Three SRA-Domain Methylcytosine-Binding Proteins Cooperate to Maintain Global CpG Methylation and Epigenetic Silencing in Arabidopsis

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Abstract

Methylcytosine-binding proteins decipher the epigenetic information encoded by DNA methylation and provide a link between DNA methylation, modification of chromatin structure, and gene silencing. VARIANT IN METHYLATION 1 (VIM1) encodes an SRA (SET- and RING-associated) domain methylcytosine-binding protein in Arabidopsis thaliana, and loss of VIM1 function causes centromere DNA hypomethylation and centromeric heterochromatin decondensation in interphase. In the Arabidopsis genome, there are five VIM genes that share very high sequence similarity and encode proteins containing a PHD domain, two RING domains, and an SRA domain. To gain further insight into the function and potential redundancy among the VIM proteins, we investigated strains combining different vim mutations and transgenic vim knock-down lines that down-regulate multiple VIM family genes. The vim1 vim3 double mutant and the transgenic vim knock-down lines showed decreased DNA methylation primarily at CpG sites in genic regions, as well as repeated sequences in heterochromatic regions. In addition, transcriptional silencing was released in these plants at most heterochromatin regions examined. Interestingly, the vim1 vim3 mutant and vim knock-down lines gained ectopic CpHpH methylation in the 5S rRNA genes against a background of CpG hypomethylation. The vim1 vim2 vim3 triple mutant displayed abnormal morphological phenotypes including late flowering, which is associated with DNA hypomethylation of the 5’ region of FWA and release of FWA gene silencing. Our findings demonstrate that VIM1, VIM2, and VIM3 have overlapping functions in maintenance of global CpG methylation and epigenetic transcriptional silencing.

Introduction

DNA cytosine methylation is an epigenetic mark important for many processes including parental imprinting, X chromosome inactivation, and the silencing of transposable elements [1–3]. In mammals, methylated cytosines are found almost exclusively in symmetrical CpG sequence contexts, and CpG methylation patterns are propagated after DNA replication by “maintenance” DNA methyltransferases (DNMT1-type) [2]. However, DNA cytosine methylation in plants can occur at CpHpG and CpHpH (where H = A, T, or C) as well as CpG sites. In Arabidopsis, these three categories of cytosine methylation are carried out by distinct activities [4]: CpG methylation is maintained primarily by the DNMT1-type methyltransferase, MET1; CHROMOMETHYLASE3 (CMT3) is responsible for CpHpG methylation; and methylation at CpHpH sites is accomplished by a de novo methyltransferase, DOMAINS REARRANGED METHYLASE (DRM). Cytosine methylation patterns in Arabidopsis are also propagated after DNA replication by “maintenance” methyltransferase, DOMAINS REARRANGED METHYLASE 1 (DRM1), and release of DNA hypomethylation and decondensation of centromeres in interphase [8].

Complex interactions between DNA methylation and histone modification articulate epigenetic gene expression states, and methylcytosine-binding proteins play an important role in interpreting the epigenetic information encoded by DNA methylation [3]. The best understood class of methylcytosine-binding proteins contains a conserved methylcytosine-binding domain (MBD) [7]. Several MBD proteins in mammals, including methyl-CpG-binding protein 1 (MeCP1), MeCP2, MBD2, and MBD4 have been identified with high affinity for methylated DNA, and the biological importance of mammalian methylcytosine-binding proteins has been shown in the wide range of severe phenotypes by mutations of these genes [8–10]. In contrast to mammalian MBD proteins, knowledge of plant MBD proteins remains relatively limited. Among 13 MBD proteins in the Arabidopsis proteome, three (AtMBD5, AtMBD6, and AtMBD7) have been shown to bind symmetrically methylated CpG sites in vitro [11–13]. Although developmental defects have been observed in lines carrying a loss-of-function mutation of AtMBD9 or a transgene directing RNAi against AtMBD11 transcripts, the role of any AtMBD proteins in epigenetic regulation remains to be defined [14,15].

Recently a novel class of methylcytosine-binding proteins have been defined that interact with the modified base through an SRA (SET- and RING-Associated) domain [16,17]. We previously reported that mutations in the Arabidopsis VIM1 gene, which encodes an SRA domain methylcytosine-binding protein, causes DNA hypomethylation and decondensation of centromeres in interphase [8]. The SRA domain of VIM1 shares amino acid...
Author Summary

Methylation of cytosine bases provides one layer of epigenetic information that is superimposed on the nucleotide sequence of a genome. Proteins that bind methylated cytosines and also help maintain that DNA modification are important linchpins in a self-propagating system mediating memory of epigenetic states. We previously demonstrated that the VIM1 (VARIANT IN METHYLATION 1) protein from the flowering plant Arabidopsis thaliana binds DNA that contains methylated cytosine and is required for complete methylation and compaction of centromeric DNA. In this study, we show that VIM1 works in concert with two related proteins, VIM2 and VIM3, to maintain cytosine methylation not only at centromeres but throughout the genome. VIM proteins act specifically in the DNA methylation pathway that targets CpG dinucleotides, which plants share with animals, rather than the plant-specific non-CpG methylation pathways. Loss of VIM1, VIM2, and VIM3 function also causes a reduction in transcriptional gene silencing at a variety of sequences, and leads to abnormal developmental phenotypes, including late flowering associated with loss of FWA gene silencing. Our results demonstrate that these three related VIM family proteins have overlapping functions in the MET1-mediated CpG methylation pathway.

