DOI: 10.1534/genetics.106.063974

# A Histone Methylation-Dependent DNA Methylation Pathway Is Uniquely Impaired by Deficiency in Arabidopsis S-Adenosylhomocysteine Hydrolase

**Lori Mull,[1] Michelle L. Ebbs[2] and Judith Bender[3]**

*Department of Biochemistry and Molecular Biology, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland 21205*

Manuscript received July 25, 2006
Accepted for publication August 14, 2006

ABSTRACT

S-adenosylhomocysteine hydrolase (SAH) is a key enzyme in the maintenance of methylation homeostasis in eukaryotes because it is needed to metabolize the by-product of transmethylation reactions, S-adenosylhomocysteine (AdoHcy), which causes by-product inhibition of methyltransferases (MTase's). Complete loss of SAH function is lethal. Partial loss of SAH function causes pleiotropic effects including developmental abnormalities and reduced cytosine methylation. Here we describe a novel partial-function missense allele of the Arabidopsis *SAH1* gene that causes loss of cytosine methylation specifically in non-CG contexts controlled by the CMT3 DNA MTase and transcriptional reactivation of a silenced reporter gene, without conferring developmental abnormalities. The CMT3 pathway depends on histone H3 lysine 9 methylation (H3 mK9) to guide DNA methylation. Our results suggest that this pathway is uniquely sensitive to SAH impairment because of its requirement for two transmethylation reactions that can both be inhibited by AdoHcy. Our results further suggest that gene silencing pathways involving an interplay between histone and DNA methylation in other eukaryotes can be selectively impaired by controlled SAH downregulation.

S-ADENOSYLMETHIONINE (AdoMet) serves as the methyl-group donor for transmethylation reactions including modification of DNA, RNA, proteins, and lipids, generating S-adenosylhomocysteine (AdoHcy) as a by-product (reviewed in Chiang 1998). In eukaryotes, AdoHcy is further metabolized by S-adenosylhomocysteine hydrolase (SAH) to yield homocysteine and adenosine. The SAH-catalyzed reaction is critical to relieve by-product inhibition of methyltransferases (MTase's) by AdoHcy. Consequently, loss of SAH function can have pleiotropic effects. For example, null mutations in the major Arabidopsis gene encoding SAH (At4g13940, hereafter referred to as *SAH1*) are embryonic lethal (Rocha *et al.* 2005). Similarly, complete loss of SAH function in the mouse is embryonic lethal (Miller *et al.* 1994). Partial inactivation of plant SAH activity by missense mutations or anti-sense RNA expression confers developmental abnormalities including slow growth, reduced fertility, and reduced size (Tanaka *et al.* 1997; Rocha *et al.* 2005). In humans partial loss of SAH function confers severe defects, including a lack of myelin in the central nervous system, implicating perturbation of lipid biosynthesis as a key component of the SAH deficiency (Baric *et al.* 2004, 2005). Nonetheless, controlled downregulation of SAH has potential beneficial outcomes, including suppression of viral replication in plants and mammals (Masuta *et al.* 1995; Chiang 1998; De Clercq 2004) and inhibition of mammalian parasites (Bujnicki *et al.* 2003; Rapp *et al.* 2006).

Among the diverse transmethylation reactions controlled by SAH, cytosine methylation has received particular attention because this modification plays a key role in regulating gene expression and genome stability via heterochromatin formation in mammals and plants. In mammalian and plant cells, suppression of SAH by chemical inhibitors or anti-sense RNA results in reduced cytosine methylation (Wolfson *et al.* 1986; Tanaka *et al.* 1997; Fojtova *et al.* 1998). Similarly, *hog1* missense mutations in the Arabidopsis *SAH1* gene cause reduced cytosine methylation on a transgene reporter and ribosomal DNA (rDNA) repeats (Rocha *et al.* 2005). One mechanism for this reduction is AdoHcy inhibition of cytosine MTase activity. Such inhibition has been demonstrated *in vitro* for the mammalian Dnmt1 enzyme (Bacolla *et al.* 1999). However, in some cases cytosine methylation is targeted by histone protein methylation. For example, in the fungus *Neurospora crassa*, mutation of either the histone H3 lysine 9 (H3 K9) MTase DIM-5 or mutation of the K9 residue of H3 confers genome-wide loss of cytosine methylation (Tamaru and Selker 2001). In Arabidopsis, mutation of the H3 K9 MTase SUVH4/KYP (hereafter referred to as SUVH4) confers

[1]*Present address:* Laboratory of Biochemistry and Genetics, NIDDK, National Institutes of Health, Bldg. 8, Room 407, Bethesda, MD 20892-0851.

[2]*Present address:* Department of Molecular Genetics and Microbiology, P.O. Box 3509, Duke University Medical Center, Durham, NC 27710.

[3]*Corresponding author:* Department of Biochemistry and Molecular Biology, Johns Hopkins University, Bloomberg School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205. E-mail: jbender@mail.jhmi.edu

reduced cytosine methylation in non-CG sequence contexts that are controlled by the DNA MTase CMT3 (Jackson *et al.* 2002; Malagnac *et al.* 2002). In mouse embryonic stem cells, double mutation of the related Suv39h1 and Suv39h2 H3 K9 MTase's, or mutation of the G9a H3 K9 MTase, results in loss of cytosine methylation from specific regions of the genome (Lehnertz *et al.* 2003; Xin *et al.* 2003). Thus, modulation of SAH function can potentially disrupt DNA methylation patterning through both cytosine MTase and histone MTase targets.

Here we describe a missense mutation in the Arabidopsis *SAH1* gene, *sah1L459F*, that confers reduced genomic cytosine methylation specifically in non-CG sequences controlled by CMT3. In contrast to the *hog1* missense mutants in *SAH1* that display phenotypic abnormalities including slow growth and reduced fertility (Rocha *et al.* 2005), the *sah1L459* mutant is morphologically normal. Nonetheless, this weak allele is sufficient to cause depletion of non-CG methylation and reactivation of a silenced reporter gene. Our results suggest that CMT3-dependent non-CG methylation is uniquely susceptible to even slight perturbations in SAH activity because it relies on a pathway involving two types of enzymes that can be inhibited by AdoHcy, the SUVH H3 K9 MTase's and the CMT3 cytosine MTase. Simultaneous inhibition of these enzymes, even at a very low level, can thus result in a synergistic effect on the DNA methylation output of the CMT3 pathway.

## MATERIALS AND METHODS

**Isolation of the *sah1L459F* mutation:** The *sah11L459F* mutation was isolated from a previously described screen for mutations that reduce *PAI2* silencing in the Wassilewskija (Ws) *pai1* background, resulting in a reduced blue fluorescence phenotype (Bartee *et al.* 2001). The *sah1L459F* allele segregated in crosses as a single recessive mutation. The mutation was mapped as previously described for *cmt3* mutations isolated in the *PAI2* silencing suppressor screen (Bartee *et al.* 2001). Briefly, the Ws *pai1 sah1L459F* mutant was crossed to the polymorphic strain Nd-0, which has a similar arrangement of methylated *PAI* genes to Ws. $F_2$ progeny of this cross with the weak fluorescent phenotype diagnostic of individuals homozygous for both the *pai1* mutation and the silencing suppressor mutation were isolated and used to identify the genomic region that cosegregated with the suppressor mutation. The mapping analysis localized the mutation to a region of ~43 kb on the lower arm of chromosome 4. Within this region, we focused on the *SAH1* gene as a likely candidate to alter methylation metabolism. Sequencing of *SAH1* from the mutant identified a single C:G to T:A mutation that changed a leucine to a phenylalanine, consistent with ethyl methanesulfonate (EMS) mutagenesis used on the Ws *pai1* strain. To facilitate genetic analysis, a PCR-based dCAPS (Neff *et al.* 1998) marker was designed, where the base change created by the *sah1L459F* mutation is combined with a mismatch at the end of a nearby PCR primer to create a restriction site polymorphism. For this marker, PCR products amplified with DRAF 5′ CAGCTC GAGCTCTGGAACGAGAAAGCAAGC 3′ and STUR 5′ GTCA GATTGGTCCTTTGACAGCTTTGTAG 3′ were cleaved with *Stu*I: *sah1L459F* yields a 144-bp fragment and the wild type yields 117- and 27-bp fragments.

