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An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation

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Cytosine methylation is critical for correct development and genome stability in mammals and plants. In order to elucidate the factors that control genomic DNA methylation patterning, a genetic screen for mutations that disrupt methylation-correlated silencing of the endogenous gene *PAI2* was conducted in *Arabidopsis*. This screen yielded seven loss-of-function alleles in a SET domain protein with histone H3 Lys9 methyltransferase activity, SUVH4. The mutations conferred reduced cytosine methylation on *PAI2*, especially in non-CG sequence contexts, but did not affect methylation on another *PAI* locus carrying two genes arranged as an inverted repeat. Moreover, an unmethylated *PAI2* gene could be methylated *de novo* in the *suvh4* mutant background. These results suggest that SUVH4 is involved in maintenance but not establishment of methylation at particular genomic regions. In contrast, a heterochromatin protein 1 homolog, LHP1, had no effect on *PAI* methylation.

Keywords: DNA methylation/epigenetics/gene silencing/ histone methylation

Introduction

Cytosine methylation is centrally important in regulating gene expression and genome stability in mammals, plants and some fungi. Changes in chromatin structure associated with methylated regions of the genome lead to transcriptional silencing. This silencing mechanism controls fundamental processes including imprinting and X chromosome inactivation in mammals, and suppression of transposon movement in plants. In mammalian genomes, the bulk of methylation is found in the symmetric dinucleotide context CG, whereas plant and fungal genomes carry methylation at both CG and non-CG cytosines.

Insights into the factors involved in DNA methylation have come from genetic approaches in model organisms. For example, three mammalian cytosine methyltransferases, Dnmt1, Dnmt3a and Dnmt3b, have been characterized by knock-out mutations in mice (Li *et al*., 1992; Okano *et al*., 1999). These studies revealed that each methyltransferase has a distinct role in genomic methylation patterning and in mouse development. Similarly, genetic studies of two *Arabidopsis* cytosine methyltransferases, MET1 and CMT3, have shown that these proteins have distinct functions. *MET1*, which is related to the mammalian *DNMT1* gene, has been implicated in maintenance of methylation in the symmetric sequence context CG (Finnegan *et al*., 1996; Ronemus *et al*., 1996). *CMT3*, which combines a chromo-domain motif with the amino acid motifs characteristic of cytosine methyltransferases, has been implicated in maintenance of CNG and other non-CG methylation patterns (Bartee *et al*., 2001; Lindroth *et al*., 2001). A related chromomethylase gene from maize, *ZMET1*, has also been implicated in maintenance of CNG methylation (Papa *et al*., 2001).

Besides cytosine methyltransferases, relatively little is known about factors that control DNA methylation. Genetic screens in *Arabidopsis* showed that the *DDM1* gene, which encodes a protein related to SWI2/SNF2 chromatin-remodeling factors, is important for maintenance of genomic methylation (Jeddeloh *et al*., 1999). Loss of DDM1 function confers globally reduced DNA methylation. Similar results have been obtained for a mouse mutant in the *DDM1*-orthologous gene *Lsh* (Dennis *et al*., 2001). DDM1 and Lsh thus indicate a relationship between chromatin structure and DNA methylation.

A genetic screen in the fungus *Neurospora* revealed that the *dim-5* gene, which encodes a SET domain protein, is required for genomic methylation (Tamaru and Selker, 2001). Like the related SU(VAR)3-9 and CLR4 SET domain proteins that have been characterized from mammalian and yeast systems (Rea *et al*., 2000; Nakayama *et al*., 2001), the *dim-5* gene product catalyzes the methylation of histone H3 at the lysine 9 (K9) position. This modification is associated with histones found in heterochromatic regions of eukaryotic genomes (Noma *et al*., 2001; Peters *et al*., 2001; Gendrel *et al*., 2002; Johnson *et al*., 2002). The finding that loss of DIM-5 function blocks both H3 methylation and genomic cytosine methylation provides another link between chromatin structure and DNA modification.

The *Arabidopsis* genome encodes nine *SU(VAR)3-9*-related genes, named *SUVH1–SUVH9* [*SU(VAR)3-9* homologs] (Baumbusch *et al*., 2001). In contrast to SU(VAR)3-9 and CLR4 proteins, the *Arabidopsis* SUVH proteins lack a chromo-domain at their N-terminus. Instead, the *Arabidopsis* SUVH proteins share a novel central sequence motif named the YDG domain, with divergent N-termini. Recently, mutations in the *SUVH4* gene were isolated as suppressors of methylation and silencing of the *SUPERMAN* floral homeotic gene (Jackson *et al*., 2002). In this analysis, the heterologously expressed protein was shown to have histone H3 K9 methyltransferase activity *in vitro*, consistent with the predicted protein function.

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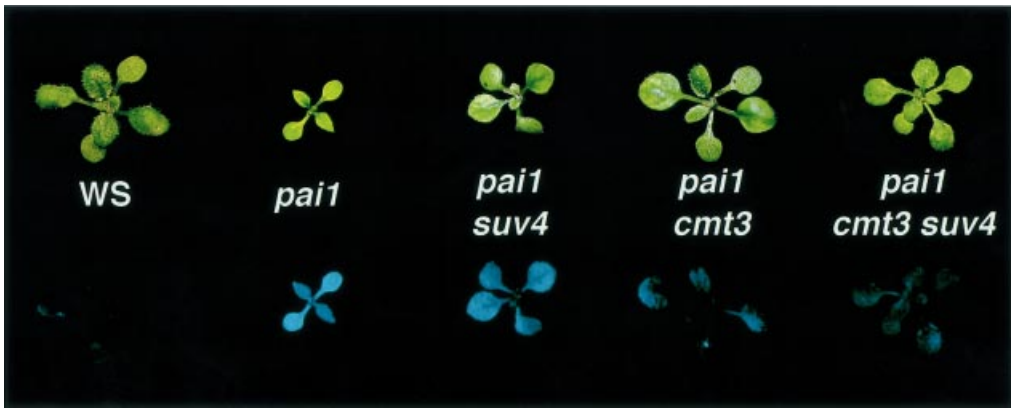

Fig. 1. *PAI2* silencing is suppressed by *suvh4* mutations. Representative 2.5-week-old plants of the indicated strains are shown under visible (upper panel) and UV (lower panel) light. The *suvh4302** and *cmt3i11a* alleles were used. The suppressed phenotype of *suvh4R302** is similar to those of the other six *suvh4* alleles.

Here we describe the isolation and characterization of seven new loss-of-function mutations in *SUVH4*. The *suvh4* mutations were recovered in a forward genetic screen for *trans*-acting factors that control DNA methylation and silencing of the endogenous gene *PAI2*. This screen previously yielded 11 loss-of-function alleles in the *CMT3* chromomethylase gene (Bartee *et al*., 2001).