We measured the steady state levels of transcripts using reverse transcriptase (RT)-PCR from all five VIM genes to obtain insight into their function and potential redundancy (Figure S1B and S1C). VIM1 was highly expressed in inflorescence tissue and to a lesser extent in two-week-old leaves of wild-type Columbia (Col) plants (Figure S1C). VIM2 [At5g39550] transcripts were found in both inflorescences and two-week-old leaves, while VIM2 was abundantly expressed in inflorescences. In contrast, VIM4 and VIM5 were absent from leaves or inflorescence tissue at this level of detection, suggesting that the steady-state levels of VIM4 and VIM5 are very low or that these may be pseudogenes. Based on our expression data, we concentrated on the functional analysis of VIM1, VIM2, and VIM3.

VIM1, VIM2, and VIM3 Function Redundantly to Maintain Centromere DNA Methylation

We first investigated the subnuclear localization of VIM1, VIM2, and VIM3 in interphase. Transgenes containing VIM1, VIM2, or VIM3 cDNAs fused with YFP at the N-terminus under the control of the strong, constitutive cauliflower mosaic virus (CaMV) 35S promoter were transformed into Col plants. Fixed nuclei from transgenic Col root cells expressing YFP-VIM1, YFP-VIM2, or YFP-VIM3 are shown in Figure 1A. YFP-VIM1, -VIM2, and -VIM3 fusion proteins were broadly distributed in the nucleus and were enriched in the heterochromatic chromocenters. The similar subnuclear localization of all three expressed VIM proteins suggests a possible functional redundancy among these VIM proteins.

We characterized T-DNA insertional mutations that disrupt the coding sequences of VIM1, VIM2, and VIM3 in the Col background. Using RT-PCR and primers flanking the T-DNA insertion sites, we did not detect expression of VIM1, VIM2, and VIM3 in vim1-2, vim2-2, and vim3-1 mutant plants, respectively, confirming that the T-DNA insertions likely destroy gene function (Figure S2 and data not shown). While the vim1-2 mutation caused a slight decrease in DNA methylation of centromeric 180-bp repeat arrays, neither the vim2-2 nor the vim3-1 allele led to a centromere repeat hypomethylation phenotype (Figure 1B). Therefore, VIM2 and/or VIM3 are not required for centromere DNA methylation, or alternatively, one or both of these genes function redundantly with VIM1 to maintain centromere DNA methylation.

To test these alternative hypotheses, we individually introduced cDNA copies of Col VIM1, VIM2 or VIM3 genes into Bor-4 plants, which carry the vim1-1 loss-of-function allele, and investigated the effect on centromere DNA methylation. VIM cDNAs fused with TFP at the N-terminus were expressed under the control of the 35S promoter (Figure S3). Expression of a wild-type Col VIM1 cDNA in Bor-4 plants fully restored DNA methylation at the centromere (Figure 1C). Over-expression of Col VIM2 or VIM3 cDNAs can also fully suppress the hypomethylation of centromeric repeats in Bor-4 vim1-1 plants, demonstrating that VIM2 and VIM3 proteins can function redundantly with VIM1.

Isolation of vim Double Mutants and Transgenic Lines with Coordinate Gene Silencing of VIM1, VIM2, and VIM3

To determine the extent of functional redundancy within the VIM gene family, we generated and characterized a set of double mutants combining two loss-of-function mutations among VIM1, VIM2, and VIM3. As an alternative approach, we also isolated transgenic plants with a coordinate decrease in the expression of VIM1, VIM2 and VIM3 genes. We took advantage of the variation in transgene expression among the Col transgenic lines expressing an YFP-VIM1 cDNA used in our nuclear localization analysis (Figure 1A). A significant proportion of the transgenic plants did

Results

The VIM Gene Family in Arabidopsis

VIM1 is a member of a small gene family, which was originally identified by a naturally-occurring null mutation in the Arabidopsis thaliana accession, Borky-4 (Bor-4) [18]. The Arabidopsis genome contains five VIM genes, each of which encodes a protein containing a PHD domain, two RING domains, and an SRA domain (Figure S1A). Interestingly, four of the VIM genes (VIM1 [At1g57820], VIM2 [At1g66050], VIM4 [At1g66040], and VIM5 [At1g57800]) are located within a 3 Mb region on the lower arm of chromosome 1. A reverse transcriptase pseudogene (At1g57810) is located between VIM1 and VIM5, and an unrelated putative pseudogene (At1g66045) is located between VIM2 and VIM4. The VIM proteins share high amino acid sequence identity (>68%) throughout their entire length, including the four previously recognized domains (Figure S1A).