The *pai1 sah1L459F* mutant was two times outcrossed with the wild-type Ws to segregate *pai1 sah1L459F* and *sah1L459F* strains used in this analysis. Other DNA methylation-deficient strains used for comparison to *sah1L459F* in Figures 2, 3, and 5 were previously described: *cmt3i11a* (Bartee *et al.* 2001), *suvh4R302** (Malagnac *et al.* 2002), the *met1-1* allele (Kankel *et al.* 2003) introgressed into the Ws background (Bartee and Bender 2001), and the Ws *drm1 drm2* double mutant (Cao and Jacobsen 2002b). Southern blot assays for DNA methylation were performed as previously described (Melquist *et al.* 1999). Previously described Southern blot probes for the *PAI* genes (Melquist *et al.* 1999), the *Mu1* DNA transposon (Lippman *et al.* 2003), the *Ta3* retrotransposon (Johnson *et al.* 2002), the *MEA-ISR* repeat (Cao and Jacobsen 2002a), and the 180-bp centromere repeat (Vongs *et al.* 1993) were used.

**Plant transformation with the T-*SAH1* and T-*SHM* genomic clones:** A 5.5-kb *Sna*BI Ws genomic fragment carrying the *SAH1* gene was cloned into the *Sma*I site of the pBIN19 plant transformation vector (Bevan 1984) to make the T-*SAH1* construct (Figure 1B). A 7.4-kb *Xba*I-*Sma*I Ws genomic fragment carrying the neighboring *SHM* gene was cloned into the *Xba*I and *Sma*I sites of pBIN19 to make the T-*SHM* control construct. Both constructs were transformed into the *pai1 sah1L459F* strain using the floral dip method (Clough and Bent 1998).

**Complementation of a yeast *sah1* mutant:** A *Saccharomyces cerevisiae* diploid strain heterozygous for an *sah1* deletion was obtained from Open Biosystems (no. 20176, YSC1021-669867) in strain background BYB4743 (*MAT***a**/α *ura3*Δ*0/ura3*Δ*0 leu2*Δ*0/leu2*Δ*0 his3*Δ*1/his3*Δ*1 met15*Δ*0/MET1 lys2*Δ*0/LYS2*). A DNA fragment containing the *S. cerevisiae SAH1* promoter and full-length coding sequence (*ScSAH1*) was amplified by PCR and cloned into the polylinker of pRS315 (*LEU2 CEN*), pRS316 (*URA3 CEN*), pRS325 (*URA3* 2μ), or pRS425 (*LEU2* 2μ) yeast vectors (Sikorski and Hieter 1989). The same *ScSAH1* promoter sequence was fused to the start codon of the Arabidopsis *SAH1* cDNA (*AtSAH1*, obtained from the Arabidopsis Biological Resource Center), the *sahlL459F* cDNA (*Atsah1L459F*, derived from the *SAH1* cDNA by site-directed mutagenesis), or the *SAH2* cDNA (*AtSAH2*, amplified from Ws RNA by reverse transcriptase–PCR). These expression cassettes were cloned into the polylinker of pRS315 or pRS425.

The heterozygous *sah1/SAH1* yeast strain was transformed with either the *ScSAH1 URA3 CEN* plasmid or the *ScSAH1 URA3* 2μ plasmid. Tetrads were dissected from sporulated transformants and analyzed for genetic and molecular markers to identify Δ*sah1* (*ScSAH1 URA3 CEN*) and Δ*sah1* (*ScSAH1 URA3* 2μ) haploid strains. These strains were supertransformed with *LEU2 CEN* or *LEU2* 2μ plasmids carrying no insert or with *ScSAH1*, *AtSAH1*, *Atsah1L459F*, or *AtSAH2* expression cassettes. Cultures were grown overnight in selective SC −Leu broth and density was determined by spectrophotometry at 600 nm. A series of 10-fold dilutions of each culture, starting at ~10,000 cells per spot and ranging down to ~10 cells per spot, were spotted on either SC −Leu medium or 0.1% 5-fluoroorotic Acid (5-FOA) (United States Biological F5050) medium. Plates were photographed after 2 days of growth at 30° (Figure 6).

**Chromatin immunoprecipitation:** Chromatin immunoprecipitation (ChIP) assays were performed using a previously described method (Gendrel *et al.* 2002) starting with 0.7 g of leaf tissue from 3-week-old plants grown in soilless potting mix (Fafard mix no. 2) under continuous illumination. Chromatin was immunoprecipitated with anti-H3 dimethyl K4 antibodies (Upstate Biotechnologies, Lake Placid, NY) or with anti-H3 dimethyl K9 antibodies (gift of T. Jenuwein, Vienna Biocenter) or carried through the protocol with no antibody added as a control (mock precipitation). PCR primer sets for the center

of the *PAI1-PAI4* inverted repeat, *Ta3*, and *ACTIN* were previously described (Ebbs and Bender 2006). PCR-amplified products from ChIP template DNA were visualized on a 2.5% agarose gel stained with Gel-Star (Cambrex). Each ChIP assay was performed in three independent experiments, with results from a representative experiment shown in Figure 7.

## RESULTS

**Isolation of the *sah1L459F* mutation as a suppressor of *PAI2* transcriptional silencing:** The *sah1L459F* allele was identified in a genetic screen for mutations that reduce cytosine methylation and silencing of the Arabidopsis *PAI2* gene, which encodes the tryptophan biosynthetic enzyme phosphoribosylanthranilate isomerase (PAI). In the Ws strain of Arabidopsis, duplicated *PAI* genes are arranged as an inverted repeat (*PAI1–PAI4*) at one locus and singlet genes (*PAI2* and *PAI3*) at two other unlinked loci (Bender and Fink 1995). These four genes are densely methylated in both CG and non-CG contexts over their regions of shared sequence identity, which includes proximal promoter sequences of each gene (Luff *et al.* 1999). The proximal promoters are thus transcriptionally silenced. Moreover, *PAI3* and *PAI4* encode nonfunctional PAI enzyme (Melquist *et al.* 1999). Nonetheless, Ws expresses sufficient PAI enzyme for normal tryptophan synthesis due to expression of *PAI1* from a promoter that lies upstream of the methylated region (Melquist and Bender 2003). Transcription from this upstream promoter is also required to maintain *PAI* cytosine methylation, indicating that the *PAI1–PAI4* inverted repeat locus generates an RNA signal for *PAI* DNA methylation (Melquist and Bender 2003).

To exploit the functional but methylated and silenced *PAI2* gene as a reporter for *PAI* cytosine methylation, we isolated a Ws *pai1* missense mutation that inactivates PAI1 enzyme without affecting the RNA signal for *PAI* DNA methylation generated by transcription of *PAI1–PAI4* (Bartee and Bender 2001). The *pai1* mutant displays a number of PAI-deficient phenotypes including blue fluorescence under ultraviolet (UV) light due to accumulation of a fluorescent tryptophan precursor, yellow–green leaf color, pointed tips on juvenile leaves, reduced plant size, and reduced fertility. In the *pai1* reporter background, mutations that decrease the transcriptional silencing of the functional *PAI2* gene can be easily detected by suppression of blue fluorescence. Using this *pai1* suppressor screening strategy, we previously isolated 7 loss-of-function alleles in the SUVH4 H3 K9 MTase (Malagnac *et al.* 2002) and 11 loss-of-function alleles in the CMT3 cytosine MTase (Bartee *et al.* 2001). Both *suvh4* and *cmt3* mutants display a strong loss of non-CG methylation from the *PAI2* reporter. *PAI2* is particularly responsive to loss of non-CG methylation because the majority of cytosines in its promoter are methylated non-CG residues (Luff *et al.* 1999).

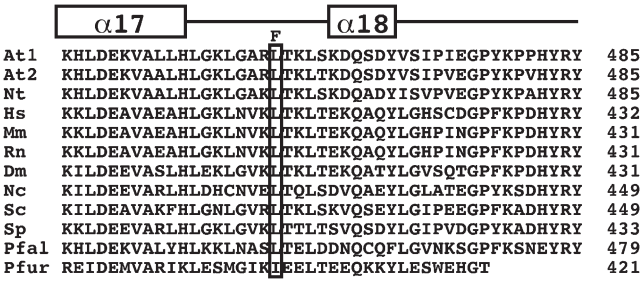


Figure 1.—The *sah1L459F* mutation affects a conserved residue in the SAH carboxy-terminal domain. The aligned predicted carboxy-terminal amino acid sequences of SAH from a variety of organisms are shown. These sequences correspond to the region extending from α-helix α17 through α-helix α18 to the carboxy terminus of the protein in the crystal structure of the human SAH protein (Turner *et al.* 1998), as indicated over the alignment. The residue corresponding to L459 in the Arabidopsis SAH1 protein is boxed, with the mutation to F indicated over this position. The coordinate number of the carboxy-terminal residue for each amino acid sequence is shown in the right margin. At1 is Arabidopsis SAH1, At2 is Arabidopsis SAH2, Nt is *Nicotiana tabacum*, Hs is *Homo sapiens*, Mm is *Mus musculus*, Rn is *Rattus norvegicus*, Dm is *Drosophila melanogaster*, Nc is *Neurospora crassa*, Sc is *Saccharomyces cerevisiae*, Sp is *Schizosaccharomyces pombe*, Pfal is *Plasmodium falciparum*, and Pfur is *Pyrococcus furiosus*.

