A group of SUVH methyl-DNA binding proteins regulate expression of the DNA demethylase ROS1 in Arabidopsis

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Abstract DNA methylation is typically regarded as a repressive epigenetic marker for gene expression. Genome-wide DNA methylation patterns in plants are dynamically regulated by the opposing activities of DNA methylation and demethylation reactions. In Arabidopsis, a DNA methylation monitoring sequence (MEMS) in the promoter of the DNA demethylase gene ROS1 functions as a methylstat that senses these opposing activities and regulates genome DNA methylation levels by adjusting ROS1 expression. How DNA methylation in the MEMS region promotes ROS1 expression is not known. Here, we show that several Su(var)3-9 homologs (SUVHs) can sense DNA methylation levels at the MEMS region and function redundantly to promote ROS1 expression. The SUVHs bind to the MEMS region, and the extent of binding is correlated with the methylation level of the MEMS. Mutations in the SUVHs lead to decreased ROS1 expression, causing DNA hypermethylation at more than 1,000 genomic regions. Thus, the SUVHs function to mediate the activation of gene transcription by DNA methylation.

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INTRODUCTION

5'-methyl-cytosine (5-mC) is a conserved epigenetic marker in mammals and plants (He et al. 2011; Zhang et al. 2018). It plays important roles in the regulation of gene expression, gene imprinting and genome stability (Chinnusamy et al. 2008; Zhu 2009; He et al. 2011; Li et al. 2017; Wei et al. 2017). In plants, de novo DNA methylation is catalyzed by Domains Rearranged Methyltransferase 2, a homolog of mammalian DNMT3, and is targeted by RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen 2010; Haag and Pikaard 2011; Matzke and Mosher 2014; Matzke et al. 2015). Once established, DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3) maintain CG and CHG (where H is A, T or C) (Lindroth et al. 2001; Saze et al. 2003) methylation levels, respectively, by copying DNA methylation patterns to the daughter strands after DNA replication. The asymmetrical CHH methylation can be maintained by CMT2 or by DRM2 through the RdDM pathway. DNA methylation can be lost passively due to compromised maintenance mechanisms or actively removed by a family of 5-mC DNA glycosylase/lyases, including ROS1, DME, DML2 and DML3 (Zhu 2009).

The SU(VAR)3-9 HOMOLOG (SUVH) family has 10 members in Arabidopsis, among which KRYPTONITE (KYP or SUVH4), SUVH5 and SUVH6 are best characterized as histone H3K9 methylases (Law and Jacobsen 2010; Li et al. 2018). SUVH proteins have a SET and RING finger-associated (SRA) domain and a pre-SET/SET/post-SET domain cassette. SUVH4, SUVH5 and SUVH6 bind
methylated CHG methylation with their SRA domain, and methylate H3K9 with the pre-SET/SET/post-SET domain. The H3K9me2 marker can be recognized by CMT3 that methylates CHG, thus forming a self-reinforcing feedback loop. SUVH2 and SUVH9 lack the thumb loop which is critical for the 5-mC flipping mechanism of methyltransferases and have no methyltransferase activity (Li et al. 2018). Nevertheless, they can still bind to methylated DNA and recruit RNA polymerase V to generate scaffold RNA in the RdDM pathway (Johnson et al. 2014; Liu et al. 2014). Recently, SUVH1 was identified as an anti-silencing factor that may function downstream of DNA methylation to promote the expression of genes with methylated promoters (Li et al. 2016). The functions of other SUVH family proteins are still not known.