Similarity with mammalian UHRF1 (also known as mouse Np95 and human ICBP90), which has been implicated in regulation of chromatin modification [19,20], transcription [21], and the cell cycle [22]. Recent reports demonstrate that UHRF1 is required for maintenance of CpG DNA methylation [23,24]. UHRF1 physically interacts with DNMT1 and has been postulated to mediate the loading of DNMT1 on to replicating heterochromatin [23–25].

In the Arabidopsis genome, there are four genes that share high sequence similarity with VIM1. To obtain further insight into the function and potential redundancy among the VIM proteins, we have investigated vim double and triple mutants, as well as transgenic vim knock-down lines that down-regulate multiple VIM family genes. Our results indicate that VIM1, VIM2 and VIM3 have overlapping functions in maintenance of cytosine methylation at CpG dinucleotides distributed throughout the genome. Moreover, VIM proteins are required for transcriptional silencing of a variety of sequences, including centromeric repeats, transposons, and the parentally imprinted FWA locus.

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not show any YFP expression and had lower steady-state transcript levels of VIM2 and VIM3 as well as VIM1 (Figure S2) presumably due to RNA interference. We chose two of these transgenic VIM family “knock-down” lines (vimKD-A and vimKD-B) for further analysis.

**DNA Methylation in Heterochromatic Regions Is Decreased in the vim1 vim3 Double Mutant and Transgenic vim Knock-Down Lines**

We tested whether DNA methylation at highly repetitive loci is dependent on the function of multiple VIM gene family members. Genomic DNA samples prepared from plants of the different vim genotypes were analyzed by DNA gel blot hybridization after digestion with methylation-sensitive restriction endonucleases. Cleavage by HpaII (5′-CCCGG-3′) is inhibited by either Cpg or ChpG methylation; MspI (5′-CCCGG-3′) digestion is blocked by ChpG methylation; and NalIII (5′-CATG-3′) activity is inhibited by CpaHP methylation. The vim1 vim2 and vim2 vim3 double mutants did not display any change in centromere or 45S rRNA gene cytosine methylation compared to vim1 and Col wild-type plants, respectively (Figure S4 and data not shown). In contrast, the vim1 vim3 double mutant and the two vim knock-down lines displayed decreased CpG and ChpG methylation at HpaII and MspI sites in both the centromere and 45S rRNA genes relative to the vim1 single mutant (Figure 2A and 2B). There was no difference among the genotypes tested after NalIII digestion at the 180-bp centromeric repeats and 45S rRNA genes (Figure S5), which might reflect a low level of CpaHP methylation. Our DNA gel blot data demonstrate that VIM proteins, particularly VIM1 and VIM3, function redundantly to maintain CpG and ChpG methylation at the 180-bp centromere repeats, as well as in repetitive sequences outside the centromere.

To evaluate the potential role of the VIM genes in shaping DNA methylation patterns in other heterochromatic regions, the DNA methylation status of two transposable elements, the MULE DNA transposon AtMUS1 and the gypsy-class LTR retroelement AtGP1, was compared among the different vim genotypes using bisulfite sequencing. We found that Cpg sites in these elements were heavily methylated in Col wild-type, vim1, and vim3 plants, but Cpg methylation was significantly decreased in the vim1 vim3 mutant and the two transgenic vim knock-down lines (Figure 2C and 2D; Table S1). In contrast, no substantial changes in CpaHP and CpaHP methylation were observed. These results further indicate that VIM proteins function redundantly to maintain cytosine methylation of heterochromatic sequences outside of the centromere.

**5S rRNA Gene Methylation Is Altered in the vim1 vim3 Mutant and Transgenic vim Knock-Down Lines**

Next, we assessed the DNA methylation pattern of 5S rRNA genes using DNA gel blot hybridization analysis and bisulfite sequencing. The vim1 vim3 mutant and the two transgenic vim knock-down lines had strongly decreased Cpg methylation at HpaII sites and reduced CpaHP methylation at MspI sites in the 5S rRNA genes (Figure 3A). This hypomethylation was accompanied by higher molecular weight hybridization signals after HpaIII (5′-GGCC-3′) digestion (Figure 3A) and NalIII digestion (Figure S3). Bisulfite sequencing of the 5S rRNA genes confirmed that Cpg methylation was significantly decreased in the vim1 vim3 mutant and the two transgenic vim knock-down lines relative to Col wild-type plants (Figure 3B; Table S1). Most of the Cpg sites are affected by the vim1 vim3 mutation combination and the coordinate knock-down of VIM gene expression in the transgenic lines, but the degree of hypomethylation at different Cpg sites varied widely (5%–70% decrease in vimKD-B) (Figure 3C and data not shown). Our bisulfite sequencing analysis also demonstrated an increase in CpaHP methylation throughout the 5S rRNA genes in the vim1 vim3 mutant and the transgenic vim knock-down lines, especially vimKD-B.