In the Wassilewskija (WS) strain of *Arabidopsis*, *PAI2* methylation is triggered by an unlinked inverted repeat *PAI* gene arrangement *PAI1–PAI4* (Luff *et al*., 1999). Mutation of *CMT3* reduces non-CG methylation on both *PAI2* and the inverted repeat *PAI* genes, and blocks the ability of the inverted repeat to establish a new methylation imprint on a 'naïve' unmethylated singlet *PAI2*. In contrast, mutation of *SUVH4* reduces non-CG methylation on *PAI2* but not on the inverted repeat *PAI* genes, and does not block the ability to establish a new *PAI2* methylation imprint. Taken together, our results lead to a model for the establishment and maintenance of methylation on unlinked repeated sequences in plant genomes: CMT3 is needed during both an initiation phase and a maintenance phase of methylation, whereas SUVH4 is only needed during the maintenance phase at targets where methylation is signaled *in trans* from an unlinked initiator locus. This system probably mirrors the mechanism of transposable element methylation as a means of genome defense (Yoder *et al*., 1997).

Results

Isolation of suvh4 mutants as suppressors of PAI2 silencing

An endogenous *Arabidopsis* gene that is subject to cytosine methylation and silencing, *PAI2*, serves as a facile reporter in genetic screens for methylation mutations. The *PAI* genes encode an intermediate enzyme in the tryptophan biosynthetic pathway. In some *Arabidopsis* strains including WS, these genes are arranged as an inverted repeat (*PAI1–PAI4*) at one locus, plus two singlet genes (*PAI2* and *PAI3*) at unlinked loci, and are cytosine methylated at both CG and non-CG cytosines across their regions of sequence identity (Luff *et al*., 1999; Melquist *et al*., 1999). Only *PAI1* and *PAI2* encode a functional enzyme, and only *PAI1* is expressed due to the presence of novel promoter sequences upstream of the methylated region (Melquist *et al*., 1999). The *PAI2* gene is silenced by methylation of its proximal promoter sequences. To exploit *PAI2* as a reporter locus, a *pai1* missense mutant variant of WS (*pai1C251Y*) was isolated (Bartee and Bender, 2001). The *pai1* strain displays PAI-deficient phenotypes, including blue fluorescence due to accumulation of a tryptophan pathway intermediate, yellow–green leaf pigmentation, reduced size, increased bushiness and reduced fertility, because the remaining functional gene, *PAI2*, is silenced by methylation. This fluorescent reporter strain was mutagenized and screened for isolates with suppression of the PAI-deficient fluorescent phenotype as a means of finding plants with reduced *PAI2* silencing.

The *PAI2* silencing suppressor screen yielded 11 loss-of-function mutations in the *CMT3* cytosine methyltransferase gene as the predominant class of silencing suppressor mutations (Bartee *et al*., 2001). Here we describe the other major complementation group of mutations recovered from this screen: seven loss-of-function *suvh4* mutations in the *SUVH4* SET domain histone methyltransferase gene. In the *pai1* reporter strain, *suvh4* mutations conferred partial suppression of fluorescence (Figure 1). These phenotypes were similar to those conferred by *cmt3* mutations, except that the suppression of fluorescence was weaker in *suvh4* than in *cmt3* mutant backgrounds. The *suvh4* mutations did not confer any obvious morphological defects, even after several generations of inbreeding. There were also no obvious morphological defects for a *suvh4* mutation crossed into the wild-type WS background.

suvh4 mutations reduce methylation at singlet PAI genes but not at the PAI1–PAI4 inverted repeat

In addition to suppression of silenced *PAI2* phenotypes, the *suvh4* mutations conferred reduced cytosine methylation on the *PAI2* reporter gene. Methylation was monitored both by Southern blot assays and by sodium bisulfite genomic sequencing. For Southern blot analysis, genomic DNA was cleaved with the isoschizomers *Hpa*II and *Msp*I, which recognize 5′-CCGG-3′, and probed with a *PAI* probe. *Hpa*II will not cleave if either the inner (CG) or the outer (CCG) cytosine of the recognition site is

methylated, whereas *Msp*I is only sensitive to methylation of the outer cytosine. Thus, if neither enzyme cleaves, the site carries outer CCG methylation, whereas if *Msp*I but not *Hpa*II cleaves, the site carries only inner CG methylation. These enzymes cleave once in each WS *PAI* locus at a site that is affected by methylation, plus cleaving at unmethylated flanking sites which lie different distances away for each locus (Figure 2A). These enzymes thus allow the methylation status of the site in each *PAI* locus to be monitored independently.

*Hpa*II/*Msp*I Southern blot analysis of WS *pai1 suvh4* DNA revealed that the recognition sites in the singlet *PAI2* and *PAI3* genes were partially demethylated and susceptible to increased cleavage by both enzymes relative to the parental strain; however, the recognition site in the *PAI1–PAI4* inverted repeat retained methylation and resisted cleavage with both enzymes (Figure 2B). This pattern of demethylation was different from the pattern previously observed for *cmt3* mutant DNA, where all three *PAI* loci are almost completely cleaved by *Msp*I (Figure 2B; Bartee *et al*., 2001). The *suvh4* pattern of reduced methylation for *PAI2* but not *PAI1–PAI4* versus the *cmt3* pattern of reduced methylation on both *PAI2* and *PAI1–PAI4* was confirmed with a second Southern blot assay using the methylation-sensitive enzyme *Hin*cII, which monitors non-CG cytosines at the translational start codons of *PAI1*, *PAI4* and *PAI2* (Supplementary figure 1 available at *The EMBO Journal* Online). The *PAI* demethylation patterns characteristic of *suvh4* persisted when the mutation was segregated away from *pai1* in a back-cross to WS (data not shown). Detailed analysis of the effects of *ddm1* and *met1* mutations on *PAI* methylation and silencing were described previously (Bartee and Bender, 2001).

To score effects of *suvh4* at the methylated 180 bp *CEN* repeats in *Arabidopsis*, the *PAI Hpa*II/*Msp*I Southern blot was reprobed with a *CEN* probe. This analysis revealed no significant enhancement of *Hpa*II cleavage and only a slight enhancement of *Msp*I cleavage relative to the parental control strain at *CEN* (Figure 2C). Other characterized methylation mutations, *ddm1*, *met1* and *cmt3*, display different alterations in the patterns of *CEN* cleavage (Figure 2C; Vongs *et al*., 1993; Bartee and Bender, 2001). The *ddm1* mutation confers increased cleavage by both *Hpa*II and *Msp*I, the *met1* mutation confers increased cleavage by *Hpa*II, and the *cmt3* mutation confers increased cleavage by *Msp*I.

The *PAI1* and *PAI2* genes were also subjected to sodium bisulfite genomic sequencing of methylation patterns (Frommer *et al*., 1992) in the regions just upstream of their translational start codons. These regions include distal heterologous sequences unique to each gene and proximal sequences that are nearly identical among the *PAI* genes (Figure 3). Sequencing of the same regions in wild-type WS showed that there is dense CG and non-CG methylation within the regions of *PAI* identity for each gene, but that there is very little methylation in the upstream heterologous regions (Luff *et al*., 1999). In a WS *pai1 suvh4* DNA sample, methylation sequencing analysis showed that the *PAI2* reporter locus had a reduction in overall methylation relative to wild-type WS, with most of the residual methylation retained at cytosines in the CG sequence context. This pattern is similar to that previously