Typically, methyl DNA readers recognize DNA methylation and recruit histone modifiers and chromatin remodelers to silence gene expression. ROS1 expression is regulated by DNA methylation at the DNA methylation monitoring sequence (MEMS) in its promoter, which is targeted by DNA methylation including RdDM and by ROS1-mediated active DNA demethylation (Lei et al. 2015; Williams et al. 2015). Unlike most other DNA methylation-regulated genes, ROS1 expression is positively correlated with the level of DNA methylation at the MEMS region. Therefore, there must be factor(s) linking DNA methylation and transcriptional activation at this locus. In this study, we identified SUVH1, SUVH3, SUVH7 and SUVH8 as DNA methylation interpreters, which bind to the MEMS region in a DNA methylation-dependent manner, and associate with DnaJ domain putative transcriptional activators, to promote ROS1 expression. Thus, our study unraveled an important mechanism wherein the SUVHs recognize DNA methylation marks to promote ROS1 gene expression, which in turn regulates genome-wide DNA methylation patterns.

RESULTS

SUVH1 and SUVH3 function redundantly to prevent gene silencing

In the SUVH family, SUVH2 and SUVH9 are involved in the RdDM pathway, while SUVH4, SUVH5 and SUVH6 methylate H3K9, enforcing CHG methylation (Law and Jacobsen 2010; Johnson et al. 2014; Liu et al. 2016; Li et al. 2018). To determine whether the other SUVH proteins may also be involved in epigenetic regulation, we mutated SUVH1 and SUVH3 with clustered regularly interspaced short palindromic repeats (CRISPR) — CRISPR-associated protein 9 (Cas9) in the 35S-SUC2 transgenic background (Figure S1). In this system, sucrose transporter 2 (SUC2) is overexpressed under the constitutive cauliflower mosaic virus 35S promoter (35S-SUC2) (Figure 1B). The transgenic plants with SUC2 expression would take up sucrose in excess from sucrose-containing media, resulting in severe growth inhibition; mutants with silenced 35S-SUC2 do not show such growth inhibition. Using this genetic system, we identified a number of cellular factors that regulate the active DNA demethylation pathway, including components of the RdDM pathway (Lei et al. 2014; Qian et al. 2014; Duan et al. 2015; Lei et al. 2015; Duan et al. 2017). Mutations in SUVH1 resulted in a partial reduction in the growth inhibition on sucrose-containing media (Figure 1A). Quantitative real-time polymerase chain reaction (PCR) analysis showed that the transcript levels of both the 35S-SUC2 and 2 × 35S-HPTII transgenes were decreased in the suvh1 mutants (Figure 1C). The result is consistent with a recent report showing that a 2 × 35S-LUC transgene was silenced in suvh1 mutants (Li et al. 2016). When both SUVH1 and SUVH3 were mutated, the expression of 35S-SUC2 and 2 × 35S-HPTII was further reduced and the double mutant exhibited even less growth inhibition than the 35S-SUC2 wild-type plants (Figure 1B, C). The expression of the methylated endogenous genes AT1G52040, AT1G54040 and AT3G14210 (Li et al. 2016) was reduced in suvh1, and the expression of AtiG54040 was even less in the suvh1 suvh3 double mutant, compared to that in the wild type (Figure 1D). These results suggest that SUVH1 and SUVH3 function redundantly in the prevention of gene silencing.