Increased CpaHP methylation levels observed in the 5S rRNA genes could result from activation of DNA methylation activity and/or inhibition of DNA demethylation activity. To address these possibilities, we first monitored the levels of sRNA species that could target de novo methylation and/or inhibition of DNA demethylation activity. To address these possibilities, we first monitored the levels of sRNA species that could target de novo methylation and/or inhibition of DNA demethylation activity.

**VIM Proteins Affect Genic DNA Methylation at Cpg Sites**

We analyzed genic cytosine methylation at three different loci ([@ref]{27}), At4g00500 (lipase class 3 family protein), At4g31560 (MEE57, maternal effect embryo arrest 57), and At4g31150 (endonuclease V family protein), to determine whether VIM proteins are involved in DNA methylation of low-copy, expressed sequences not associated with heterochromatin. First, a PCR-based method was used to assay DNA methylation of these loci in the vim mutant lines. After HpaII treatment, unmethylated DNA will be digested and therefore not amplified by PCR. In wild-type Col, vim1, and vim3 samples, all three tested genic regions were easily amplified after HpaII digestion. In contrast, the abundance of PCR product for At4g00500 and At4g31150 (and to a lesser extent At4g31560) amplified from the vim1 vim3 and transgenic vim knock-down samples was lower than that from the wild-type Col sample, indicating a reduction in Cpg methylation (Figure 4A).

To verify and extend the results obtained by the PCR-based method, bisulfite sequencing was carried out for the At4g31150 gene (Figure 4B; Table S1). DNA methylation at At4g31150 in wild-type Col plants was predominantly localized in Cpg dinucleotides. The vim1 vim3 mutant and the transgenic vim knock-down lines showed a two- to three-fold reduction in Cpg methylation at the locus. In wild-type Col, vim1, and vim3 samples, 75–91% of the Cpg dinucleotide in

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**Figure 1. DNA methylation of the centromeric repeats in vim mutants.** (A) Subnuclear localization of VIM proteins. Localization of VIM proteins were detected in Col root nuclei expressing a YFP-VIM1, YFP-VIM2, or YFP-VIM3 transgene under the control of the 35S promoter. Propidium iodide (PI) was used as a DNA counterstain; chromocenters are more intensely stained. Bar, 5 μm. (B) 180-bp centromeric repeat methylation phenotype in different vim single mutants. (C) Suppression of centromere DNA hypomethylation phenotype in the bor-4 strain, which contains the natural vim1-1 allele, by overexpressing VIM1 cDNA clones. Bor-4 plants were transformed with VIM1, VIM2, or VIM3 transgene under the control of the 35S promoter. Genomic DNA samples of the indicated genotypes were digested with HpaII and used for DNA gel blot analysis with a probe for the 180-bp centromere repeats. doi:10.1371/journal.pgen.1000156.g001
the \textit{HpaI} site of \textit{At4g31150} monitored in our PCR-based assay was methylated. Notably, less than 17\% of those sites were methylated in \textit{vimKD-A}, and the CpG in the \textit{HpaI} site was completely unmethylated in the \textit{vim1 vim3} mutant and the \textit{vimKD-B} line. In contrast to CpG methylation, wild-type Col samples had very low levels (<4\%) of CpHpG and CpHpH methylation at \textit{At4g31150}, and none of the tested genotypes displayed an effect on CpHpG and CpHpH methylation in the indicated genotypes.

Transcriptional Silencing Is Released in the \textit{vim1 vim3} Mutant and Transgenic \textit{vim} Knock-Down Lines

RT-PCR was carried out to determine whether VIM deficiency affects gene expression. First, we investigated the level of transcripts generated from the 180-bp centromeric repeats in different \textit{vim} genotypes. Elevated levels of 180-bp repeat transcripts from both strands were detected in the \textit{vim1 vim3} mutant and the two transgenic \textit{vim} knock-down lines, suggesting that DNA hypomethylation of the 180-bp centromeric repeats caused by VIM deficiency is associated with a loss of transcriptional silencing of these repeats (Figure 5A). Next, we focused on the 5S rRNA genes, where loss of VIM function leads to dramatic changes in cytosine methylation. 5S rRNA genes are organized into pericentromeric tandem repeat arrays, and only a subset of the repeats is transcribed with the remainder being epigenetically silenced. To detect the release of 5S rRNA repeat silencing, we used primer pairs that detect two 5S transcript variants, resulting in amplification of 140 and 210 nucleotide products from cDNA templates prepared from wild-type Col plants. 5S-140 transcripts accumulated to a higher level in the \textit{vim1 vim3} mutant and the transgenic \textit{vim} knock-down lines compared to the wild-type Col genotype, but no significant increase in 5S-210 was observed (Figure 5B). This result demonstrates that a decrease of 5S rRNA gene methylation at CpG sites in the \textit{vim1 vim3} mutant and the transgenic \textit{vim} knock-down lines is associated with loss of 5S rRNA gene silencing of the smaller variant. We also examined the expression of the two loci representing methylated genes: \textit{At4g00500} and \textit{At4g31150}. We detected equivalent levels of
transcription for \textit{At4g00500} and \textit{At4g31150} in each genotype (Figure S7). These data suggest that the genic CpG methylation found in these genes does not lead to a general repression of gene expression and that VIM proteins are not playing an active role in regulating expression of these genes.