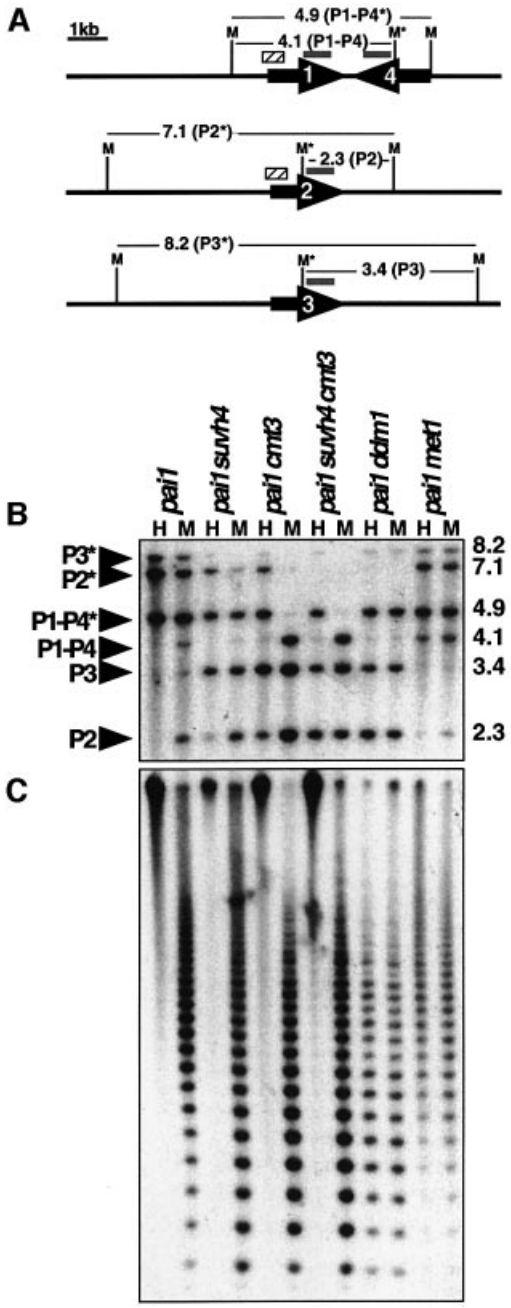

Fig. 2. *suvh4* mutations confer reduced *PAI2* methylation. (**A**) The *Hpa*II (H) and *Msp*I (M) restriction map of each WS *PAI* locus is shown, with the probed regions indicated by gray bars and the bisulfite sequenced regions indicated by hatched bars. (**B**) Genomic DNAs prepared from 4-week-old plants of the indicated genotypes were cleaved with either *Hpa*II or *Msp*I and used for Southern blot analysis with a *PAI* probe. P1-P4 is *PAI1–PAI4*, P2 is *PAI2*, and P3 is *PAI3*, with asterisks indicating the positions of species methylated at internal *Hpa*II/*Msp*I sites. Note that *PAI3* is divergent from the probe sequence and thus gives a weaker signal than other *PAI* genes. (**C**) The blot shown in (B) was reprobed with a 180 bp *CEN* repeat probe. The phenotypes of the WS *suvh4R302** allele shown are representative of the phenotypes observed with six other *suhv4* alleles. The phenotypes of the WS *pai1 suvh4R302* cmt3i11a* double mutant line shown are representative of the phenotypes observed with three other independent double mutant lines.

observed in a *cmt3* mutant background (Bartee *et al*., 2001; Figure 3B; Supplementary figure 2). In contrast, for the *PAI1* locus, there was no significant change in