SUVH1, SUVH3, SUVH7 and SUVH8 prevent DNA hypermethylation

To characterize the potential involvement of SUVH1 and SUVH3 in DNA methylation, we used chop-PCR to examine the DNA methylation status at the 3′ region of AT1G26400 and the promoter of AT4G18650, which are known to be hypermethylated in DNA demethylation mutants such as rosi and idmi (Qian et al. 2012). We found that both loci were mildly hypermethylated in the suvh1 single mutant and the methylation levels were
higher in the suvh1 suvh3 double mutant than in the suvh1 single mutant (Figure S2). We also mutated SUVH3 in a suvh1 T-DNA insertion mutant to generate a suvh1 suvh3 double mutant in a background without the 35S-SUC2 transgene (Figure S1). Our chop-PCR assay on this mutant confirmed that SUVH1 and SUVH3 prevent DNA hypermethylation (Figure 2A). Although both loci were hypermethylated in suvh1 suvh3 double mutants, the methylation level was still much lower than that in the rost-4 mutant. We mutated two more genes, SUVH7 and SUVH8, and generated a suvh1 suvh3 suvh7 suvh8 quadruple mutant. Chop-PCR results showed that the methylation level was higher in the quadruple mutant than in the double mutant (Figure 2A). Genomic bisulfite sequencing also confirmed that the 3′ region of AT1G26400 and the promoter of AT4G18650 were hypermethylated in the double and quadruple mutants, although the methylation levels were still much lower than that in rost-4 mutant plants (Figure 2B). Consistent with the notion that DNA methylation in most promoters represses gene expression, the expression of AT4G18650 was decreased in the double and quadruple mutants (Figure 2C). We compared the genome-wide DNA methylation profiles of suvh1 suvh3 double and suvh1 suvh3 suvh7 suvh8 quadruple mutants with Columbia-0 wild type. There were 746 hypermethylated genomic regions in the suvh1 suvh3 double mutant, while the numbers of hypermethylated genomic regions were 1,672 in the suvh1 suvh3 suvh7 suvh8 quadruple mutant and 3,542 in the rost-4 mutant. We also identified 713, 660 and 404 hypomethylated genomic regions in suvh1 suvh3, suvh1 suvh3 suvh7 suvh8
and ROS1-4 mutants, respectively. The hypermethylated genomic regions were distributed throughout the five chromosomes in the mutants, and overlapped with genic (48%), transposable elements (TE) (24%) and intergenic (28%) regions (Figure S3).

The SUVHs bind to the MEMS in ROS1 promoter and regulate ROS1 expression

About 67% of the 1,672 hypermethylated genomic regions in the suvh1 suvh3 suvh7 suvh8 quadruple mutant were also hypermethylated in the ROS1-4 mutant (Figure 3A). This significant overlap prompted us to explore the relationship between the SUVHs and ROS1. While ROS1 gene expression was decreased in suvh1 and suvh3 single mutants, the decrease was more pronounced in the suvh1 suvh3 double and suvh1 suvh3 suvh7 suvh8 quadruple mutants (Figure 3B). However, the expression of IDM1 and ROS3 was not affected in the mutants (Figure S4). The results indicated that SUVH1, SUVH3, SUVH7 and SUVH8 function redundantly to promote ROS1 gene expression. Previously, we reported that DNA methylation in the MEMS region in the ROS1 promoter is required for ROS1 expression (Lei et al. 2015). We hypothesized the presence of cellular factors that can recognize the methylation in MEMS and promote ROS1 transcription. As the SUVH family of proteins have an SRA domain known to bind methylated DNA, we tested whether the SUVH proteins could bind the MEMS. GST-fused SUVH3 protein was expressed and purified from Escherichia coli for

Figure 2. SUVH1, SUVH3, SUVH7 and SUVH8 prevent DNA hypermethylation
(A) Analysis of DNA methylation levels at AT1G26400 and AT4G18650 loci in Columbia-0 (Col-0), suvh1 suvh3 and suvh1 suvh3 suvh7 suvh8 quadruple mutants by chop-polymerase chain reaction (PCR). Undigested DNA was used as a control. (B) Levels of DNA methylation at AT1G26400 and AT4G18650 loci in Col-0, double, quadruple and ROS1-4 mutants as screenshots from Integrative Genomics Viewer. (C) The expression of AT1G26400 and AT4G18650 was examined in double and quadruple mutants with quantitative real-time PCR. Values are mean ± SE of three biological replicates and normalized to transcript level in Col-0 wild type. Means with different letters are significantly different at P < 0.01. (D) Chromosomal distribution of hypermethylated (blue) and hypomethylated (red) loci in double, quadruple and ROS1-4 mutants.
electrophoretic gel mobility shift assays (Figure S5). The results showed that SUVH3 could only bind to methylated MEMS (Figure 3C) and the binding was competitively blocked by unlabeled methylated MEMS (Figure 3C). To test whether SUVH3 may bind to the ROS1 promoter in vivo, we introduced FLAG-tagged SUVH3 driven by its native promoter into the suvh1 suvh3 double mutant. In the transgenic plants, ROS1 expression was de-repressed, indicating that the fusion protein was functional (Figure S6). Chromatin immunoprecipitation (ChIP) assays showed that SUVH3 was enriched at the ROS1 promoter (Figure 3D). Taken together, these results show that the SUVHs can bind to MEMS in the ROS1 promoter and stimulate ROS1 transcription.