In addition to multiple alleles of *cmt3* and *suvh4*, the *pai1* suppressor screen yielded a single *sah1L459F* allele of *SAH1*. This mutation was cloned on the basis of its map position (materials and methods). The mutated leucine residue is conserved in SAH enzymes from a variety of organisms including humans, Drosophila, and fungi, although thermophilic archaea maintain an isoleucine at the analogous position (Porcelli *et al.* 2005, Figure 1). This residue occurs near the carboxy terminus of the protein. In the three-dimensional structure of the human SAH tetramer solved by X-ray crystallography, the analogous leucine residue lies in a linker region between two α-helicies (α17 and α18, Figure 1) that reach across and make contacts with the nicotinamide adenine dinucleotide (NAD) cofactor-binding domain on the adjacent monomer (Turner *et al.* 1998).

In the *pai1* background, the *sah1L459F* mutation conferred a partial suppression of fluorescence similar to *suvh4*, but not as strong as *cmt3* (Figure 2A). Like *suvh4* and *cmt3* mutations (Bartee *et al.* 2001; Malagnac *et al.* 2002), the *sah1L459F* mutation did not confer any obvious morphological defects, either in the *pai1* background or in the wild-type Ws background. In contrast, previously described *hog1* missense alleles of *SAH1* confer slow growth and reduced fertility (Rocha *et al.* 2005).

The *sah1L459F* mutation was confirmed as the *PAI2* silencing suppressor mutation by complementation with a transgene carrying a genomic clone of the *SAH1* locus (T-*SAH1*). Transgenic *pai1 sah1L459F* (T-*SAH1*) plants displayed strong fluorescence similar to that of

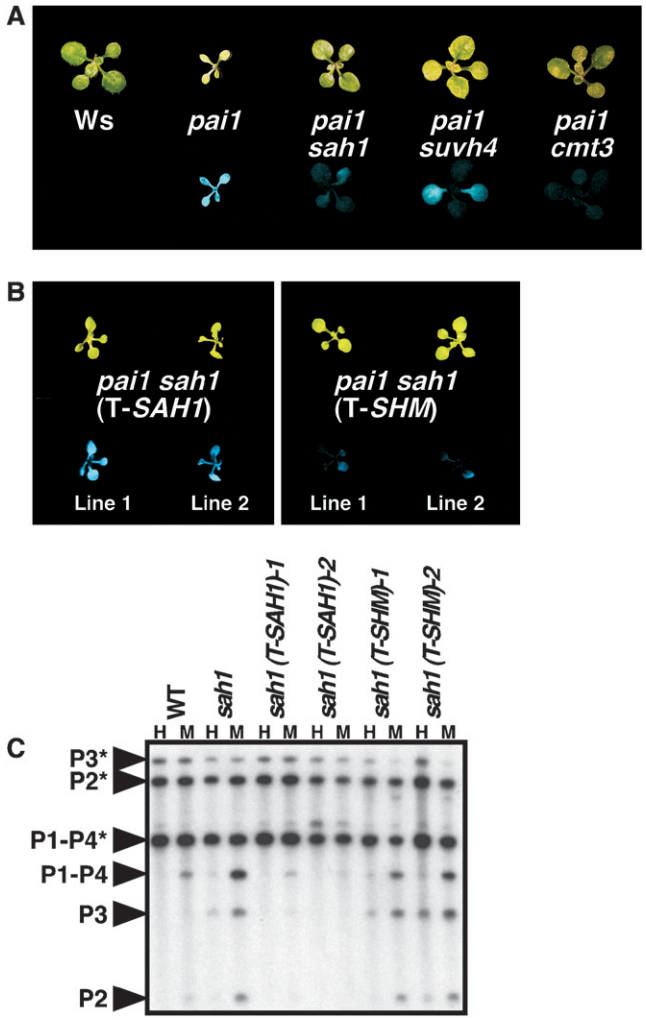


FIGURE 2.—The *sah1L459F* mutation suppresses *PAI2* silencing. (A and B) Two-week-old seedlings of the indicated genotypes grown on agar medium were photographed under visible (top) or UV (bottom) light, with *sah1* indicating *sah1L459F*. (A) Representative seedlings of mutants isolated from the *PAI2* silencing suppressor screen. (B) Representative T2 generation seedlings of Ws *pai1 sah1L459F* transformed with either an *SAH1* (T-*SAH1*) or a control *SHM* (T-*SHM*) genomic transgene. (C) Genomic DNAs prepared from 4-week-old plants of the indicated genotypes were cleaved with *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 *PAI* internal sites. WT is Ws *pai1*, *sah1* is Ws *pai1 sah1L459F*, and the remaining strains are Ws *pai1 sah1L459F* transformed with the indicated transgenes, corresponding to the transgenic lines shown in B.

the *pai1* parental strain (Figure 2B). In contrast, *pai1 sahlL459F* mutant plants transformed with a control transgene carrying a genomic clone of a neighboring gene At4g13930 encoding a serine hydroxymethyltransferase (T-*SHM*) displayed weak fluorescence similar to that of the untransformed *pai1 sah1L459F* strain. The *pai1 sah1L459F* (T-*SAH1*) transgenic plants also displayed remethylation of the *PAI2* reporter gene as assessed by *Hpa*II and *Msp*I Southern blot assays for DNA methylation (Figure 2C). These assays are described in detail below.

**The *sah1L459F* mutation reduces *PAI* cytosine methylation in non-CG contexts:** The suppression of PAI-deficient phenotypes in the *pai1* background by the *sah1L459F* mutation correlated with reduced methylation on the *PAI2* reporter gene as well as the *PAI3* and *PAI1–PAI4* loci. The *PAI* demethylation conferred by this mutation occurred primarily in non-CG sequence contexts, as determined by Southern blot assays with methylation-sensitive restriction enzymes and by bisulfite genomic sequencing.

For Southern blot analysis, genomic DNA was cleaved with different enzymes that monitor methylation in non-CG or CG sequence contexts. For comparison, we included *suvh4* and *cmt3* mutants previously recovered from the *PAI2* silencing suppressor screen (see above). The *cmt3* mutation confers a strong reduction of non-CG methylation on all three *PAI* loci, whereas CG methylation patterning is only slightly reduced (BARTEE *et al.* 2001). The *suvh4* mutation also confers a loss of non-CG methylation on the singlet genes *PAI2* and *PAI3*, but does not alter the CG plus non-CG methylation patterning on the *PAI1–PAI4* inverted repeat (MALAGNAC *et al.* 2002) because histone H3 lysine 9 methylation (H3 mK9) at this locus is uniquely controlled by the combined action of SUVH4 and a related enzyme SUVH6 (EBBS *et al.* 2005; EBBS and BENDER 2006).

To assess methylation in non-CG contexts, genomic DNA was cleaved with *Hinc*II, which has recognition sites at the translational start codons of *PAI1*, *PAI4*, and *PAI2* (but not *PAI3* due to a polymorphism) (MALAGNAC *et al.* 2002). *Hinc*II monitors methylation of cytosines in the contexts 5′ CAG 3′ and 5′ CAT 3′on each of the two DNA strands of its recognition site. In this assay, the *sah1L459F* mutant displayed cleavage of *PAI1–PAI4* and *PAI2* intermediate between the wild type and the *cmt3* mutant, implying a partial loss of non-CG methylation (Figure 3A). As a second assay for non-CG contexts, genomic DNA was cleaved with *Msp*I, which has recognition sites in the second introns of *PAI2*, *PAI3*, and *PAI4* (but not *PAI1* due to a polymorphism) (MALAGNAC *et al.* 2002) and monitors methylation of cytosines in the context 5′ CCG 3′. The *sah1L459F* mutant displayed *Msp*I cleavage of all three *PAI* loci intermediate between the wild type and the *cmt3* mutant (Figure 3B), similar to the patterns observed for *Hinc*II cleavage.