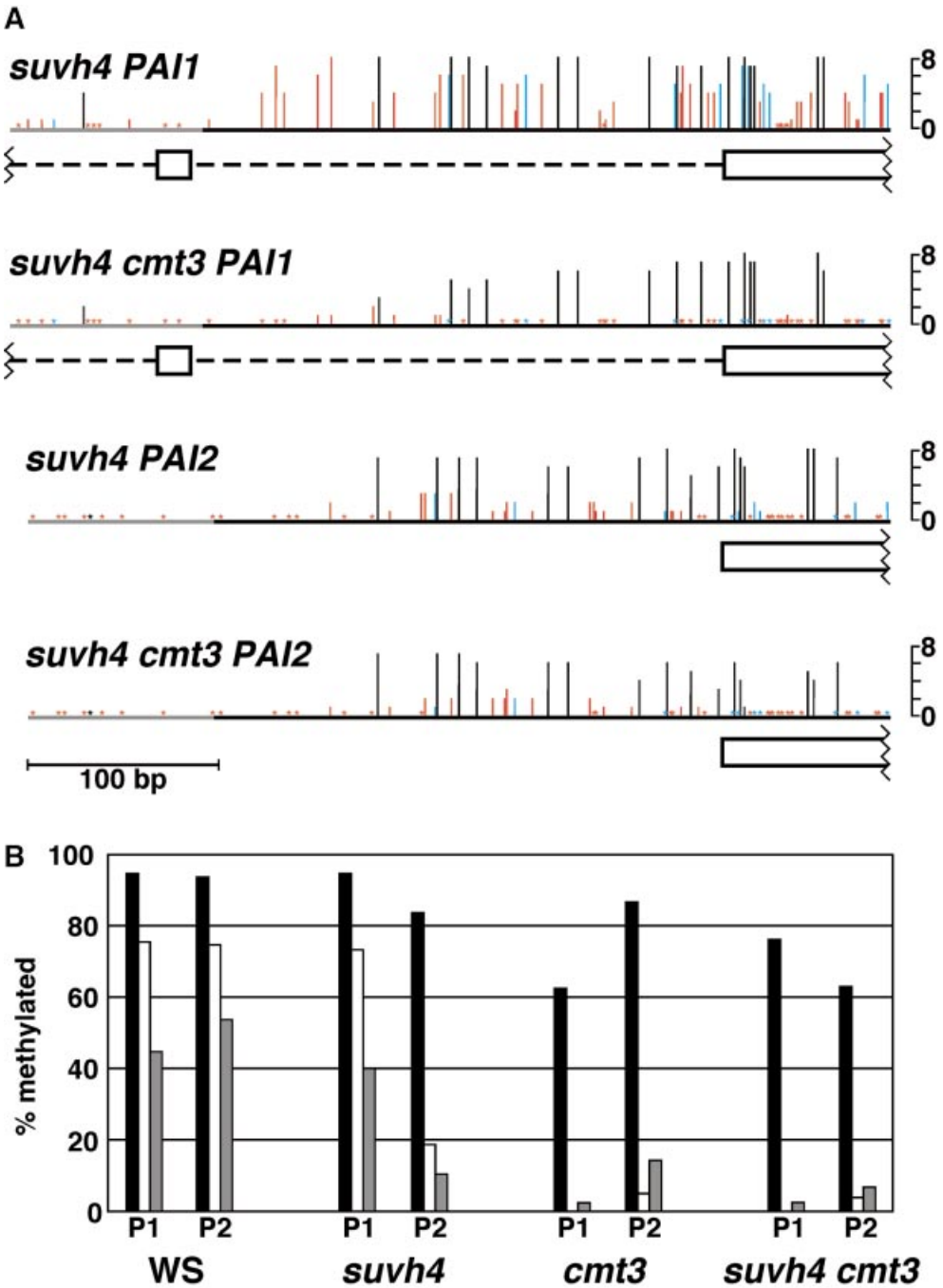

Fig. 3. Bisulfite sequencing analysis of the *PAI* genes in a *suvh4* mutant background. (**A**) Bisulfite genomic methylation sequencing was performed for the top strands of the *PAI1* and *PAI2* upstream regions in WS *pai1 suvh4R302** or WS *pai1 suvh4R302* cmt3i11a* DNA. For each region, eight independent molecules were sequenced. Vertical lines indicate positions of cytosines, with the height of each line representing how many sequenced molecules had 5-methylcytosine (5-Me-C). Black indicates CG cytosines, blue indicates CNG cytosines, and red indicates other cytosines. Asterisks indicate sites with no methylation. The black horizontal line indicates the region of *PAI* identity, and the gray horizontal line indicates flanking upstream heterologous sequence unique to each gene. The exon and intron structures of *PAI1* and *PAI2* are shown as open boxes and dashed lines, respectively, under each sequence. These structures are based on full-length cDNA sequences for each gene (Melquist *et al*., 1999). (**B**) Within the region of *PAI* identity (black horizontal line in A), the percentages of available cytosines that are methylated in CG (black bars), CNG (white bars) or other (gray bars) sequence contexts are shown for either *PAI1* (P1) or *PAI2* (P2) in the indicated strains. Data for wild-type WS and WS *pai1 cmt3* are from previous publications (Luff *et al*., 1999; Bartee *et al*., 2001). See also Supplementary figure 2.

methylation relative to wild-type WS. This pattern is different from the *PAI1* pattern observed in the *cmt3* mutant background, where overall methylation is reduced and the residual methylation occurs primarily at cytosines in the sequence context CG.

Positional cloning of the suvh4 mutations

The *suvh4* mutations were mapped as previously described for the *cmt3* mutations isolated in the *PAI2* silencing suppressor screen (Bartee *et al*., 2001; see Materials and methods). Mapping analysis showed linkage to a region on

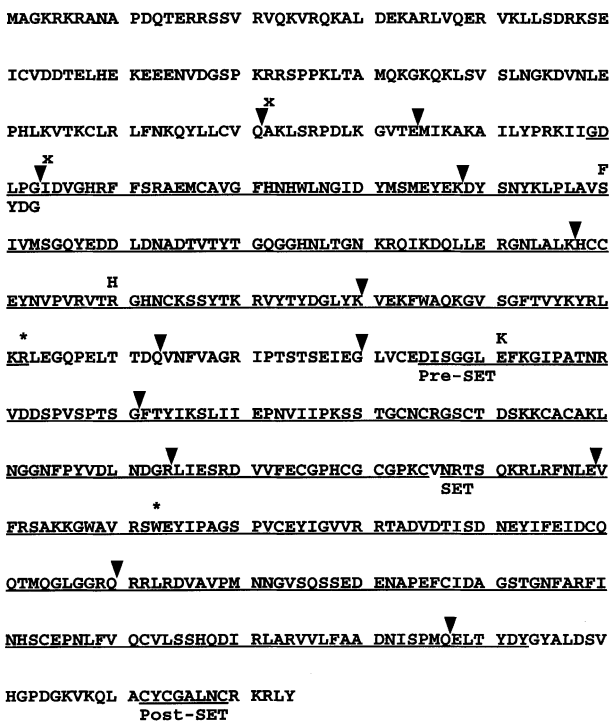

Fig. 4. Positions of seven *suvh4* alleles recovered as *PAI2* silencing suppressors. The predicted amino acid sequence of WS SUVH4 is shown, with the positions of introns marked with black arrowheads. Missense mutations are indicated by the new amino acid above the affected codon. Nonsense mutations are indicated by an asterisk above the affected codon. Splice mutations are indicated by an x at the appropriate junction of the affected intron. The YDG, pre-SET, SET and post-SET domains as previously defined for SUVH4 relative to related proteins (Baumbusch *et al*., 2001; Jackson *et al*., 2002) are underlined. The WS genomic sequence of *SUVH4* is available as DDBJ/EMBL/GenBank accession No. AF538715.

the upper arm of chromosome 5, between markers that lie ~830 kb apart. We focused on the *SUVH4* gene in this region as a candidate for the suppressor locus acting on information that suppressors of methylation and silencing of the floral homeotic gene *SUPERMAN* affect this locus (Jackson *et al*., 2002). The *SUVH4* gene was confirmed as the mutant locus with two approaches. First, the gene was cloned and sequenced from the seven *PAI2* silencing suppressor alleles. Each mutant isolate carried a single C:G to T:A transition mutation in the *SUVH4* coding region predicted to abrogate protein function (Figure 4; Supplementary figure 3). The *suvh4* alleles included two premature termination codons, two splice junction mutations and three missense mutations. To understand the consequences of the splice junction mutations, RNA prepared from mutant plants was used as a template for RT–PCR of the *SUVH4* transcript, and the products were analyzed by cloning and sequencing. This analysis revealed that each mutation resulted in at least three different mis-spliced transcript species that created either frameshifts with premature termination of the protein-coding region, or in-frame internal deletions of the protein-coding region (Supplementary figure 4). Thus, both splice site mutations are likely to be null alleles. The *S200F* and *R260H* missense mutations alter residues that lie in the YDG domain, and the *E341K* missense mutation alters a residue in the pre-SET domain of the *Arabidopsis* SUVH group of proteins (Baumbusch *et al*., 2001). These residues are conserved in all nine members of the SUVH family, with the exception of SUVH7, which carries a cysteine rather than a serine at the position analogous to S200 in SUVH4. The conserved nature of the mutated residues and the observation that the missense mutations have phenotypes of similar severity to the nonsense and splice mutations suggest that the missense alleles are strong loss-of-function alleles.

As a second approach to verify the *SUVH4* gene as the site of the mutations, the *pai1 suvh4* isolates were transformed with a genomic clone of the wild-type *SUVH4* gene. Strongly fluorescent transformants with phenotypes similar to that of the parental *pai1 SUVH4* strain were recovered, showing that the cloned *SUVH4* gene can complement the silencing defect (Figure 5). Transformant lines assayed by Southern blotting in the T_2 generation showed remethylation of *PAI2* to the levels observed in WS and WS *pai1*, although in some cases *PAI3* was refractory to remethylation, as previously observed (Luff *et al*., 1999). In contrast, when the *pai1 suvh4* mutant was transformed with a *CMT3* genomic clone previously shown to complement the *cmt3* mutation (Bartee *et al*., 2001), all of the transformed progeny retained the suppressed *pai1 suvh4* phenotype. This result suggests that extra copies of *CMT3* in *suvh4* are not sufficient to bypass the mutant defect.

The suvh4 cmt3 double mutant retains partial PAI2 silencing and methylation

The genetic screen for suppressors of *PAI2* silencing revealed that both the histone methyltransferase SUVH4 and the DNA methyltransferase CMT3 are important for maintenance of DNA methylation at this locus, particularly in CNG and other non-CG sequence contexts. A simple interpretation of these results is that SUVH4-mediated histone modifications at the *PAI2* locus facilitate CMT3 action at *PAI2*. To examine the relationship between SUVH4 and CMT3 functions further, we constructed a double *suvh4 cmt3* mutant in the WS *pai1* background and examined its cytosine methylation and silencing phenotypes relative to each of the parental single mutants.