RdDM and ROS1 regulate ROS1 expression through SUVH complex

The SUVH1, SUVH3, SUVH7 and SUVH8 proteins have an SRA domain to bind to methylated DNA but lack methyltransferase activity (Li et al. 2018). These proteins may regulate ROS1 expression by recruiting histone modifiers or chromatin remodelers to alter the chromatin states, or by recruiting transcriptional regulators to more directly control ROS1 expression. To identify these factors, we immunoprecipitated (IP) SUVH3-3xFlag with anti-Flag antibody, and analyzed the IP product using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Three DnaJ-domain proteins were co-precipitated with SUVH3 in pSUVH3::SUVH3-3xFlag transgenic plants, but not in
pHD2C::HD2C-3XFlag control plants (Figure 4A). These three DnaJ-domain proteins were also pulled down, along with SUVH3 and SUVH1 in a DNA-trap experiment using methylated MEMS as a probe, with unmethylated MEMS as a control (Figure 4B). During the preparation of this manuscript, Harris and colleagues also identified SUVH1, SUVH3 and DnaJ proteins as components of a DNA methylation reader complex that activates gene expression (Harris et al. 2018). It is interesting to note that in this issue of the Journal, Zhao and colleagues show SUVH1 and SUVH3 function synergistically with these DnaJ proteins to prevent promoter methylated genes from silencing (Zhao et al. 2019).

Methylation through RdDM and demethylation through ROS1 in the MEMS region in ROS1 promoter regulates ROS1 expression (Lei et al. 2015; Williams et al. 2015). We observed a decrease in the enrichment of SUVH3 at the MEMS in the nrpd1-3 mutant where ROS1 gene expression was compromised. On the other hand, SUVH3 binding to the MEMS was enhanced in the ros1-4 mutant where ROS1 gene expression was increased. (Figures 4C, S5B). We examined SUVH3 transcript and protein levels in

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**Figure 4.** RNA-directed DNA methylation (RdDM) and DNA demethylation pathways regulate ROS1 expression through the SUVH complex

(A) Detection of SUVH3-associated proteins in vivo by immunoprecipitation (IP) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). (B) Identification of methylation monitoring sequence (MEMS)-binding proteins using affinity trapping. Biotin-labeled methylated MEMS was incubated with protein extract from Columbia-0 (Col-0) wild type and binding proteins were analyzed by LC-MS/MS. (C) Association of SUVH3-3xFlag protein with MEMS region as revealed by chromatin IP-quantitative polymerase chain reaction in nrpd1-3 and ros1-4 mutants. Values are mean ± SE of three biological replicates. Means with different letters are significantly different at P < 0.01. (D) The proposed methylstat model. RdDM pathway methylates loci genome-wide, including MEMS. With the increase of DNA methylation, SUVH proteins interpret the MEMS methylation and bring transcriptional activator proteins to the ROS1 promoter and activate ROS1 gene expression. Increased active demethylation activity erases DNA methylation. Decrease in DNA methylation causes MEMS hypomethylation which compromises the binding of the SUVH complex and subsequently decreases ROS1 gene expression. Thus, the plant cells can maintain DNA methylation homeostasis through this dynamic regulation.
DISCUSSION