We also examined dispersed transposable elements for loss of transcriptional silencing. Specifically, we investigated the transcription status of the non-LTR retroelement \textit{AtLINE1-4}, the LTR retrotransposon \textit{AtGP1}, and the class II element \textit{AtMU1} using an RT-PCR assay on RNA samples derived from \textit{vim} mutants (Figure 5C). No \textit{AtGP1} transcripts were detected in any of the \textit{vim} mutant lines despite the loss of CpG methylation. However, we found that transcription of \textit{AtMU1} and \textit{AtLINE1-4} elements were activated in the \textit{vim1 vim3} mutant and the transgenic \textit{vim} knock-down lines. These findings indicate that VIM proteins function redundantly to silence different types of transposable elements.

The Late Flowering Phenotype of the \textit{vim1 vim2 vim3} Triple Mutant Is Associated with Ectopic \textit{FWA} Expression

Despite the changes in DNA methylation and the release of gene silencing, the \textit{vim1} single mutant, the \textit{vim1 vim3} double

Figure 3. DNA methylation patterns at 5S rRNA genes. (A) DNA methylation was determined by DNA gel blot analysis with genomic DNA samples; genomic DNA digested with \textit{HpaI}, \textit{MspI}, or \textit{HaeIII} was hybridized to a 5S rRNA probe. (B) Analysis of DNA methylation by bisulfite sequencing. Histograms represent the percentage of CpG, CpHpG, and CpHpH methylation in the indicated genotypes. (C) Detailed DNA methylation profile for 5S rRNA gene in Col (top) and \textit{vimKD-B} (bottom). The percentages of methylation of each cytosine residue were calculated. The x-axis represents cytosine positions in the analyzed region, and the y-axis represents methylation levels in each genotype. The black bar in the middle shows a coding region of 5S rRNA gene. CpG, CpHpG, and CpHpH methylation are indicated by blue, green, and red bars, respectively. (D) ROS1 mRNA accumulation was determined by RT-PCR. Total RNA from 3-week-old leaves of the indicated plant genotypes, and then RT-PCR was carried. First-strand cDNA synthesis was carried out with or without RT; we did not detect transcripts from the ‘minus RT’ samples. Amplification of \textit{GAPC} (glyceraldehyde-3-phosphate dehydrogenase) was used to normalize RNA template amounts.

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The vim1 vim2 vim3 Triple Mutant Displays DNA Hypomethylation Phenotypes Comparable to Those Exhibited by a met1 Null Mutant

As seen in Figure 6, the vim1 vim2 vim3 triple mutant showed a stronger phenotype than that observed in the vim1 vim3 double mutant, indicating VIM2 is important in repressing FWA expression and hypermethylation of the upstream region of the FWA gene in vegetative tissues. To investigate whether VIM2 has a redundant function with VIM1 and VIM3 outside of the FWA locus, we examined DNA methylation in other genic regions. The abundance of PCR product for At4g00500 and At4g13610 amplified from the HpaII-digested vim1 vim2 vim3 mutant sample was significantly less than that from the vim1 vim3 mutant sample, indicating that the vim triple mutant displayed a more severe reduction in genomic methylation compared to the vim1 vim3 mutant (Figure 7A). As the vim triple mutant phenocopied met1 mutants in several aspects, including preference for CpG hypomethylation, ectopic CpHpH hypermethylation, reduced ROS1 expression, and late flowering associated with ectopic expression of FWA, we further explored the severity of phenotypes displayed by vim mutants relative to met1 mutants. Specifically, we compared transcriptional activation of transposons and DNA hypomethylation of highly repetitive heterochromatic regions in vim1 vim2 vim3 and met1 mutants. First, we re-examined the transcription status of AtLINE1-4 and AtMu1 using an RT-PCR assay. Loss of transcriptional silencing of AtMu1 and AtLINE1-4 elements was more severe in the vim1 vim2 vim3 mutant compared to the vim1 vim3 mutant (Figure 7B). Transcription of both transposons in the vim1 vim2 vim3 mutant was similar or higher than that observed in the hypomorphic met1-1 mutant. We next assessed the DNA methylation pattern of the 180-bp centromere repeats and the 5S rRNA genes by DNA gel blot hybridization analysis after digestion with HpaII. The vim1 vim2 vim3 triple mutant displayed stronger hypomethylation at HpaII sites relative to the vim1 vim3 double mutant for both repeat families (Figure 7C). Interestingly, the DNA hypomethylation phenotypes in the vim1 vim2 vim3 triple mutant were significantly stronger than those observed in the hypomorphic met1-1 mutant, but similar to the phenotypes exhibited by the met1-3 null mutant. Taken together, these findings indicate that VIM1, VIM2 and VIM3 function redundantly to silence different types of transposable elements and to maintain CpG methylation in the heterochromatic as well as genic regions. Further, our results indicate that simultaneous loss of VIM1, VIM2 and VIM3 function almost completely blocks CpG methylation.