We additionally tested cleavage with *Hpa*II, an isoschizomer of *Msp*I that is sensitive to methylation of either cytosine in the recognition site 5′ CCGG 3′ (CG and CCG methylation). In this assay a slight increase in *PAI2* cleavage was evident in *sah1L459F* relative to the wild type, similarly to *suvh4* and *cmt3* (Figure 3B).

To monitor methylation specifically in a CG context we used the enzyme *Cla*I, which recognizes the sequence 5′ ATCGAT 3′ in the third intron of each of the four *PAI*

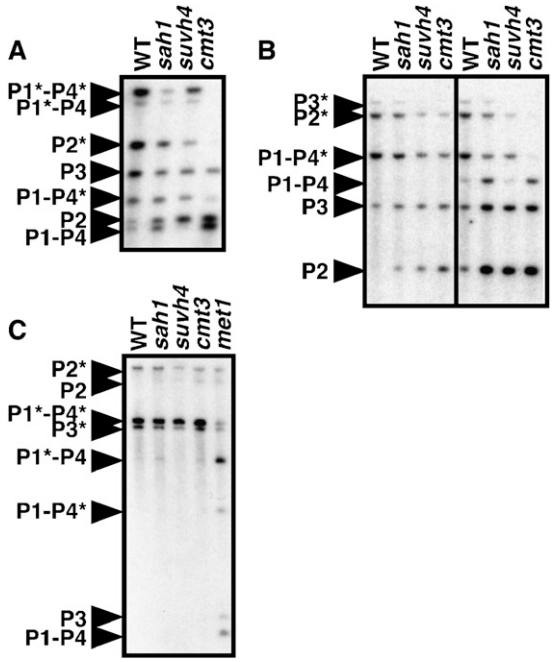


FIGURE 3.—The *sah1L459F* mutation reduces *PAI* non-CG methylation. Genomic DNA prepared from 4-week-old plants of the indicated genotypes was cleaved with (A) *Hinc*II, (B) *Hpa*II (left) or *Msp*I (right), or (C) *Cla*I and used for Southern blot analysis with a *PAI* probe. P1–P4 is *PAI1–PAI4*, P2 is *PAI2*, and P3 is *PAI3*, with asterisks indicating the positions of species methylated at *PAI*-internal sites. All strain backgrounds carried the *pai1* mutation. WT is Ws *pai1* and *sah1* is Ws *pai1 sah1L459F*.

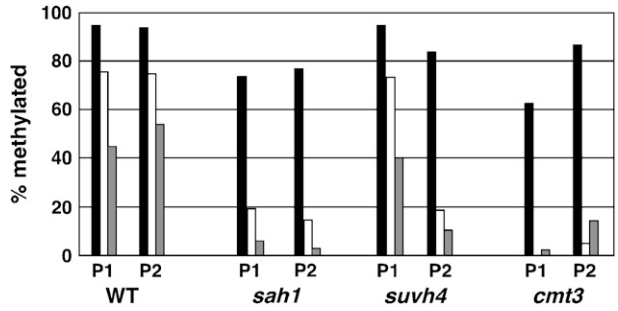


FIGURE 4.—Genomic bisulfite methylation sequencing data for *PAI1* and *PAI2* proximal promoter regions in the *sah1L459F* mutant. Eight independent top strand clones were sequenced for *PAI1* or *PAI2* from bisulfite-treated DNA of Ws *pai1 sah1L459F* (*sah1*) as previously described (MELQUIST and BENDER 2003). The percentage of 5-methyl-cytosines detected out of total cytosines available within the region of *PAI* sequence identity (344 bp for *PAI1* or 338 bp for *PAI2*) is shown, divided into the contexts CG (solid bars), CNG (open bars), and other contexts (shaded bars). For comparison, previously determined methylation patterns for wild-type Ws (WT) (LUFF *et al.* 1999), Ws *pai1 suvh4R302** (*suvh4*) (MALAGNAC *et al.* 2002), and Ws *pai1 cmt3G456D* (*cmt3*) (BARTEE *et al.* 2001) are shown.

genes. For comparison, we included a *met1* mutant deficient in the major CG MTase (BARTEE and BENDER 2001; KANKEL *et al.* 2003). The *sah1L459F*, *suvh4*, and *cmt3* mutants displayed *PAI Cla*I cleavage patterns similar to the wild type (Figure 3C). The *met1* mutant displayed partial *Cla*I cleavage diagnostic of reduced CG methylation at all three *PAI* loci. Together, the Southern blot analyses indicate that the *sah1L459F* mutation primarily reduces non-CG methylation patterning on the *PAI* genes.

In addition to Southern blot assays, we monitored methylation patterning on the proximal promoter regions of *PAI1* and *PAI2* in the *sah1L459F* mutant background using genomic bisulfite sequencing. This analysis showed reduced methylation at both *PAI1* and *PAI2* relative to the wild type, with a stronger reduction in CNG and other non-CG contexts than in CG contexts (Figure 4). Overall, the methylation patterns at the *PAI2* reporter locus were similar among *sah1L459F*, *suvh4*, and *cmt3*. However, the *sah1L459F* mutant had a slightly stronger depletion of CG methylation at *PAI2* than either *suvh4* or *cmt3*, suggesting additional weak inhibition of the MET1 CG methylation pathway specifically in this background.

**The *sah1L459F* mutation reduces non-CG methylation on transposon sequences:** Our finding that the *sah1L459F* allele causes demethylation of the *PAI* genes at non-CG residues, similarly to *suvh4* and *cmt3* mutations, suggested that the CMT3-controlled methylation pathway might be uniquely susceptible to SAH perturbations. To further investigate this issue, we monitored methylation patterning at known endogenous targets for the three cytosine methylation pathways characterized in Arabidopsis—SUVH4/CMT3, DRM, or MET1 (reviewed in MATHIEU and BENDER 2004)—in the *sah1L459F* mutant background.

Some Arabidopsis transposon sequences including the *Mu1* mutator-related DNA transposon and the *Ta3* retrotransposon are densely methylated at both CG and non-CG cytosines (JOHNSON *et al.* 2002; LIPPMAN *et al.* 2003; EBBS and BENDER 2006). Like the *PAI* genes, these transposons are partially demethylated in a *suvh4* mutant background and strongly demethylated in a *cmt3* mutant background in CCG contexts monitored by *Msp*I cleavage. Using *Msp*I Southern blot analysis, we found that the *sah1L459F* mutation caused partially increased cleavage of both of these transposons relative to the wild-type parental strain (Figure 5). Therefore SUVH4/CMT3 target loci in addition to the *PAI* genes are partially demethylated in non-CG contexts by the *sah1L459F* mutation.

The Arabidopsis DRM1 and DRM2 cytosine MTase's control establishment of new methylation imprints, as well as maintenance of non-CG methylation at some loci (CAO and JACOBSEN 2002a,b; CAO *et al.* 2003). In particular, the *MEA-ISR* direct repeat sequence is methylated and inhibited from cleavage by *Msp*I in the wild type, but is demethylated and completely cleaved in the *drm1 drm2* mutant (CAO and JACOBSEN 2002a). We