DNA methylation can be recognized by the methyl-CpG binding domain (MBD) or SRA domain. Generally, MBD proteins bind to the symmetrically methylated CpGs and recruit a variety of histone deacetylases and chromatin remodelers to compact chromatin resulting in transcriptional gene silencing (Ng et al. 1999). However, MBD7 in Arabidopsis recognizes highly methylated CpGs and tethers the histone acetyltransferase IDM1 and its associated proteins to facilitate DNA demethylation and prevent gene silencing (Lang et al. 2015; Wang et al. 2015). Similarly, all SUVH proteins have an SRA domain which binds to methylated DNA, but their functions are dependent on the pre-SET/SET/post-SET domain. Pre-SET/SET/post-SET domains in SUVH4, SUVH5 and SUVH6 have methyltransferase activities and catalyze H3K9 methylation, which reinforces CHG methylation. In contrast, pre-SET/SET/post-SET domains in SUVH2 and SUVH9 have no methyltransferase activity, but interact with the DDR complex and mediate Pol V recruitment in the RdDM pathway (Johnson et al. 2014; Liu et al. 2016). They also interact with MORC proteins to recruit a chromatin-remodeling complex to condense chromatin (Jing et al. 2016). Our work here showed that SUVH1, SUVH3, SUVH7 and SUVH8 have critical roles in anti-silencing. They function redundantly to recognize DNA methylation in the MEMS in the ROS1 promoter and perhaps in other genomic regions as well, and recruit transcriptional regulators such as the DnaJ domain proteins to activate transcription. The MEMS in ROS1 promoter acts as a methylstat that senses both DNA methylation and demethylation activities. RdDM increases MEMS methylation, ensuring the recruitment of the SUVH complex to activate ROS1 transcription. ROS1 causes the demethylation of MEMS, and the decrease in methylation prevents SUVH binding to MEMS, leading to reduced ROS1 transcription, thus inhibiting active demethylation (Figure 4D).

MATERIALS AND METHODS

Plant materials and mutant generation
All plants used in this study were Arabidopsis in Columbia-0 (Col-0) genetic background. A 35S-SUC2 transgene line, which contains the CaMV 35S promoter-driven sucrose transporter 2 and 2 × 35S promoter-driven HPTII transgenes in Col-0 genetic background, was reported previously for screening anti-silencing factors (Lei et al. 2014; Lang et al. 2015). To check the anti-silencing phenotype, suvh1 single and suvh1 suvh3 double mutants were generated in 35S-SUC2 background through the CRISPR/Cas9 methods as described (Zhang et al. 2016). The root phenotype of wild type, suvh1 and suvh1 suvh3 in 35S-SUC2 background was observed on 1/2 strength Murashige and Skoog (MS) solid medium plates with 0.5% or 1% (wt/vol) sucrose. As a control and to avoid the side effects of sucrose accumulation, sucrose was replaced with 1% glucose. After 2 d of stratification at 4 °C, plates were moved to a growth chamber at 22 °C with 100 μmol/m2/s for 7 d. To establish the function of SUVHs in Col-0 background, we used the CRISPR-Cas9 system (Zhang et al. 2016) to generate the suvh3 mutant in Col-0 plants and the double suvh1 suvh3 and quadruple mutants suvh1 suvh3 suvh7 suvh8 in suvh1 (SALK_003675), which was in the Col-0 background (without 35S-SUC2 and 35S-HPTII transgene). Seeds from T2 or T3 plants that contain various homozygous suvh mutations and no Cas9 transgene were used. T-DNA mutant suvh1 (SALK_003675), suvh7 (GK-037C06-015009) and suvh8 (SAIL_713_A11) were ordered from the Arabidopsis Biological Resource Center.

Plasmid construction and mutant complementation
SUVH3 genomic DNA with about 2 kb promoter region was amplified from Col-0 genomic DNA and cloned into the pENTR/D-TOPO entry vector (Invitrogen). After confirmation by sequencing, the genomic DNA was recombined into the pEarly302 binary vector with C-terminal FLAG tag (Earley et al. 2006) using LR Clonase (Invitrogen). Agrobacterium strain GV3101 carrying various constructs was used to transform the Col-0 and suvh1 suvh3 mutant by the standard floral dip method (Clough and Bent 1998).