Overall, the *pai1 suvh4 cmt3* double mutant displayed suppressed *PAI2* silencing and methylation phenotypes similar to those of the *cmt3* single mutant. Double mutant plants had a weakly fluorescent phenotype like that of the *pai1 cmt3* single mutant (Figure 1). In Southern blot assays for methylation, double mutant DNA displayed increased cleavage of *PAI2* with *Hpa*II relative to either single mutant parent, whereas patterns of *PAI1–PAI4*, *PAI3* and *CEN* cleavage were similar to those displayed by *cmt3* single mutant DNA (Figure 2). *Hin*cII Southern blot analysis showed no difference between double mutant and *cmt3* single mutant patterns of cleavage in the *PAI1–PAI4* and *PAI2* loci (Supplementary figure 1). Sodium bisulfite genomic sequencing of the *PAI1* and *PAI2* upstream regions in double mutant DNA revealed patterns similar to those observed previously for the *cmt3* single mutant, with the bulk of residual methylation retained at cytosines in the context CG (Figure 3). However, the density of methylation was slightly lower in the *PAI2* upstream region of

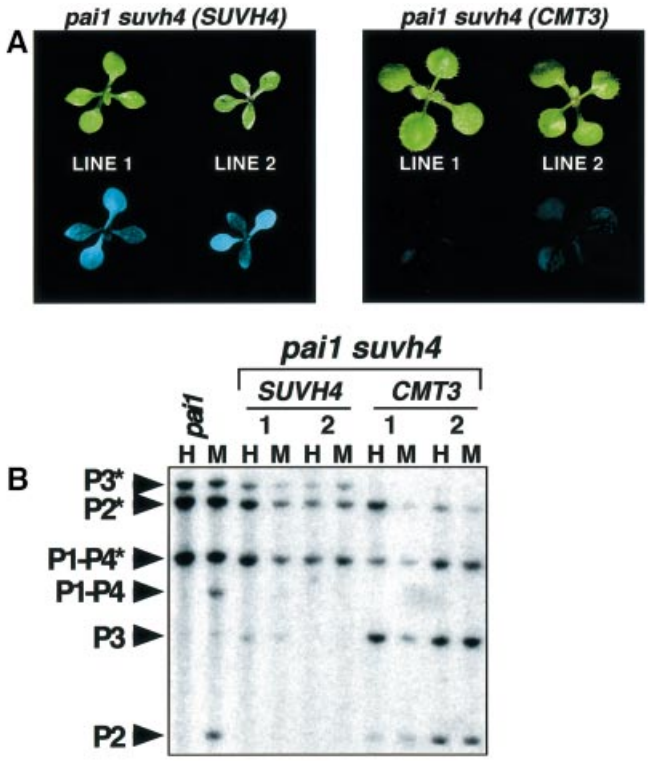

Fig. 5. A *SUVH4* transgene can complement the *suvh4* mutant defect. (**A**) Representative T_2 2-week-old seedlings of the *suvh4R302** mutant transformed with either a *SUVH4* genomic clone or a *CMT3* genomic clone (two independent lines each) are shown under visible (upper panel) or UV (lower panel) light. (**B**) Genomic DNAs prepared from single leaves of 4-week-old T_2 plants of the same lines shown in (A) were cleaved with either *Hpa*II (H) or *Msp*I (M) and used for Southern blot analysis with a *PAI* probe. P1-P4 is *PAI1–PAI4*, P2 is *PAI2*, and P3 is *PAI3*, with asterisks indicating the positions of species methylated at internal *Hpa*II/*Msp*I sites.

double mutant DNA than in *cmt3* DNA. These results are consistent with the model that SUVH4 is involved primarily in facilitating CMT3 action, with little effect on other factors that contribute to maintenance of *PAI* or *CEN* methylation.

An Arabidopsis HP1 homolog is not required for PAI methylation

Heterochromatin protein 1 (HP1) has been implicated as a component of heterochromatin in animal and fungal systems (reviewed in Jenuwein and Allis, 2001; Richards and Elgin, 2002). This protein contains a chromo-domain which binds to histone H3 methylated at K9. The *Arabidopsis* genome encodes a single protein with HP1 homology, called like HP1 (LHP1) (Gaudin *et al*., 2001). The LHP1 protein was shown to interact with the CMT3 protein *in vitro*, leading to the proposal that LHP1 serves as a bridge between the SUVH4-catalyzed histone modification and the targeting of CMT3 (Jackson *et al*., 2002). This model predicts that loss of LHP1 function should lead to loss of methylation from CMT3 target loci. To test this prediction, we assayed an *lhp1* mutant isolated in the WS strain (Gaudin *et al*., 2001) for changes in *PAI* and *CEN* methylation using Southern blot analysis with *Hpa*II and *Msp*I (Figure 6), or for changes in *PAI* methylation with *Hin*cII (Supplementary figure 1). These analyses revealed no differences between parental WS and WS *lhp1* for *PAI* or *CEN* methylation, suggesting that LHP1 protein is not involved in maintaining DNA methylation at these loci.

CMT3, but not SUVH4, is required for establishment of methylation of PAI2

In previous work, we showed that the *PAI1–PAI4* inverted repeat locus provides a signal for methylation of *PAI* sequences. In the genomes of most commonly used strains of *Arabidopsis*, including Columbia (Col) and Landsberg *erecta* (Ler), there are three singlet *PAI* genes (*PAI1*, *PAI2* and *PAI3*), and these genes have no detectable cytosine methylation (Melquist *et al*., 1999). Only strains such as WS that carry a *PAI1–PAI4* inverted repeat gene structure at the *PAI1* locus display dense methylation of their *PAI* genes. If the *PAI1–PAI4* inverted repeat from WS is combined with an unmethylated *PAI2* locus from Col via genetic crosses, the *PAI2* locus becomes methylated *de novo* (Luff *et al*., 1999). This methylation increases progressively upon inbreeding: the Col *PAI2* methylation reaches a density similar to that found on WS *PAI2* by the F_4 generation in such WS $\times$ Col hybrid lines.

We wanted to determine whether inverted repeat-triggered *PAI2* methylation can occur in strains that are deficient in either SUVH4 or CMT3. To address this question, we performed crosses between a WS *pai1 suvh4* mutant and a Ler *suvh4* mutant, or between a WS *pai1 cmt3* mutant and a Ler *cmt3* mutant, and examined whether the Ler *PAI2* could acquire silencing and cytosine methylation when combined in a hybrid genome with the WS *pai1–PAI4* inverted repeat. As a control, we performed the analogous wild-type cross between WS *pai1* and Ler. The use of the WS *pai1–PAI4* locus with a

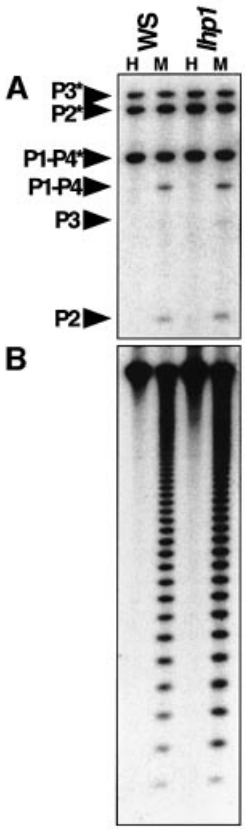

Fig. 6. The *lhp1* mutation does not affect *PAI* or *CEN* methylation in WS. (**A**) Genomic DNAs prepared from 4-week-old plants of the indicated genotypes were cleaved with either *Hpa*II (H) or *Msp*I (M) and used for Southern blot analysis with a *PAI* probe. P1-P4 is *PAI1–PAI4*, P2 is *PAI2*, and P3 is *PAI3*, with asterisks indicating the positions of species methylated at internal *Hpa*II/*Msp*I sites. (**B**) The blot shown in (A) was reprobed with a 180 bp *CEN* repeat probe. The *lhp1-1* allele, which displays characteristic developmental defects, was used for this analysis (Gaudin *et al*., 2001).