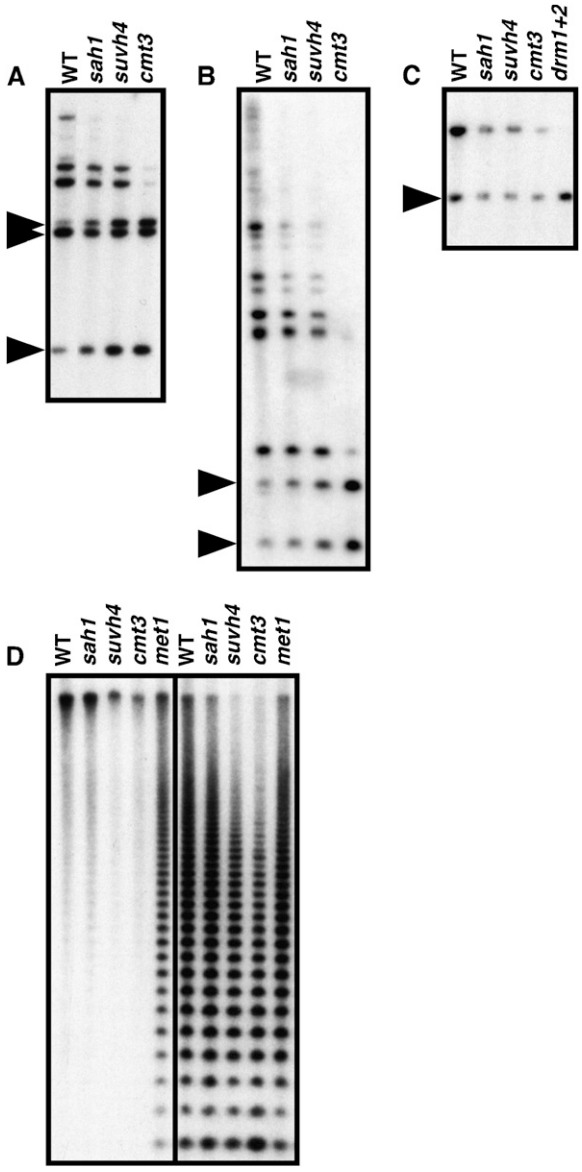


FIGURE 5.—The *sah1L459F* mutation reduces non-CG methylation on transposon sequences, similarly to *suvh4* and *cmt3* mutations. Genomic DNA prepared from 4-week-old plants of the indicated genotypes was cleaved with the indicated restriction enzymes and used for Southern blot analysis with the indicated probes. (A) DNA digested with *Msp*I and *Hind*III and probed for *Mu1*. (B) DNA digested with *Msp*I and probed for *Ta3*. (C) DNA digested with *Msp*I and probed for *MEA-ISR*. (D) DNA digested with *Hpa*II (left) or *Msp*I (right) and probed for the 180-bp centromere repeat. WT is the wild-type Ws, *sah1* is *sah1L459F*, and *drm1+2* is Ws *drm1 drm2*. Arrowheads in left margins indicate the positions of fully cleaved species.

found that in the *sah1L459F* mutant there was no increase in *Msp*I cleavage of *MEA-ISR* relative to the wild type (Figure 5C). This result suggests that DRM-mediated non-CG methylation is not affected by the *sah1L459F* mutation.

A major target for cytosine methylation in the Arabidopsis genome is the 180-bp centromere-associated repeat sequence (VONGS *et al.* 1993). This sequence carries mainly CG methylation and is demethylated by mutation of the MET1 CG-specific MTase, as indicated by increased cleavage with the CG-sensitive enzyme *Hpa*II (KANKEL *et al.* 2003, Figure 5D). *Hpa*II Southern blot analysis showed no increase in centromere repeat cleavage for the *sah1L459F* mutant relative to the wild type (Figure 5D). Thus, the *sah1* defect does not impair MET1-controlled CG methylation at the centromere repeats. The centromere repeats also carry non-CG methylation, as indicated by increased cleavage with *Msp*I in *suvh4* and *cmt3* mutant backgrounds relative to the wild type (Figure 5D). However, *Msp*I Southern blot analysis showed no obvious increase in centromere repeat cleavage for the *sah1L459F* mutant relative to the wild type. The lack of a centromere demethylation phenotype might reflect the limited sensitivity of the Southern blot assay for this highly repetitive sequence and an overall weaker effect on CCG methylation for *sah1L459F* relative to *suvh4* or *cmt3*.

**The *sah1L459F* mutation is a weak allele of *SAH1*:** To understand the degree to which the *sah1L459F* allele disrupts SAH1 function, we determined whether the Arabidopsis SAH1 *vs.* sah1L459F enzymes could complement the lethality of a yeast *sah1* deletion mutant. We also included Arabidopsis SAH2 (At3g23810) in this analysis to determine whether it encodes a functional enzyme. The predicted SAH2 protein is 96% identical to SAH1 (ROCHA *et al.* 2005). Both *SAH1* and *SAH2* are represented in databases by multiple cDNA and expressed sequence tag clones from a range of different tissue sources. Moreover, RNA gel blot analysis with gene-specific probes indicates that both *SAH1* and *SAH2* transcripts accumulate to readily detectable levels in total RNA prepared from adult plants (data not shown). However, an insertion mutation in SAH2 was previously reported to confer no morphological or DNA methylation phenotypes, in contrast to the lethal phenotype of SAH1 insertion mutations (ROCHA *et al.* 2005). A possible explanation for this result is that *SAH2* does not encode a functional enzyme. Alternatively, both SAH1 and SAH2 might be functional, but SAH1 might serve the dominant role in the plant due to its expression patterns or enzymatic properties.

To test the activities of the Arabidopsis SAH enzymes in yeast, we used a plasmid shuffle strategy. For this strategy, we constructed a haploid yeast strain where a deletion of the genomic *SAH1* coding region is complemented by the yeast *SAH1* gene expressed from its own promoter carried on a *URA3*-marked plasmid. We then supertransformed this strain with *LEU2*-marked plasmids, where each of the Arabidopsis *SAH* cDNAs, or as a positive control the yeast *SAH1* gene, is expressed from the yeast *SAH1* promoter. Serial dilutions of cultures grown from each strain were plated on medium containing 5-FOA, which selects against cells expressing *URA3*. Thus, 5-FOA-resistant colonies recovered in this

assay are those where the yeast *SAH1* gene on the *URA3* vector has segregated away and where the plant *SAH* gene on the *LEU2* vector is sufficient to complement the genomic *sah1* deletion; an absence of 5-FOA-resistant colonies indicates that the plant *SAH* gene is not able to complement the genomic *sah1* deletion. We found that when the Arabidopsis *SAH1*, *SAH2*, or *sah1L459F* cDNAs were expressed from a high copy 2μ yeast vector, they were all able to support growth on 5-FOA medium (Figure 6). However, when the Arabidopsis *SAH* cDNAs were expressed from a low-copy centromeric (*CEN*) plasmid, only wild-type *SAH1* and *SAH2* were able to support growth on 5-FOA medium. The plasmid shuffle data indicate that the *sah1L459F* allele confers partial loss of function that can be compensated for by overexpression. The data also indicate that both *SAH1* and *SAH2* encode functional SAH enzyme. Thus, the dominant role of *SAH1* in the plant is likely due to differences in expression patterns between *SAH1* and *SAH2*.

**Histone H3 methylation is maintained near wild-type levels in the *sah1L459F* mutant:** H3 dimethylated at K9 (H3 2mK9) catalyzed by SUVH histone MTase's is required for CMT3-mediated DNA methylation (Jackson *et al.* 2002, 2004; Malagnac *et al.* 2002; Ebbs *et al.* 2005; Ebbs and Bender 2006). The *sah1L459F* mutation could thus selectively impair the CMT3 pathway by combined inhibition of SUVH MTase's and the CMT3 MTase. To estimate the severity of the *sah1L459F* mutation toward H3 K9 MTase's, we performed ChIP analysis of H3 2mK9 levels on heterochromatic loci in *sah1L459F* relative to the wild type and *suvh* mutants. We also used ChIP analysis to monitor levels of H3 dimethylated at lysine 4 (H3 2mK4), a modification associated with transcriptionally active genes (Gendrel *et al.* 2002; Lippman *et al.* 2004).

H3 mK9 can be lost either through direct impairment of SUVH activity or as an indirect consequence of transcriptional activation (Johnson *et al.* 2002; Ebbs *et al.* 2005). To avoid this ambiguity, we assayed H3 2mK9 levels at two heterochromatic loci where transcription is not activated by reduced DNA methylation: the *Ta3* retrotransposon and the *PAI1–PAI4* constitutively transcribed inverted repeat. In the wild type, *Ta3* is transcriptionally silenced and enriched for H3 2mK9; in *suvh4*, *Ta3* is only partially demethylated and remains transcriptionally silenced, but is strongly depleted for H3 2mK9 (Johnson *et al.* 2002; Ebbs *et al.* 2005; Ebbs and Bender 2006). In the wild type, *PAI1–PAI4* is both DNA methylated and transcriptionally active due to an unmethylated promoter that lies upstream of *PAI1* (Melquist and Bender 2003). *PAI1–PAI4* is enriched for H3 2mK9 despite its transcriptional activity (Ebbs *et al.* 2005; Ebbs and Bender 2006). In addition, the accumulation of transcripts from the locus is not significantly altered by reduced DNA methylation of the internal inverted repeat sequences. Interestingly, H3 2mK9 at *PAI1–PAI4* is not reduced in the *suvh4* mutant