RNA analysis and chop-PCR
Total RNA was extracted from 12-d-old seedlings using the RNeasy Plant Kit (Qiagen). An aliquot of 1 μg of total
RNA was used for cDNA synthesis with a final volume of 20 μL using a Reversal Transcription Reagent Kit (TaKaRa). Quantitative real-time PCR was performed using the cDNAs as templates and running in a CFX96 real-time PCR detection system (Bio-RAD). ACT2 gene was used as an internal control. The primers used in quantitative PCR are listed in Table S1.

Genomic DNA was extracted from 12-d-old whole seedlings using DNeasy Plant Kit (Qiagen). Twenty nanograms of DNA were digested by 0.2 μL methylation-sensitive enzymes HhaI (NEB) or 0.2 μL H2O (control) in 20 μL reactions for 12 h, and 1 μL of reaction mixture was used as templates for PCR amplification. The methylation levels of target genes were visualized through agarose gel electrophoresis.

Whole-genome bisulfite sequencing and data analysis
Twelve-d-old seedlings of Col-0, suvh1 suvh3, suvh1 suvh3 suvh7 suvh8 and ros1-4 were collected for extraction of genomic DNA. Bisulfite conversion, library construction, and deep sequencing were performed by the Genomic Platform of Shanghai Center for Plant Stress Biology (PSC), Chinese Academy of Science (CAS). DMRs were identified as described (Lang et al. 2015). The raw data of bisulfite sequencing of Col-0, suvh1 suvh3 double, suvh1 suvh3 suvh7 suvh8 quadruple and ros1-4 mutants have been deposited in the GEO database (accession no. GSE124635).

Electrophoretic mobility shift assay (EMSA)
Truncated SUVH3 (containing SRA and SET domain) was cloned into pGEX-4T-1 (GE Healthcare) for fusion expression with a GST tag. Recombinant proteins or GST protein (control) produced in E. coli BL21 were purified using Glutathione Sepharose 4 Fast Flow (GE Healthcare). 5mC methylated or non-methylated MEMS DNA fragments were amplified using biotin-labeled primers by adding d5mCTP or dCTP. Methylated MEMS amplified with non-biotin-labeled primers was used as a competitor probe. The PCR products were purified through agarose gel electrophoresis and collected using a gel extraction kit (OMEGA). EMSA was performed with a LightShift Chemiluminescent EMSA Kit (Pierce). Migration of biotin-labeled probes was detected using ECL (Pierce) and ChemDoc imaging system (Bio-Rad). All of the primers used are listed in Table S1.

ChIP assay
The ChIP assay was performed as described (Wierzbicki et al. 2008). Dynabeads (Invitrogen, 10004D) were used for pre-clearing samples and antibody binding. Antibodies used in ChIP were a commercial anti-FLAG antibody (Sigma, F1804). ChIP products were diluted with 50 μL of ddH2O, and 1 μL product was used for each quantitative PCR reaction.

DNA affinity trapping
DNA affinity trapping was performed as previously described (Shaikhali et al. 2012). DNA fragment from −85 to +29 of ROS1 gene was used to isolate MEMS binding proteins. Biotinylated fragment was generated by PCR with primers: MEMS-F, biotin-5′-CTAGGAGATTGTAGAAAAG-3′ and MEMS-R, 5′-CAC-TATTTTCTACAACCGTTTGTTT-3′. To get the methylated fragment, dCTP was substituted to d5mCTP in the PCR reaction mix.

Immunoprecipitate-mass spectrometry (IP-MS)
To find the interacting protein, IP-MS was performed as described (Lang et al. 2015b). The transgenic line that contains the SUVH3 gene with FLAG tag driven by native promoter in Col-0 background was used for protein IP followed by MS performed by the Proteomics platform of Shanghai Center for Plant Stress Biology (PSC), CAS. Col-0 plant was used as a negative control. FLAG antibody pre-connecting agarose beads (Sigma) were used for enrichment of SUVH3-FLAG and candidate interacting proteins.