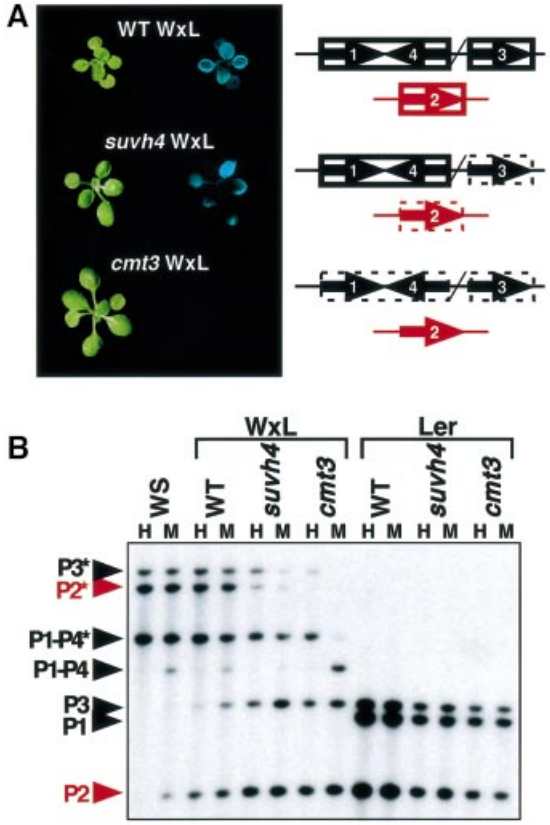

Fig. 7. The WS *pai1–PAI4* inverted repeat locus triggers *de novo* methylation and silencing of an unmethylated Ler *PAI2* gene in a SUVH4-deficient but not a CMT3-deficient background. (**A**) To the left, representative WxL wild-type (F_4), WxL *suvh4* (F_5) or WxL *cmt3* (F_5) 2.5-week-old seedlings are shown under visible (left panel) or UV (right panel) light. To the right, diagrams show the *PAI* genotypes and methylation phenotypes in these lines. *PAI* genes inherited from WS are indicated as black arrows. Note that none of the three WS *PAI* genes encodes functional PAI enzyme. The WS *PAI1–PAI4* and *PAI3* loci both lie on the upper arm of chromosome 1, indicated by a slash separating the two loci. The Ler *PAI2* gene is indicated by a red arrow. Methylation is indicated by boxes around the affected genes, with a solid line representing dense methylation and a dashed line representing partial methylation. (**B**) Genomic DNAs prepared from 4-week-old plants of the indicated strains were cleaved with either *Hpa*II (H) or *Msp*I (M) and used for Southern blot analysis with a *PAI* probe. P1-P4 is WS *PAI1–PAI4*, P1 is Ler *PAI1*, P2 is *PAI2*, and P3 is *PAI3*, with asterisks indicating the positions of species methylated at internal *Hpa*II/*Msp*I sites. WxL wild-type, WxL *suvh4* and WxL *cmt3* DNAs were prepared from F_4, F_5 and F_5 generation plants, respectively.

missense mutation in the *PAI1* gene allowed us to monitor silencing of Ler *PAI2* via acquisition of a blue fluorescent PAI-deficient phenotype.

There is almost no methylation on *PAI2* sequences in wild-type Ler, as determined both by *Hpa*II/*Msp*I Southern blot analysis (Figure 7; Melquist *et al*., 1999) and by bisulfite genomic sequencing of the *PAI2* upstream region (two methylated sites out of 584 total cytosines monitored). Similarly, *Hpa*II/*Msp*I Southern blot analysis shows that the *PAI* genes are not methylated in SUVH4- or CMT3-deficient Ler backgrounds (Figure 7).

In the wild-type, the SUVH4-deficient and the CMT3-deficient WS *pai1* × Ler experiments, populations of F_2 progeny plants were scored for their genotypes at the *PAI1* and *PAI2* loci as previously described (Luff *et al*., 1999) to identify individuals homozygous for both the trigger WS *pai1–PAI4* inverted repeat and the target Ler *PAI2* gene. We examined six independent 'WxL' wild-type control lines, two independent 'WxL *suvh4*' lines and four independent 'WxL *cmt3*' lines.

In the WxL wild-type control experiment, three lines were non-fluorescent and three lines were fluorescent in the F_2 generation. All six lines segregated all or almost all fluorescent progeny in the F_3 generation. By the F_4 generation, all six lines were strongly fluorescent, similar to the parental WS *pai1* strain (Figures 1 and 7). The fluorescent phenotype correlated with partial methylation of the Ler *PAI2* gene, as determined by *Hpa*II/*Msp*I Southern blotting of DNA prepared from F_4 generation plants (Figure 7). These WS *pai1* × Ler *PAI2 de novo* methylation patterns show that the single base missense mutation in the *pai1* gene does not impair the ability of the inverted repeat to trigger methylation. However, these strains did not display complete remethylation of *PAI2* to WS levels by the F_4 generation, as previously observed in the analogous WS × Col experiment (Luff *et al*., 1999). This difference might represent a selection against the most strongly silenced PAI-deficient female gametes in the *pai1* background (Bender and Fink, 1995).

In the SUVH4-deficient experiment, neither of the two WxL *suvh4* lines was detectably fluorescent in the F_2 generation. However, both lines segregated ~30% F_3 progeny that were fluorescent, suggesting that there was progressive partial methylation of *PAI2* during the F_3 generation. Two individual non-fluorescent F_3 plants of each line gave rise to F_4 progeny that segregated all or almost all fluorescent plants, consistent with a progressive trend towards increased methylation and silencing. In fact, DNA prepared from progeny F_5 populations showed slight *PAI2* methylation by *Hpa*II/*Msp*I Southern blot analysis, similar to the patterns observed in the WS *pai1 suvh4* strain (Figures 2 and 7). WxL *suvh4* F5 plants also displayed an intermediate fluorescence phenotype similar to the WS *pai1 suvh4* strain (Figures 1 and 7). These results indicate that the Ler *PAI2* gene can acquire and maintain methylation and silencing despite a SUVH4 deficiency.

In the CMT3-deficient experiment, none of four WxL *cmt3* lines was detectably fluorescent in the F_2 generation. In contrast to the SUVH4-deficient experiment, none of these lines segregated fluorescent progeny plants in the F_3 generation. Furthermore, two individual F_3 plants from each line yielded F_4 progeny populations that were completely non-fluorescent. Similarly, two individual F_4 plants from each line yielded F_5 progeny populations that were completely non-fluorescent. DNA prepared from these F_5 populations showed no significant *PAI2* methylation by *Hpa*II/*Msp*I Southern blot analysis (Figure 7); in comparison, WS *pai1 cmt3* strains display intermediate fluorescence and a readily detectable methylated *PAI2* species in a *Hpa*II digest (Figures 1 and 2; Bartee *et al*., 2001). The *pai1–PAI4* locus and the *PAI3* locus were inherited from the WS *pai1 cmt3* parent in all four WxL *cmt3* lines, and these loci maintained CG methylation to the same levels as observed in the parent strain (Figures 2 and 7). These results suggest that although existing methylation imprints can be maintained in the absence of CMT3 function, new *PAI2* methylation imprints cannot be propagated to the point where they result in methylation or silencing phenotypes like those observed for the parental *pai1 cmt3* strain.

