essentially the same results were obtained when we examined double mutants of *met1* and another *cmt3* mutant allele, *cmt3-7*, which is in the Landsberg *erecta* ecotype background; the double mutation specifically induced high-frequency

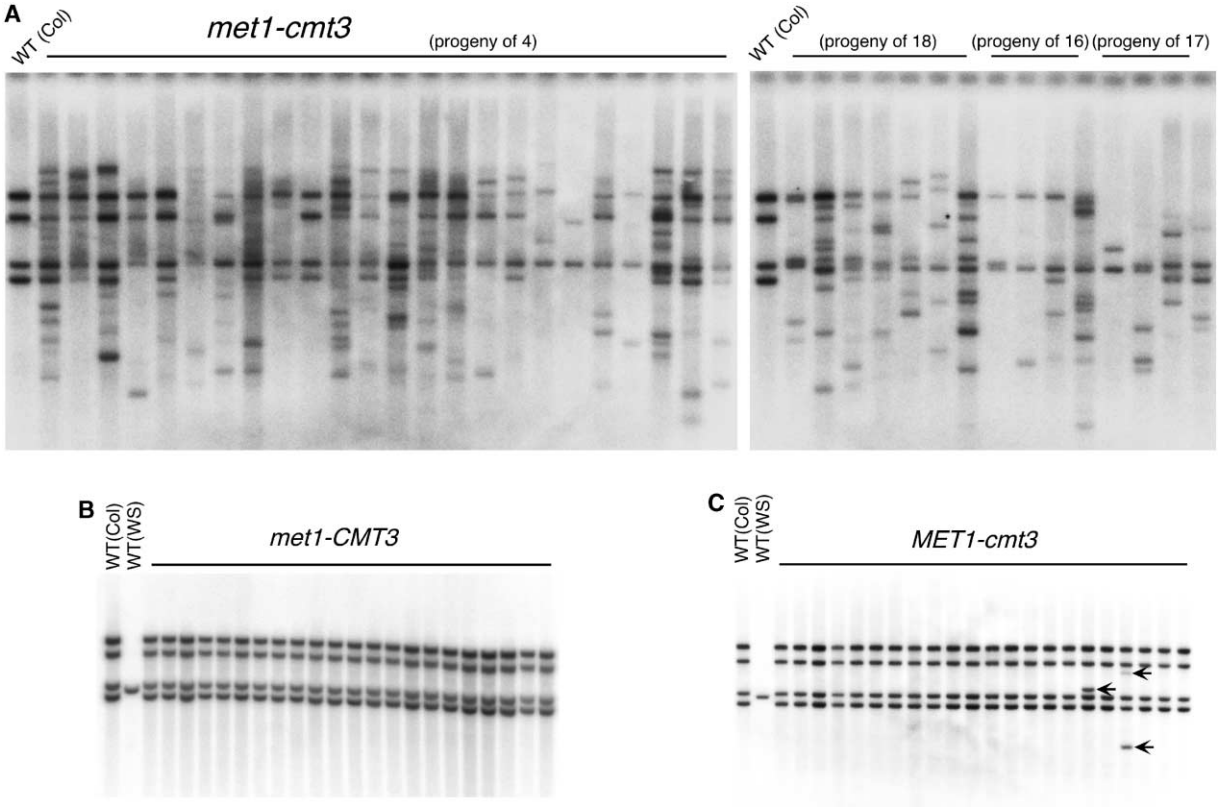

Figure 3. Transposition in the F_3 Generation

For scoring transposition in the F_3 generation (Table 1), self-pollinated progeny from plants keeping all the original Col copies (plants 4, 6, 9, 15, 22, 33, and 36 in Figure 2C) were used. For *met1-cmt3* double mutants, in addition to plant 4, we used plants 16, 17, and 18. However, we did not use plant 21 because it already showed many transpositions in the F_2 generation (Figure 2C).

(A) Transposition in self-pollinated progeny from *met1/met1-cmt3/cmt3* plants (plants 4, 18, 16, and 17 of Figure 2C).

(B) Self-pollinated progeny from a *met1/met1-CMT3/CMT3* plant (progeny from plant 22 in Figure 2C).

(C) Self-pollinated progeny from a *MET1/MET1-cmt3/cmt3* plant (progeny from plant 9 in Figure 2C). Arrowheads indicate unique bands.

mobilization of *CACTA* elements in the segregating F_2 family (not shown).

Interestingly, although the transposition frequency in single methyltransferase mutants (*met1* or *cmt3*) was much less than that induced in the double mutant, we could detect significant accumulation of *CACTA* transcript in both *met1* and *cmt3* single mutants (Figures 1A and 2B). Although our semiquantitative estimation of the transcript level suggests that the double mutant accumulated more transcript than the single mutants, elevation of the transposition frequency in the double mutants is much greater than the increase in transcript levels. One possible explanation for this apparent discrepancy is that accumulation of the transcript over a threshold level might induce the high frequency of transposition observed in the double mutant. Alterna-

Table 1. Transposition of *CACTA* Elements in *ddm1*, *met1*, *cmt3*, and *met1-cmt3* Double Mutants