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AUTHOR CONTRIBUTIONS
M.L. and X.X. designed the experiments. X.X. performed most of the experiments and analyzed the data with M.L. Other authors assisted in experiments and discussed the results. X.X., V.S., J.K.Z. and M.L wrote the manuscript.
REFERENCES


The CRYPTOCHROME1-dependent response to excess light is mediated through the transcriptional activators ZINC FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM LIKE1 and ZMML2 in *Arabidopsis*. *Plant Cell* 24: 3009–3025


The black boxes marked the NGG or reverse comple-

**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12768/suppinfo

**Figure S1.** Schematic diagram of various suvh mutants generated with CRISPR-cas9 systems

(A) Diagram showing the mutation sites (single base or T-DNA insertion) located in 5′ end of CDS for each SUVH allele. (B) DNA sequencing results show that the single base insertion of SUVH1 or/and SUVH3 gene in 35S-SUC2 background. (C) Double mutant suvh1/3 in Col-0 background was generated in suvh1 (SALK_003675) by single base insertion in exon of SUVH3 gene. (D) Quadruple mutant suvh1/3/7/8 in Col-0 background was generated in suvh1 (SALK_003675) by single base insertion in exon of SUVH3, SUVH7 and SUVH8 gene. The black boxes marked the NGG or reverse complement CCN. The black arrows marked the base insertion. The asterisks marked the artificially altered base in PCR product for generating a restriction enzyme cutting site.

**Figure S2.** SUVH1 and SUVH3 function redundantly to prevent DNA hypermethylation

DNA methylation level at AT1G26400 and AT4G18650 loci in WT, suvh1, suvh1/3, ros1-11 were detected by chomp-PCR. Genomic DNA was digested by the DNA methylation-sensitive restriction enzymes HhaI. Undigested DNA was used as a control. These four genotypes are all in 35S-SUC2 background.

**Figure S3.** Composition of the hypermethylated (upper) and hypomethylated (lower) loci in suvh1 suvh3 double, suvh1 suvh3 suvh7 suvh8 and ros1-4 mutants

**Figure S4.** The effect of suvh1/3 and suvh1/3/7/8 on the gene IDM1, ROS1, and ROS3 expression

The RNA transcripts of IDM1, ROS1, and ROS3 were detected by RT-qPCR in Col-0, suvh1/3, and suvh1/3/7/8. ACT2 was amplified as an reference gene. Values are mean ± SE of three biological replicates and normalized to transcript level in Col-o wild type. Means with different letters are significantly different at *P* < 0.01.

**Figure S6.** Depressed ROS1 gene expression in suvh1/3

was rescued by native promoter driven SUVH3:FLAG

The seedlings of Col-o, suvh1, suvh1/3 and three homozygous transgene lines which transform SUVH3- pro:SUH3:FLAG to suvh1/3 were used to detect ROS1 gene expression by RT-qPCR. Values are mean ± SE of three biological replicates and normalized to transcript level in Col-o wild type. Means with different letters are significantly different at *P* < 0.01.

**Figure S7.** RNA transcript level and protein levels of SUVH1 and SUVH3 were not induced in the mutant nrpd1-3 and ros1-4 with different DNA methylation levels

(A) RNA relative transcript level in Col-o, nrpd1-3, nrpe1-11, and ros1-4 were detected by RT-qPCR. ROS1 gene which was induced in RdDM mutants was used as a positive control. Values are mean ± SE of three biological replicates and normalized to transcript level in Col-o wild type. Means with different letters are significantly different at *P* < 0.01. (B) SUVH3 protein levels in Col-o, nrpd1-3, and ros1-4 were detected by Western blot. α-tubulin was used as a protein loading control.

**Table S1.** List of primers used in this study

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