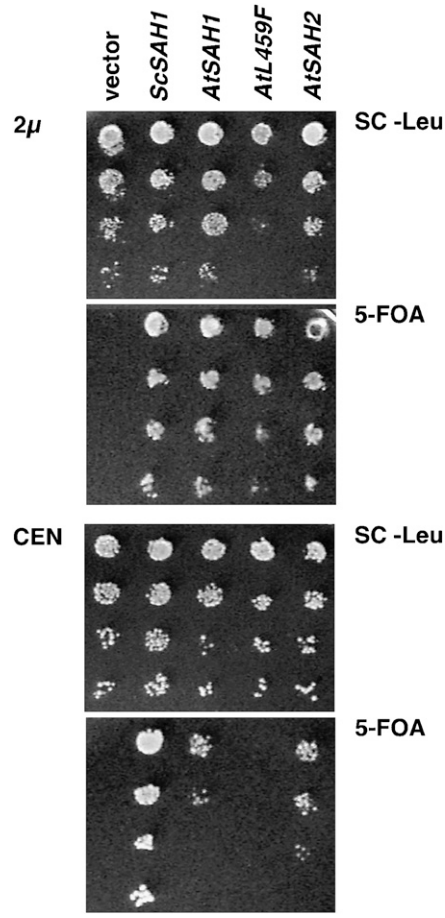


Figure 6.—Arabidopsis sah1L459F can complement a yeast SAH deficiency when overexpressed. Haploid yeast deleted for *SAH1* and carrying a complementing yeast *SAH1* (*ScSAH1*) gene on a *URA3* vector were supertransformed with *ScSAH1* or the Arabidopsis *SAH1* (*AtSAH1*), *sah1L459F* (*AtL459F*), or *SAH2* (*AtSAH2*) genes all expressed from the yeast *SAH1* promoter carried on a *LEU2* vector. As a negative control the strain was also transformed with the empty *LEU2* vector. Both high-copy 2μ and low-copy *CEN* vectors were tested. Serial dilutions of cultures grown from each strain were spotted in parallel on medium lacking leucine to indicate the total number of supertransformed cells plated in each spot (SC −Leu) or on medium containing 5-FOA to select against *URA3* cells.

because a related H3 K9 MTase SUVH6 is also active at this locus; both SUVH4 and SUVH6 must be mutated to cause loss of *PAI1–PAI4* H3 2mK9 and non-CG methylation (Ebbs *et al.* 2005; Ebbs and Bender 2006). However, the effects of the *suvh6* mutation are locus specific: partial demethylation of *Ta3* in *suvh4* is not enhanced in *suvh4 suvh6*.

ChIP analysis showed that the *sah1L459F* mutant maintained similar levels of H3 2mK9 relative to the wild type at both *Ta3* and *PAI1-PAI4* (Figure 7). In contrast, H3 2mK9 was lost from *Ta3* in *suvh4* or *suvh4 suvh6* mutant backgrounds and from *PAI1–PAI4* in the *suvh4 suvh6* mutant background. Neither *Ta3* nor *PAI1–PAI4* carried H3 2mK4 in any of the strains assayed, consistent with a lack of transcriptional activation. These results

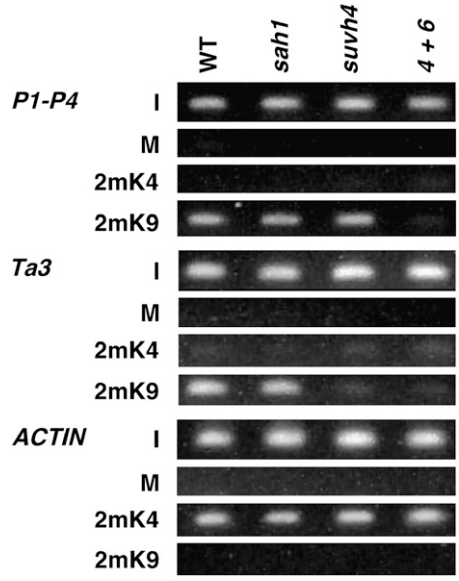


Figure 7.—The *sah1L459F* mutation does not deplete H3 2mK9 or H3 2mK4 from modified regions. ChIP analysis of the indicated mutants is shown. WT is Ws *pai1*, *sah1* is Ws *pai1 sah1L459F*, *suvh4* is Ws *pai1 suvh4*, and *4+6* is *pai1 suvh4 suvh6* (Ebbs *et al.* 2005). Primer sets specific for each locus were used to amplify PCR products from total-input chromatin (I), no antibody mock precipitation control (M), chromatin immunoprecipitated with H3 anti-dimethyl K9 antibodies (2mK9), or chromatin immunoprecipitated with H3 anti-dimethyl K4 antibodies (2mK4) from the indicated mutants. GelStar-stained PCR products are shown. P1–P4 indicates the *PAI1–PAI4* inverted repeat.

indicate that the *sah1L459F* mutation does not inhibit the CMT3 DNA methylation pathway by strongly impairing SUVH H3 K9 MTase activity. However, it should be noted that a subtle modulation in H3 2mK9 levels would not be detectable within the sensitivity of the ChIP assay.

The *ACTIN* gene is enriched for H3 2mK4 in the wild-type and DNA methylation-deficient backgrounds including *suvh4* and *cmt3* (Johnson *et al.* 2002; Ebbs *et al.* 2005; Ebbs and Bender 2006). The levels of *ACTIN* H3 2mK4 were similar in the wild-type, *sahlL459F*, and *suvh* mutant backgrounds (Figure 7). This result suggests that as for SUVH H3 K9 MTase's, Arabidopsis H3 K4 MTase activity is not strongly inhibited by the *sah1L459F* mutation.

## DISCUSSION

The addition of a methyl group to the 5-position of cytosine is a key epigenetic modification in mammals and plants, signaling heterochromatin formation and transcriptional silencing. Cytosine methylation functions in genome defense against invasive sequences (Yoder *et al.* 1997; Walsh *et al.* 1998; Miura *et al.* 2001; Kato *et al.* 2003; Lippman *et al.* 2003, 2004; Bourc'his and Bestor 2004), in regulation of gene dosage through parental imprinting (da Rocha and Ferguson-Smith 2004; Gehring *et al.* 2004), and in centromere compaction (Xu *et al.* 1999; Soppe *et al.* 2002). However, aberrant methylation and silencing of tumor suppressor genes is a common event in the progression of human cancers (Jones and Baylin 2002). In addition, methylation and silencing of newly integrated transgenes often presents a technical barrier to the expression of novel traits in transgenic plants and animals. Here we show that weak impairment of the Arabidopsis SAH enzyme that controls overall methylation homeostasis can specifically impair cytosine methylation and silencing without other phenotypic consequences.

We recovered the *sah1L459F* missense allele of the Arabidopsis *SAH1* gene through a genetic screen designed to monitor transcriptional silencing of the *PAI2* promoter sequence, which contains mainly methylated non-CG cytosines (Luff *et al.* 1999). In the non-CG methylation maintenance pathway H3 mK9 mediated by SUVH4 is required for DNA methylation mediated by CMT3 (Jackson *et al.* 2002; Malagnac *et al.* 2002). The *sahlL459F* mutation confers similar partial non-CG demethylation of *PAI2* and transposon sequences to a *suvh4* null mutation, but both *sah1L459F* and *suvh4* confer weaker non-CG demethylation than a *cmt3* null mutation (Figures 3 and 5). The *sah1L459F* mutation does not cause demethylation of sequences controlled by the DRM and MET1 MTase's (Figures 3 and 5). Together these results indicate that the SUVH4/CMT3 cytosine methylation pathway is preferentially inhibited by impairment of SAH1 relative to the DRM or MET1 cytosine methylation pathways.

The sah1L459F mutant protein can complement a yeast *sah1* deletion mutant when expressed from a high-copy plasmid, demonstrating that the protein retains partial enzymatic activity (Figure 6). Consistent with this weak defect in enzyme activity, the *sah1L459F* mutation does not confer developmental abnormalities. In contrast, previously described *hog1* missense mutations in *SAH1* cause slow growth and decreased fertility (Rocha *et al.* 2005). The *hog1* morphological defects might be caused by partial impairment of MTase's that catalyze essential metabolic functions and/or by dysregulation of gene expression due to loss of cytosine methylation. For example, Southern blot and thin layer chromatography-based assays show that the *hog1-1* mutant has reductions in both CG and non-CG methylation (Rocha *et al.* 2005). Loss of methylation in CG contexts, such as in a *met1* mutant, is associated with developmental abnormalities including reduced size, abnormal flower development, and delayed time to flowering (Kankel *et al.* 2003; Saze *et al.* 2003; Xiao *et al.* 2006). Furthermore, loss of methylation in both CG and non-CG contexts, such as in a *met1 cmt3* double mutant, is associated with transposon reactivation (Kato *et al.* 2003).