Discussion

The Arabidopsis genome contains five VIM genes, each of which encodes a protein containing a PHD domain, two RING domains, and an SRA domain. Previously, we reported that VIM1 is an unconventional methylcytosine-binding protein and that loss-of-function vim1 mutations cause cytosine hypomethylation and decondensation of centromeres [18]. Here we describe a broader analysis of the VIM gene family in Arabidopsis. Our results indicate that the expressed VIM proteins cooperate to maintain global CpG methylation and epigenetic transcriptional silencing.

Functional Redundancy in the VIM Gene Family

We previously reported that VIM1 binds methylated CpG and CpHpG in vivo and similarly Johnson et al. [16] showed that ORTH1/VIM3 and ORTH2/VIM1 can bind methylated CpG, CpHpG, or CpHpH substrates. Our results provide additional support for the hypothesis that VIM2, VIM1, and VIM3 function...
redundantly. First, all three expressed VIM proteins showed a similar subnuclear localization. When ectopically expressed in transgenic plants under control of the 35S promoter, YFP-VIM1, VIM2, and VIM3 protein fusions were broadly distributed in the nucleus and were enriched in the heterochromatic chromocenters (Figure 1A). Second, VIM1, VIM2, and VIM3 were abundantly expressed in leaves and inflorescence tissue – an overlapping expression pattern that supports the possibility of functional redundancy (Figure S1C). Third, the heterologous overexpression of wild-type Col VIM2 or VIM3 coding sequences compensated for the loss of VIM1 with regard to centromere DNA methylation when transformed into Bor-4 vim1-1 plants (Figure 1C). Fourth, combining vim mutations or suppressing the expression of multiple VIM genes in transgenic lines resulted in stronger DNA hypomethylation than those exhibited by vim single mutants (Figure 2, 3, 4, 6, and 7). A release of gene silencing also occurred at the 180-bp centromeric repeats, 5S rRNA repeats, and some transposable elements in the vim1 vim3 double mutants and the transgenic vim knock-down lines (Figure 5). Simultaneous disruption of VIM1, VIM2 and VIM3 led to a loss of FWA gene silencing in vegetative tissues, as well as a more severe reduction in genic and tandem repeat methylation compared to the vim1 vim3 double mutant (Figure 6 and 7). These results indicate that VIM proteins play important, overlapping roles in maintenance of cytosine methylation and transcriptional silencing throughout the Arabidopsis genome.

Although VIM proteins have overlapping functions, a hierarchy exists among VIM proteins. The vim1 vim3 mutant displayed a strong synergistic effect on cytosine methylation compared to either single mutant, whereas the vim1 vim2 mutation combination showed no significant enhancement of DNA hypomethylation compared to the vim1 single mutant (Figure S4 and Figure 7A). The importance of VIM2 is demonstrated by the more severe cytosine hypomethylation and loss of transcriptional silencing displayed by the vim1 vim2 vim3 triple mutant compared to the vim1 vim3 double mutant (Figure 6 and 7). These results indicate that VIM1 is the major functional member of the VIM family with regards to DNA methylation and epigenetic silencing, while VIM3 and VIM2 (in descending order) play lesser roles in the examined loci. This functional hierarchy might reflect qualitative differences among VIM proteins or relative gene expression levels (VIM1 is the most highly transcribed member of the gene family based on public databases: http://mpss.udel.edu/at and http://bbc.botany.utoronto.ca/efp).

### Specificity of Cytosine Hypomethylation in vim Mutants

VIM proteins affect CpHpG as well as CpG methylation at the 180-bp repeats and 45S rRNA genes (Figure 2), while VIM
proteins specifically affect CpG methylation in other loci examined. One possibility is that CpgHpg methylation in the centromere and 45S rRNA genes may be reduced as a secondary consequence of a loss of Cpg methylation, as reported in met1 mutants [29]. Alternatively, VIM proteins may have locus-specific regulatory mechanisms for maintaining DNA methylation. One indication of locus specificity is the preferential effect of vim1 mutations on centromere methylation and compaction [18], which might result from varying levels of functional redundancy at different genomic locations—for instance, a more diminished role for VIM2 and VIM3 at the centromere. The specificity may be determined by the primary sequence itself or sequence copy number. In addition, the activities of VIM proteins could be influenced by other proteins that have sequence specificity.