Discussion

Genomic DNA methylation is catalyzed by cytosine methyltransferase enzymes, but the factors that control the targeting and activity of these enzymes at different genomic sites are not well understood. Here we show that a SET domain histone methyltransferase protein, SUVH4, is important for maintenance of particular cytosine methylation patterns in the *Arabidopsis* genome. SUVH4 is involved mainly in maintenance of non-CG methylation at a subset of the sites controlled by the predicted cytosine methyltransferase CMT3.

In *Arabidopsis*, two cytosine methyltransferases, MET1 and CMT3, have been characterized by genetic analysis. Loss of MET1 function reduces methylation primarily in the sequence context CG, which is the predominant methylation context in the *Arabidopsis* genome, and leads to developmental pleiotropy (Finnegan *et al*., 1996; Ronemus *et al*., 1996). In contrast, loss of CMT3 function reduces methylation primarily in CNG and other non-CG contexts and does not confer obvious developmental alterations (Bartee *et al*., 2001; Lindroth *et al*., 2001).

The WS *PAI* genes display a high level of CG and non-CG methylation across their regions of shared sequence identity (Luff *et al*., 1999), suggesting that they are preferred substrates for both MET1 and CMT3. Indeed, loss of function in either cytosine methyltransferase gene leads to partially reduced methylation on the *PAI1–PAI4* inverted repeat methylation trigger locus and on two unlinked singlet genes *PAI2* and *PAI3* (Figure 2; Bartee and Bender, 2001; Bartee *et al*., 2001). In the case of *cmt3* mutations, the three *PAI* loci display a weak reduction in CG methylation and a strong reduction in non-CG methylation relative to parental WS. Our finding that *suvh4* mutations cause a similar loss of mostly non-CG methylation from the singlet *PAI2* gene (Figures 2 and 3) suggests that the SUVH4 histone-modifying activity is necessary for CMT3 to act at this region. Previous *in vitro* studies showed that heterologously expressed CMT3 does not interact directly with K9-methylated H3 peptides (Jackson *et al*., 2002), and the proposal that the *Arabidopsis* HP1 homolog LHP1 is a bridging factor for this interaction is not supported by our observation that an *lhp1* mutation has no effect on *PAI* or *CEN* methylation (Figure 6). Therefore, SUVH4 might facilitate CMT3 action at *PAI2* through an indirect effect on overall chromatin structure.

Strikingly, in contrast to *cmt3* mutations, *suvh4* mutations do not affect maintenance of dense CG and non-CG methylation on the *PAI1–PAI4* inverted repeat locus (Figures 2 and 3). This result suggests that SUVH4-mediated histone methylation is not needed for CMT3 or other cytosine methyltransferases to act at *PAI1–PAI4*. Similarly, *suvh4* mutations only partially reduce CCG methylation at *CEN* repeats relative to *cmt3* mutations (Figure 2; Jackson *et al*., 2002), suggesting only a partial requirement for SUVH4 at these repeats to facilitate CMT3 action. One explanation for these differences in *suvh4* and *cmt3* DNA methylation phenotypes is that other SUVH proteins (Baumbusch *et al*., 2001) contribute to histone modifications in the *PAI1–PAI4* and *CEN* regions of the genome. However, recent chromatin immunoprecipitation experiments with antibodies against histone H3 methylated at K9 showed that *suvh4* mutant chromatin is depleted for K9 methylation at *CEN* and other DNA methylated genomic sites, arguing that SUVH4 is the major H3 K9-modifying activity for heterochromatin in *Arabidopsis* (Johnson *et al*., 2002). An alternative view is that repeated sequences like *PAI1–PAI4* and *CEN* might have chromatin structure features that attract CMT3 independently of K9 methylation status.

Like our screen for suppressors of hypermethylated *PAI2* silencing, a screen for suppressors of hypermethylated *SUPERMAN* silencing yielded loss-of-function mutations in CMT3 and SUVH4 (Lindroth *et al*., 2001; Jackson *et al*., 2002). Analysis of the effects of these mutations at methylated transposon sequences and the *CEN* repeats revealed the general pattern that *cmt3* mutations confer stronger effects than *suvh4* mutations on CNG methylation, consistent with our findings. The parallel results from the two different systems indicate that CMT3 and SUVH4 are major factors in the control of non-CG methylation genome-wide. However, a notable differ-

ence between the two systems is the effect of *suvh4* mutation on inverted repeat methylation. In the *SUPERMAN* screen, an inverted repeat transgene carrying *SUPERMAN* genomic sequences was used to stabilize hypermethylation of the endogenous singlet *SUPERMAN* gene. Collective bisulfite genomic sequencing of these three *SUPERMAN* copies revealed strong demethylation by mutation of *SUVH4* (Jackson *et al*., 2002), implying that the inverted repeat copies and the singlet gene are affected uniformly. This demethylation of the *SUPERMAN* inverted repeat by *suvh4* could reflect its lower CG content or its transgenic nature versus the endogenous *PAI1–PAI4* inverted repeat.

The maintenance of CG methylation in *suvh4* mutants suggests that the MET1 DNA methyltransferase does not require SUVH4-mediated histone methylation. It will be interesting to determine whether the MET1-related mammalian methyltransferase Dnmt1 might be similarly independent of H3 K9 methylation. In *Neurospora*, loss of H3 K9 methylation confers complete loss of CG and non-CG methylation (Tamaru and Selker, 2001). This finding suggests that the mechanistic relationship between histone modification and DNA methylation has evolved differently in *Neurospora* from that in plants. In this regard, DNA methylation in *Neurospora* is controlled by the DIM-2 methyltransferase, which is structurally distinct from both MET1- and CMT3-related enzymes (Kouzminova and Selker, 2001).

In plants, certain aberrant RNA species such as double-stranded RNAs and RNA virus replication intermediates can direct DNA methylation of identical sequences (reviewed in Wassenegger, 2000; Bender, 2001). Because RNA-directed DNA methylation is typically densely patterned with a high proportion of non-CG methylation, it has been proposed that CMT3 is the methyltransferase that acts in response to an RNA signal (Martienssen and Colot, 2001; Matzke *et al*., 2001). In the WS *PAI* system, read-through RNA species from the *PAI1–PAI4* inverted repeat could provide a trigger for *PAI*-directed dense DNA methylation. Our finding that loss of CMT3 function blocks the ability of the *PAI1–PAI4* inverted repeat to promote methylation on an unmethylated *PAI2* target (Figure 7) is consistent with a central role for CMT3 in this potentially RNA-directed process.