The methylated transgene reporter used to isolate the *hog1* alleles can be reactivated by *met1* or *cmt3* mutations, but not by a *suvh4* mutation (Rocha *et al.* 2005). Since

the *sahlL459F* mutation causes similar or weaker loss of non-CG methylation compared to *suvh4* and has minimal effects on CG methylation, weak *sah1* alleles would perhaps not be recovered from the *hog1* mutant screen. Conversely, the *pai1* mutant reporter background used to isolate the *sah1L459F* allele might have selected against stronger *hog1*-like *sah1* mutations due to pleiotropic effects of combined tryptophan deficiency and SAH1 impairment. Selection against strong *sah1* alleles, together with a limited spectrum of EMS-induced mutations, could account for why we recovered only one *sah1* allele from a screen that also yielded multiple loss-of-function alleles for *suvh4* and *cmt3* (Bartee *et al.* 2001; Malagnac *et al.* 2002). In addition, SAH is highly conserved across eukaryotes (Figure 1), indicating that there are strong structural constraints on SAH function.

In the crystal structure of human SAH, the L459-analogous residue (L406) lies in the center of a linker region connecting two α-helicies (Figure 1) that make contacts with the NAD cofactor-binding domain of the adjacent monomer (Turner *et al.* 1998). The substitution of the bulky phenylalanine side chain at this position might thus impair optimal intersubunit contacts. In comparison, the strongest of the *hog1* alleles, *hog1-1* (T414I) (Rocha *et al.* 2005), mutates a conserved threonine residue (T363 in the human SAH) to isoleucine at a position adjacent to a conserved phenylalanine (F362 in human SAH) that forms the base of a hydrophobic binding pocket for the substrate adenine ring in the human SAH structure (Turner *et al.* 1998).

The CMT3 pathway for maintenance of non-CG methylation presents at least two types of MTase enzymes that can be inhibited by accumulation of AdoHcy in an *sah* mutant background: SUVH H3 K9 MTase's and the CMT3 cytosine MTase. In particular, SUVH-catalyzed dimethylation of H3 K9 is the epigenetic mark associated with CMT3-mediated cytosine methylation (Jackson *et al.* 2004). However, ChIP analysis at the *Ta3* retrotransposon and the *PAI1–PAI4* transcribed inverted repeat targets of SUVH4 and the related enzyme SUVH6 showed that these targets maintain similar levels of H3 2mK9 between the wild type and the *sah1L459F* mutant (Figure 7). Thus, SAH impairment does not preferentially inhibit SUVH MTase's to cause strong depletion of H3 2mK9, with loss of CMT3-mediated cytosine methylation occurring as a secondary effect.

Another possible explanation for preferential inhibition of the CMT3 pathway by SAH impairment is that the CMT3 cytosine MTase is uniquely susceptible to inhibition by AdoHcy. However, this possibility would require that CMT3 has a significantly different active site architecture than the DRM or MET1 cytosine MTase's, which are not strongly inhibited in the *sah1L459F* background (Figures 3 and 5). The chromomethylase family to which CMT3 belongs is defined by the insertion of a chromodomain between conserved cytosine MTase catalytic motifs (Goll and Bestor 2005). Thus, this extra domain has the potential to alter the catalytic properties of CMT3 relative to other cytosine MTase's. Future elucidation of the three-dimensional structures of plant cytosine MTase's will clarify this possibility.

Similarly, a histone MTase other than the SUVH enzymes required specifically in the CMT3 pathway could be uniquely susceptible to inhibition by AdoHcy. For example, *in vitro* studies have implicated H3 mK27 as well as H3 mK9 in CMT3 binding (Lindroth *et al.* 2004). However, this possibility would require that the putative susceptible histone MTase has a significantly different active site architecture than the SUVH H3 K9 MTase's or the MTase's that maintain H3 2mK4, which are not strongly inhibited in the *sah1L459F* background (Figure 7).

The simplest explanation for the preferential inhibition of the SUVH/CMT3 DNA methylation pathway by weak impairment of SAH1 is that simultaneous weak inhibition of the SUVH- and CMT3-mediated transmethylation reactions results in a synergistic strong inhibition of the output of the pathway. Even though SUVH activity is not impaired at the limit of detection in a ChIP assay in the *sah1L459F* background (Figure 7), a slight defect in this step of the pathway could combine with a slight defect in the CMT3-catalyzed step of the pathway to create a substantial pathway bottleneck. If this explanation indeed accounts for the preferential inhibition of SUVH/CMT3 *vs.* the DRM or MET1 DNA methylation pathways by *sah1L459F,* it also suggests that the DRM and MET1 pathways do not require histone methylation signals.

In Neurospora H3 mK9 mediated by the DIM-5 histone MTase is required for DNA methylation mediated by the DIM-2 cytosine MTase (Tamaru and Selker 2001). It would thus be interesting to determine whether weak mutations in the Neurospora SAH gene (such as a leucine-to-phenylalanine mutation at the residue analogous to the Arabidopsis SAH L459 residue, Figure 1) would cause substantial genomic demethylation without other phenotypic consequences. This result would support the view that weak SAH impairment causes preferential inhibition of histone methylation-dependent cytosine methylation because the pathway depends on sequential transmethylation reactions, rather than because one of the enzymes in the pathway is uniquely susceptible to inhibition by AdoHcy.

In the mouse, cells deleted for the related Suv39h1 and Suv39h2 H3 K9 MTase's display reduced cytosine methylation at major satellite repeats (Lehnertz *et al.* 2003), and cells deleted for the G9a H3 K9 MTase display a loss of methylation at an imprinted locus (Xin *et al.* 2003). SAH impairment might thus be a useful tool in further dissecting which regions of the mammalian genome are under control of histone methylation-dependent DNA methylation pathways. Controlled SAH impairment by chemical inhibitors or RNA interference could also serve as an alternative to inhibition of

cytosine MTase's by cytidine analogs in probing the relationship between chromatin structure and gene silencing.

We thank the Arabidopsis Biological Resource Center for the *SAH1* cDNA clone and Thomas Jenuwein (Vienna Biocenter) for H3 antidimethyl K9 antibodies. We also thank Eric Grote and Martin Romeo (Johns Hopkins University) for assistance with yeast strain constructions. This work was supported by National Institutes of Health grant GM61148 to J.B., by National Cancer Institute (NCI) training grant T32 CA09110 to L.M., and by National Institute of Environmental Health Sciences training grant T32 ES07141 and NCI training grant T32 CA09110 to M.L.E.

## LITERATURE CITED

Bacolla, A., S. Pradhan, R. J. Roberts and R. D. Wells, 1999 Recombinant human DNA (cytosine-5) methyltransferase. II. Steady-state kinetics reveal allosteric activation by methylated dna. J. Biol. Chem. **274:** 33011–33019.

Baric, I., K. Fumic, B. Glenn, M. Cuk, A. Schulze *et al.*, 2004 S-adenosylhomocysteine hydrolase deficiency in a human: a genetic disorder of methionine metabolism. Proc. Natl. Acad. Sci. USA **101:** 4234–4239.

Baric, I., M. Cuk, K. Fumic, O. Vugrek, R. H. Allen *et al.*, 2005 S-adenosylhomocysteine hydrolase deficiency: a second patient, the younger brother of the index patient, and outcomes during therapy. J. Inherit. Metab. Dis. **28:** 885–902.

Bartee, L., and J. Bender, 2001 Two *Arabidopsis* methylation-deficiency mutations confer only partial effects on a methylated endogenous gene family. Nucleic Acids Res. **29:** 2127–2134.

Bartee, L., F. Malagnac and J. Bender, 2001 *Arabidopsis cmt3* chromomethylase mutations block non-CG methylation and silencing of an endogenous gene. Genes Dev. **15:** 1753–1758.

Bender, J., and G. R. Fink, 1995 Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of Arabidopsis. Cell **83:** 725–734.

Bevan, M., 1984 Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. **12:** 8711–8721.

Bourc'his, D., and T. H. Bestor, 2004 Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature **431:** 96–99.

Bujnicki, J. M., S. T. Prigge, D. Caridha and P. K. Chiang, 2003 Structure, evolution, and inhibitor interaction of S-adenosyl-L-homocysteine hydrolase from *Plasmodium falciparum*. Proteins **52:** 624–632.

Cao, X., and S. E. Jacobsen, 2002a Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proc. Natl. Acad. Sci. USA **99**(Suppl. 4): 16491–16498.

Cao, X., and S. E. Jacobsen, 2002b Role of the *Arabidopsis DRM* methyltransferases in de novo DNA methylation and gene silencing. Curr. Biol. **12:** 1138–1144.