	F_2		F_3: Self-Pollinated Progeny from F_2	
Family Number[a] and Parental Genotype	Genotype	*CACTA1* Transcript	Number Examined[b]	Number of Transposants[c]
1, *DDM1/ddm1*	*ddm1/ddm1*	+	29	17 (59%)
	DDM1/DDM1	−	35	0
2, *MET1/met1*	*met1/met1*	+	42	0
	MET1/MET1	−	12	0
3+4, *MET1/met1-CMT3/cmt3*	*cmt3-met1*	+	39	38 (97%)
	CMT3-met1	+	45	0
	cmt3-MET1	+	42	4 (10%)
	CMT3-MET1	−	43	0

[a] All results of Southern analysis in F_3 plants in families 3 and 4 are shown in the Figure S2 of the Supplementary Materials available online.
[b] Total number of plants examined.
[c] Number of plants with new bands, which reflect transpositions.

tively, DNA methylation might also affect transposition efficiency through steps other than transcript accumulation. For example, accessibility of transposase to transposon ends might be affected by DNA methylation [39]. In addition, if the host recombination/repair machinery is involved in transposon excision and integration, this machinery might also be affected by DNA methylation [7].

Our results indicate that loss of DNA methylation is sufficient for transposon mobilization. However, changes in chromatin structure may function as downstream factors. It was recently shown that *met1* or *cmt3* mutations caused reduced histone H3 lysine 9 methylation on some retrotransposon sequences, although not on untranscribed heterochromatic sequences [35]. In addition, cytogenetic studies indicate that the *met1* mutation also affects heterochromatin formation in pericentromeric sequences, where the *CACTA1* sequence is located [38]. A combination of these effects on chromatin might also affect transposon mobility. Irrespective of mechanisms downstream of DNA methylation to suppress transposons, the high-frequency transposition in the double methyltransferase mutant strongly suggests that CG and non-CG methylation redundantly contribute as epigenetic tags for immobilization of transposons.

Arabidopsis and mouse mutants with reduced CG methylation display several types of developmental defects, which are associated with changes in host gene transcription [9, 27–30, 40–42]. In contrast, *Arabidopsis cmt3* mutations do not induce any morphological phenotype, despite the global loss of non-CG methylation [10, 11]. Interestingly, CMT3 preferentially methylates transposon-related sequences [12], suggesting that CMT3-mediated non-CG methylation might have evolved specifically to reinforce transposon control. Although CHROMOMETHYLASE genes have been found only in the plant kingdom, non-CG methylation is connected to more universal epigenetic processes such as RNA interference and histone methylation [43, 44], which might also contribute to transposon control [45].

Experimental Procedures

Plant Materials and Genotyping

The isolation of the *ddm1-1*, *cmt3-i11*, and *cmt3-7* mutants was previously reported by Vongs et al. [23], Bartee et al. [11], and Lindroth et al. [10], respectively. The *met1-1* mutant and antisense *MET1* line were kind gifts from Eric Richards and Jean Finnegan, respectively. The *cmt3/cmt3* mutant (*cmt3i11*) is in the WS background. It is a presumed null mutation that causes missplicing of the transcript, resulting in premature termination of the protein-coding region upstream of the catalytic sequences in methyltransferase motif IV. Sequences of primer pairs and restriction enzymes used to distinguish *ddm1*, *met1*, and *cmt3* mutations from the wild-type alleles are 5′-ATTTGCTGATGACCAGGTCCT-3′+5′-CATAAAC CAATCTCATGAGGC-3′ (*Ns*iI), 5′-ACTTTGGCTTCCCTTCTCGA-3′+ 5′-TCACGGTGATTGGACGGA-3′ (*Hae*III), and 5′-GTTCTGCGTCAG TTAATTG-3′+5′-GTGACCACTGATTCCTTGGC-3′ (*Mse*I), respectively.

Detection of *CACTA1* Transcripts

RNA was prepared from leaf tissue using the RNeasy Plant Mini Kit with RNase-Free DNase Set (QIAGEN, Germany). cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR conditions: 94°C for 3 min, for 40 cycles (94°C for 30 s, 58°C for 30 s, 72°C for 45 s), 72°C for 3 min. The PCR product was then separated by electrophoresis on 1.5% agarose. The primer pair to detect *CACTA1* transcripts is SP15 (5′-AACAAAAGCATCATTCTACTT AAC-3′) and SP40 (5′-AGGCCTACAATGGAAATGACG-3′). For the control reaction, the constitutively expressed *GAPC* [46] gene was amplified, using the primers GapC1(5′-CACTTGAAGGGTGGTGCC AAG-3′) and GapC2(5′-CCTGTTGTCGCCAACGAAGTC-3′).

Scoring Transposition

Transposition frequency was scored after one generation of self-pollination after becoming homozygous for the mutation(s). Transposition in the *MET1* antisense line was examined in the F_2 generation from a cross between the transgenic and Col wild-type plants. Plant genomic DNA was prepared from mature leaves, cleaved with *Eco*RV (which is insensitive to cytosine methylation), and used for Southern blot analysis with a *CACTA* probe (probe B in [36]).

Suppression PCR

Transpositions were confirmed by directly sequencing flanking regions of *CACTA1* for *cmt3* single and *met1-cmt3* double mutants. Experimental conditions for suppression PCR were as described in [36].

Supplementary Material

Supplementary material, including additional figures and results, can be found at http://images.cellpress.com/supmat/supmatin.htm.

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Note Added in Proof

The *cmt-i11* single mutation mobilized *CAC* elements only when the transposons were introduced from *MET1/met1* heterozygotes, not when introduced from wild-type Col plants (Figure S3 in the Supplementary Material), further supporting our proposal that MET1 and CMT3 function redundantly in the immobilization of the *CAC* elements.