One possibility is that CMT3 could act as both a *de novo* and a maintenance methyltransferase for *PAI* methylation. During the initiation of methylation, CMT3 would be required for a primary methylation imprint. The primary imprint would then be propagated and maintained at CG cytosines by MET1 and at non-CG cytosines by CMT3. In this model, SUVH4 would be needed to facilitate CMT3 action during the maintenance phase. However, another class of putative cytosine methyltransferases, the DRMs, has recently been shown to be required for *de novo* methylation of other *Arabidopsis* methylation target loci (Cao and Jacobsen, 2002). Therefore, an alternative possibility is that the DRMs provide the primary methylation imprint, but that CMT3 is required to propagate this imprint up to the point where MET1 will effectively maintain CG methylation. A third possibility is that the *PAI1–PAI4* methylation trigger locus must itself be densely methylated at CG and non-CG cytosines in order to produce a methylation signal that can act *in trans* on *PAI2*; the absence of this dense methylation in a *cmt3* background would block production of the signal. This third possibility is consistent with the observation that *PAI2 de novo* methylation can be triggered in a *suvh4* mutant background (Figure 7), where dense methylation on *PAI1–PAI4* is retained (Figures 2 and 3).

Mammalian genomes contain mostly CG DNA methylation patterning, and this limited methylation sequence context is sufficient to mediate effective gene silencing. The CG methylation context is also the predominant pattern found in the *Arabidopsis* genome. The question then arises of why *Arabidopsis* and other plants have evolved the capacity to maintain additional non-CG methylation patterns via CMT3-related cytosine methyltransferases. Our results suggest that non-CG methylation acts as a reinforcement to CG methylation in promoting an additional level of gene silencing. In the WS *pai1* strain where *PAI2* is densely methylated at both CG and non-CG residues, *PAI2* is strongly silenced and the plant has a severely PAI-deficient phenotype; when non-CG methylation is lost from *PAI2* via *suvh4* or *cmt3* mutation, *PAI2* silencing persists but at a reduced level (Figure 1). Because dense non-CG patterning is associated with RNA-directed DNA methylation, an attractive hypothesis is that DNA sequences perceived as being related to RNA viruses or mobile elements due to production of aberrant RNAs are targeted for an extra level of methylation and silencing as an extra level of genome defense. In fact, mutation of *CMT3* reactivates transcription of some retroelement-related sequences (Lindroth *et al*., 2001), and CMT3-mediated methylation has been shown to be targeted preferentially to some transposable element sequences (Tompa *et al*., 2002). Thus, CMT3 and SUVH4 are likely to be necessary for the long-term fitness of the plant genome in the face of invasive virus and transposon sequences.

Materials and methods

Mutant isolation and sequencing

Seeds of WS *pai1C251Y* were mutagenized with ethylmethane sulfonate (EMS), and M_2 progeny seedlings were grown on agar medium and scored at 2 weeks for reduced fluorescence as previously described (Bartee *et al*., 2001). Putative mutants were transplanted to soil, and genomic DNA prepared from a single leaf was used for *Hpa*II/*Msp*I Southern blot analysis of methylation patterns. The *PAI* and *CEN* Southern blot probes were as previously described (Bartee and Bender, 2001). From the screen of 20 000 M_2 seedlings, seven independent isolates with the Southern blot pattern characteristic of *suvh4* mutants (Figure 2) were recovered, and these isolates proved to be *suvh4* mutants by mapping, sequencing and complementation analysis with a *SUVH4* transgene.

The *suvh4* suppressor isolates were mapped by crossing with the polymorphic strain Niederzenz (Nd-0), which has an arrangement of densely methylated *PAI* genes similar to that found in WS (Melquist *et al*., 1999). F_2 progeny of the cross with a weak fluorescent phenotype indicative of homozygosity for both the *pai1* mutation and the *suvh4* mutation were selected, and confirmed as having the suppressor mutation by an *Msp*I Southern blot methylation assay. A mapping population of confirmed *pai1 suvh4* F_2 plants was then scored with standard cleaved amplified polymorphic sequence (CAPS) (Konieczny and Ausubel, 1993) and simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994) markers that are polymorphic between WS and Nd-0. This analysis showed linkage to a single locus on the upper arm of chromosome 5. The locus was narrowed down to an ~830 kb region between the *Arabidopsis* BAC clones MSH12 and T21H19. Information about the markers used at these loci is provided in the Supplementary data.

Mutant alleles of *SUVH4* were amplified by PCR from genomic DNA. Products from two independent PCRs were cloned and sequenced for each allele. To facilitate genetic analysis, a PCR-based marker was designed where the base change created by the *suvh4R302** mutation is combined with a mismatch at the end of a nearby PCR primer to create a restriction site polymorphism (see Supplementary data).

The WS *pai1 suvh4 cmt3* double mutant strain was made by crossing *pai1 suvh4R302** with a *pai1 cmt3* allele (intron 11 acceptor site, *cmt3i11a*) previously isolated as a *PAI2* silencing suppressor (Bartee *et al*., 2001), and screening the progeny plants for genotype by PCR-based assays. The *suvh4R302** mutation was scored as described above, and the *cmt3i11a* mutation was scored by a restriction enzyme site change created by the mutation (see Supplementary data). The *cmt3i11a* mutation is a G to A transition at the splice acceptor site of the intron immediately upstream of the active site motif of CMT3 that creates an *Mse*I site. The splice mutation causes mis-splicing to a cryptic acceptor site 1 bp downstream of the normal splice junction, causing a frameshift that disrupts the active site sequence and results in premature termination at codon 476. This *cmt3* allele is thus likely to be a null mutation. Four independent double mutant lines were pursued by inbreeding and Southern blot methylation assays, and each gave identical phenotypes. A representative line was used for the methylation analyses shown in Figures 2 and 3.

For the Ler *PAI2 de novo* methylation experiment shown in Figure 7, the WS *pai1 suvh4R302**, WS *pai1 cmt3i11a*, Ler *kyp-2* (Jackson *et al*., 2002) and Ler *cmt3-7* (Lindroth *et al*., 2001) alleles were used.

Sodium bisulfite genomic sequencing of methylation patterns

Mutant effects on *PAI* methylation were monitored by sequencing the top strands of the regions upstream of the start codons of the *PAI1* and *PAI2* genes in bisulfite-modified genomic DNA. The DNA samples used were prepared from the representative *pai1 suvh4R302** allele that had been backcrossed to WS once and inbred for three generations, and from a *pai1 suvh4R302* cmt3i11a* double mutant that had been inbred for two generations. The same DNA samples were used for Southern blot analysis of DNA methylation patterns (Figure 2). Bisulfite treatment was performed as previously described (Jeddeloh *et al*., 1998; Luff *et al*., 1999), except that denaturation of genomic DNA was performed in 0.3 M NaOH for 20 min at 37°C. Products were amplified by PCR from the modified genomic DNA template and cloned into the pGEM T-EASY (Promega) vector for sequencing.

Plant transformation with the SUVH4 genomic clone

The *SUVH4* transgene is an 11.8 kb Col genomic fragment extending from a *Sal*I site that lies 6.4 kb upstream of the start codon to a *Sal*I site that lies 1.0 kb downstream of the stop codon subcloned into the *Sal*I site of the pBIN19 transformation vector (Bevan, 1984). This clone was isolated by hybridization from a Col genomic λ plaque library (Bender and Fink, 1998). The *CMT3* transgene was described previously (Bartee *et al*., 2001). Transgenes were introduced into the WS *pai1C251Y suvh4R302** strain by an *in planta* transformation method (Clough and Bent, 1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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