Cao, X., W. Aufsatz, D. Zilberman, M. F. Mette, M. S. Huang *et al.*, 2003 Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. Curr. Biol. **13:** 2212–2217.

Chiang, P. K., 1998 Biological effects of inhibitors of S-adenosylhomocysteine hydrolase. Pharmacol. Ther. **77:** 115–134.

Clough, S. J., and A. F. Bent, 1998 Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

da Rocha, S. T., and A. C. Ferguson-Smith, 2004 Genomic imprinting. Curr. Biol. **14:** R646–R649.

De Clercq, E., 2004 Antivirals and antiviral strategies. Nat. Rev. Microbiol. **2:** 704–720.

Ebbs, M. L., and J. Bender, 2006 Locus-specific control of DNA methylation by the *Arabidopsis* SUVH5 histone methyltransferase. Plant Cell **18:** 1166–1176.

Ebbs, M. L., L. Bartee and J. Bender, 2005 H3 lysine 9 methylation is maintained on a transcribed inverted repeat by combined action of SUVH6 and SUVH4 methyltransferases. Mol. Cell. Biol. **25:** 10507–10515.

Fojtova, M., A. Kovarik, I. Votruba and A. Holy, 1998 Evaluation of the impact of S-adenosylhomocysteine metabolic pools on cytosine methylation of the tobacco genome. Eur. J. Biochem. **252:** 347–352.

Gehring, M., Y. Choi and R. L. Fischer, 2004 Imprinting and seed development. Plant Cell **16**(Suppl.): S203–S213.

Gendrel, A. V., Z. Lippman, C. Yordan, V. Colot and R. A. Martienssen, 2002 Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. Science **297:** 1871–1873.

Goll, M. G., and T. H. Bestor, 2005 Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. **74:** 481–514.

Jackson, J. P., A. M. Lindroth, X. Cao and S. E. Jacobsen, 2002 Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416:** 556–560.

Jackson, J. P., L. Johnson, Z. Jasencakova, X. Zhang, L. PerezBurgos *et al.*, 2004 Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. Chromosoma **112:** 308–315.

Johnson, L., X. Cao and S. Jacobsen, 2002 Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. Curr. Biol. **12:** 1360–1367.

Jones, P. A., and S. B. Baylin, 2002 The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. **3:** 415–428.

Kankel, M. W., D. E. Ramsey, T. L. Stokes, S. K. Flowers, J. R. Haag *et al.*, 2003 Arabidopsis *MET1* cytosine methyltransferase mutants. Genetics **163:** 1109–1122.

Kato, M., A. Miura, J. Bender, S. E. Jacobsen and T. Kakutani, 2003 Role of CG and non-CG methylation in immobilization of transposons in *Arabidopsis*. Curr. Biol. **13:** 421–426.

Lehnertz, B., Y. Ueda, A. A. Derijck, U. Braunschweig, L. Perez-Burgos *et al.*, 2003 *Suv39h*-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr. Biol. **13:** 1192–1200.

Lindroth, A. M., D. Shultis, Z. Jasencakova, J. Fuchs, L. Johnson *et al.*, 2004 Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. EMBO J. **23:** 4146–4155.

Lippman, Z., B. May, C. Yordan, T. Singer and R. Martienssen, 2003 Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification. PLoS Biol. **1:** E67.

Lippman, Z., A. V. Gendrel, M. Black, M. W. Vaughn, N. Dedhia *et al.*, 2004 Role of transposable elements in heterochromatin and epigenetic control. Nature **430:** 471–476.

Luff, B., L. Pawlowski and J. Bender, 1999 An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*. Mol. Cell **3:** 505–511.

Malagnac, F., L. Bartee and J. Bender, 2002 An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. EMBO J. **21:** 6842–6852.

Masuta, C., H. Tanaka, K. Uehara, S. Kuwata, A. Koiwai *et al.*, 1995 Broad resistance to plant viruses in transgenic plants conferred by antisense inhibition of a host gene essential in S-adenosylmethionine-dependent transmethylation reactions. Proc. Natl. Acad. Sci. USA **92:** 6117–6121.

Mathieu, O., and J. Bender, 2004 RNA-directed DNA methylation. J. Cell Sci. **117:** 4881–4888.

Melquist, S., and J. Bender, 2003 Transcription from an upstream promoter controls methylation signaling from an inverted repeat of endogenous genes in Arabidopsis. Genes Dev. **17:** 2036–2047.

Melquist, S., B. Luff and J. Bender, 1999 Arabidopsis *PAI* gene arrangements, cytosine methylation and expression. Genetics **153:** 401–413.

Miller, M. W., D. M. Duhl, B. M. Winkes, F. Arredondo-Vega, P. J. Saxon *et al.*, 1994 The mouse lethal *nonagouti (a(x))* mutation deletes the *S-adenosylhomocysteine hydrolase* (*Ahcy*) gene. EMBO J. **13:** 1806–1816.

Miura, A., S. Yonebayashi, K. Watanabe, T. Toyama, H. Shimada *et al.*, 2001 Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature **411:** 212–214.

Neff, M. M., J. D. Neff, J. Chory and A. E. Pepper, 1998 dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J. **14:** 387–392.

Porcelli, M., M. A. Moretti, L. Concilio, S. Forte, A. Merlino *et al.*, 2005 S-adenosylhomocysteine hydrolase from the archaeon *Pyrococcus furiosus*: biochemical characterization and analysis of protein structure by comparative molecular modeling. Proteins **58:** 815–825.

Rapp, M., T. A. Haubrich, J. Perrault, Z. B. Mackey, J. H. McKerrow *et al.*, 2006 Antitrypanosomal activity of 5′-deoxy-5′-(iodomethylene)adenosine and related 6-N-cyclopropyladenosine analogues. J. Med. Chem. **49:** 2096–2102.

Rocha, P. S., M. Sheikh, R. Melchiorre, M. Fagard, S. Boutet *et al.*, 2005 The Arabidopsis *HOMOLOGY-DEPENDENT GENE SILENCING1* gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. Plant Cell **17:** 404–417.

Saze, H., O. M. Scheid and J. Paszkowski, 2003 Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat. Genet. **34:** 65–69.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Soppe, W. J., Z. Jasencakova, A. Houben, T. Kakutani, A. Meister *et al.*, 2002 DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. EMBO J. **21:** 6549–6559.

Tamaru, H., and E. U. Selker, 2001 A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. Nature **414:** 277–283.

Tanaka, H., C. Masuta, K. Uehara, J. Kataoka, A. Koiwai *et al.*, 1997 Morphological changes and hypomethylation of DNA in transgenic tobacco expressing antisense RNA of the S-adenosyl-L-homocysteine hydrolase gene. Plant Mol. Biol. **35:** 981–986.

Turner, M. A., C. S. Yuan, R. T. Borchardt, M. S. Hershfield, G. D. Smith *et al.*, 1998 Structure determination of selenomethionyl S-adenosylhomocysteine hydrolase using data at a single wavelength. Nat. Struct. Biol. **5:** 369–376.

Vongs, A., T. Kakutani, R. A. Martienssen and E. J. Richards, 1993 *Arabidopsis thaliana* DNA methylation mutants. Science **260:** 1926–1928.

Walsh, C. P., J. R. Chaillet and T. H. Bestor, 1998 Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nat. Genet. **20:** 116–117.

Wolfson, G., J. Chisholm, A. H. Tashjian, Jr., S. Fish and R. H. Abeles, 1986 Neplanocin A. Actions on S-adenosylhomocysteine hydrolase and on hormone synthesis by GH4C1 cells. J. Biol. Chem. **261:** 4492–4498.

Xiao, W., K. D. Custard, R. C. Brown, B. E. Lemmon, J. J. Harada *et al.*, 2006 DNA methylation is critical for *Arabidopsis* embryogenesis and seed viability. Plant Cell **18:** 805–814.

Xin, Z., M. Tachibana, M. Guggiari, E. Heard, Y. Shinkai *et al.*, 2003 Role of histone methyltransferase G9a in CpG methylation of the Prader-Willi syndrome imprinting center. J. Biol. Chem. **278:** 14996–15000.

Xu, G. L., T. H. Bestor, D. Bourc'his, C. L. Hsieh, N. Tommerup *et al.*, 1999 Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature **402:** 187–191.

Yoder, J. A., C. P. Walsh and T. H. Bestor, 1997 Cytosine methylation and the ecology of intragenomic parasites. Trends Genet. **13:** 335–340.

Communicating editor: J. A. Birchler