VIM and Flowering Time Control

The vim1 vim2 vim3 triple mutant showed delayed flowering, and we uncovered FWA hypomethylation and ectopic FWA expression as one possible mechanism for this developmental phenotype. Liu et al. reported that plants overexpressing a VIM1-GFP fusion protein leads to delayed flowering and an elevated level of a key repressor of flowering, FLOWERING LOCUS C (FLC) transcripts [30]. One possibility is that the late flowering phenotypes in vim1 vim2 vim3 triple mutant plants and plants overexpressing VIM1 result from different mechanisms. Another possibility is that the late flowering phenotype in the VIM1 overexpression line might be caused by a dominant negative mechanism. The overexpressed VIM1-GFP fusion proteins could sequester other components for maintaining DNA methylation and epigenetic silencing. Alternatively, the VIM1 transgene induce silencing of endogenous VIM genes, similar to the situation in our vimKD-A and vimKD-B transgenic lines.

Possible Mechanisms of VIM Action

The precise role of VIM proteins in epigenetic regulation remains an open question, but two plausible models are supported by previous reports and this study. The mammalian counterparts of VIM proteins exhibit a variety of activities that either directly modify histones [17,19] or specifically recognize histone modifications [31]. Accordingly, we originally proposed that VIM1 affects DNA modification by acting at the chromatin—cytosine methylation interface, possibly by modifying chromatin substrates for DNA methyltransferase activity. The preference for CpG hypomethylation in the vim mutants suggests that the MET1-mediated pathway is primarily affected by any alteration of the chromatin substrates. In the alternative model, which is based on the physical interaction of mammalian VIM homologs with DNMT1 [23–25], VIM proteins tether the DNMT1-class CpG methyltransferase MET1 to the replication fork. This model predicts that inactivation of the redundant VIM family proteins would phenocopy met1 mutants—a prediction supported by several observations. First, the vim1 vim3 mutant and the transgenic vim knock-down lines showed a preference for CpG hypomethylation, similar to met1 mutants. Second, these vim mutant lines exhibited CpgHpg hypermethylation and a reduction in ROS1 transcript level, two recently described characteristics of met1 null mutants [32,33]. Third, the developmental phenotypes of the vim1 vim2 vim3 mutant, including late flowering, resembled that of met1 homozygotes [28,29]. The late flowering phenotype is likely to be caused in part by the ectopic expression of FWA associated with DNA hypomethylation of the upstream repeats observed in both met1 and vim1 vim2 vim3 mutants. Fourth, the reduction in 180-bp centromere and 5S rRNA gene repeat methylation in the vim1 vim2 vim3 triple mutant matched the extreme hypomethylation observed in the met1-3 null mutant. The parallels between the vim1 vim2 vim3 mutant and met1 mutants argue that the VIM proteins are essential components of the MET1-mediated cytosine methylation pathway.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of vim T-DNA insertion mutants were obtained from the SALK T-DNA collection [34] through the Arabidopsis Biological Resources Center at The Ohio State University. The vim1-2 allele (SALK_050903), the vim2-2 allele (SALK_135677), and the vim3-1
allele (SALK_088570) carry a T-DNA insertion in the fourth exon, the third exon, and the fourth exon of the corresponding gene, respectively. Dr. Jerzy Paszkowski kindly donated the met1-3 mutant seeds. Plants were grown in a controlled environmental chamber at 22°C under long-day conditions (16 h light per day).

**Construction of Plant Expression Vectors and Generation of Transgenic Plants**

Full-length VIM1, VIM2, and VIM3 cDNA clones were PCR-amplified from a wild-type Col first-strand cDNA preparation using primers VIM1-F/VIM1-R, VIM2-F/VIM2-R, and VIM3-F/VIM3-R, respectively. The fragments were cloned into pENTR-D TOPO (Invitrogen, USA) and the resulting VIM inserts were recombined into pEarlyGate104 [35] using Gateway technology (Invitrogen, USA). These constructs were transformed into Agrobacterium tumefaciens (LBA4404) and were introduced into Col WT or Bor-4 vim1-1 plants by *in planta* transformation [36].

**Localization of YFP-VIM Proteins**

T2 generation transgenic seeds were germinated on 1× MS (Murashige and Skoog) media and grown for 6–10 days in 16 h/8 h (light/dark) growth conditions at 22°C. Seedlings were fixed in a 1× PBS solution containing 4% paraformaldehyde for 1 hour at room temperature and subsequently stained with 10 μg/mL propidium iodide. Images of root nuclei were acquired with a Leica SP2 laser scanning confocal microscope equipped with a
DNA Gel Blot Hybridization

Genomic DNA was digested with *Hpa*II, *Msp*I, *Nde*II or *Hind*III (New England Biolabs, USA) according to the manufacturer's instructions. Radiolabeled probes were generated by random priming, and blots were prepared and hybridized using standard methods. The following probes were generated from purified priming, and blots were prepared and hybridized using standard instructions. Radiolabeled probes were generated by random (New England Biolabs, USA) according to the manufacturer's protocols. PCR products were TA-cloned into pGEM-T Easy (Promega, USA) and individual clones were sequenced with the T7 primer. Approximately 24 individual clones were sequenced for each locus from two independent bisulfite sequencing experiments. Detailed bisulfite sequencing data, including the average methylation content for each clone, are provided in Table S1.

Bisulfite Sequencing

Genomic DNA samples were modified by sodium bisulfite using the EpiTech Bisulfite kit (Qiagen, USA) according to the manufacturer's protocols. PCR products were TA-cloned into pGEM-T Easy (Promega, USA) and individual clones were sequenced with the T7 primer. Approximately 24 individual clones were sequenced for each locus from two independent bisulfite sequencing experiments. Detailed bisulfite sequencing data, including the average methylation content for each clone, are provided in Table S1.

HpaII- or HhaI-Based Cytosine Methylation Assay

1 μg of genomic DNA was digested with *Hpa*II or *Hha*I (no enzyme for controls). Distillations of DNA from the digestion reaction were then used for each PCR reaction. PCR conditions were 2 min at 94°C, followed by 27 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min for each primer set.

RT-PCR

To check the expression of the *VIM* genes, total RNA was isolated from 2-week-old leaves and inflorescence tissues from wild-type Col plants. 3-week-old leaves from each genotype were used for checking the expression of other genes. Two or three independent RNA extractions were performed per genotype and a pool of plants was used for each extraction. Aliquots of 1 μg of total RNA were treated with DNaseI (Invitrogen, USA) and 300 ng of DNase-treated total RNA was used as input in RT-PCR reactions using the Superscript III RT (Invitrogen, USA). For the 180-bp centromere repeat, we used ‘GAPC-R & CEN-F’, ‘GAPC-R & CEN-R’, or ‘no primers’ for strand-specific first-strand cDNA synthesis, and all reactions were performed with RT. The ‘no primers’ control was used to detect trace amounts of contaminating DNA, which is a particular problem due to the high-copy number of the repeat templates. For the 5S rRNA genes, we used ‘GAPC-R & 5S-R’ primers for first-strand cDNA synthesis and minus RT negative controls were performed. For other sequences, oligo(dT) primers were used for first-strand cDNA synthesis and minus RT negative controls were performed with primers specific to each sequence. Amplification of *GAPC* or *ACT2* RNA was used as an internal control. All primers used for RT PCR and the other analyses are listed in Table S2.

Small RNA Analysis

Small RNA gel blot analysis was performed using the mirVana miRNA isolation kit (Ambion, USA) as described previously [18]. The siR1003 [39] and mir163 riboprobes were generated according to the mirVana probe construction kit (Ambion, USA) and labeled by T7 polymerase transcription in the presence of α-32P UTP.
**Figure S5** DNA methylation patterns monitored by *NaI*III digestion. DNA methylation was determined by DNA gel blot analysis; genomic DNA was digested with *NaI*III and blots were hybridized with radiolabeled probes corresponding to the 180-bp centromeric repeats (left), 435 rRNA genes (middle), or 3S rRNA genes (right).

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**Figure S6** The abundance of 5S rRNA siRNA is not significantly changed in vim mutants or vimKD-B. A small RNA gel blot was hybridized with a radiolabeled riboprobe (siR1003). As a loading control, the filter was rehybridized with a probe recognizing miR163. An ethidium bromide (EtBr) stained image of the samples before transfer to the membrane is shown in the bottom row.

Found at: doi:10.1371/journal.pgen.1000156.s006 (1.07 MB PDF)

**Figure S7** Transcriptional analysis of genic regions in the vim1 vim3 mutant and the vim knock-down lines. RT-PCR was carried out using RNA samples purified from plants of the indicated genotypes, with or without RT. ACT2 and GAPC were used for loading controls.

Found at: doi:10.1371/journal.pgen.1000156.s007 (0.34 MB PDF)

**Figure S8** Late flowering phenotype of vim1 vim2 vim3 mutant plants. Wild-type Col (Col WT), vim1 Vim2 vim3, and vim1 vim2 vim3 plants are shown at the same developmental age, at the initiation of flowering. Note the elongating floral inflorescence at the center of the rosette and the larger number of rosette leaves produced by the vim1 vim2 vim3 triple mutant. The chronological ages of the plants are indicated (20 or 30 days post-germination). The plants were grown in parallel under the same environmental conditions (22°C; long-day conditions).

Found at: doi:10.1371/journal.pgen.1000156.s008 (1.43 MB PDF)

**Table S1** Percentage of methylated cytosines in different sequence contexts within the *AtMU1*, *AtGP1*, 5S rRNA, and *Ath431150* genes.

Found at: doi:10.1371/journal.pgen.1000156.s009 (0.64 MB DOC)

**Table S2** Oligonucleotide primers used in this study.

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**Author Contributions**

Conceived and designed the experiments: HRW TAD EJR. Performed the experiments: HRW TAD. Analyzed the data: HRW TAD EJR. Contributed reagents/materials/analysis tools: HRW TAD. Wrote the paper: HRW TAD EJR.

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