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

High-Impact Article

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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 $2 \times$ *35S-HPTII* transgenes were decreased in the *suvh1* mutants (Figure 1C). The result is consistent with a recent report showing that a $2 \times$ *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 $2 \times$ *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 At1G54040 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 *ros1* and *idm1* (Qian et al. 2012). We found that both loci were mildly hypermethylated in the *suvh1* single mutant and the methylation levels were

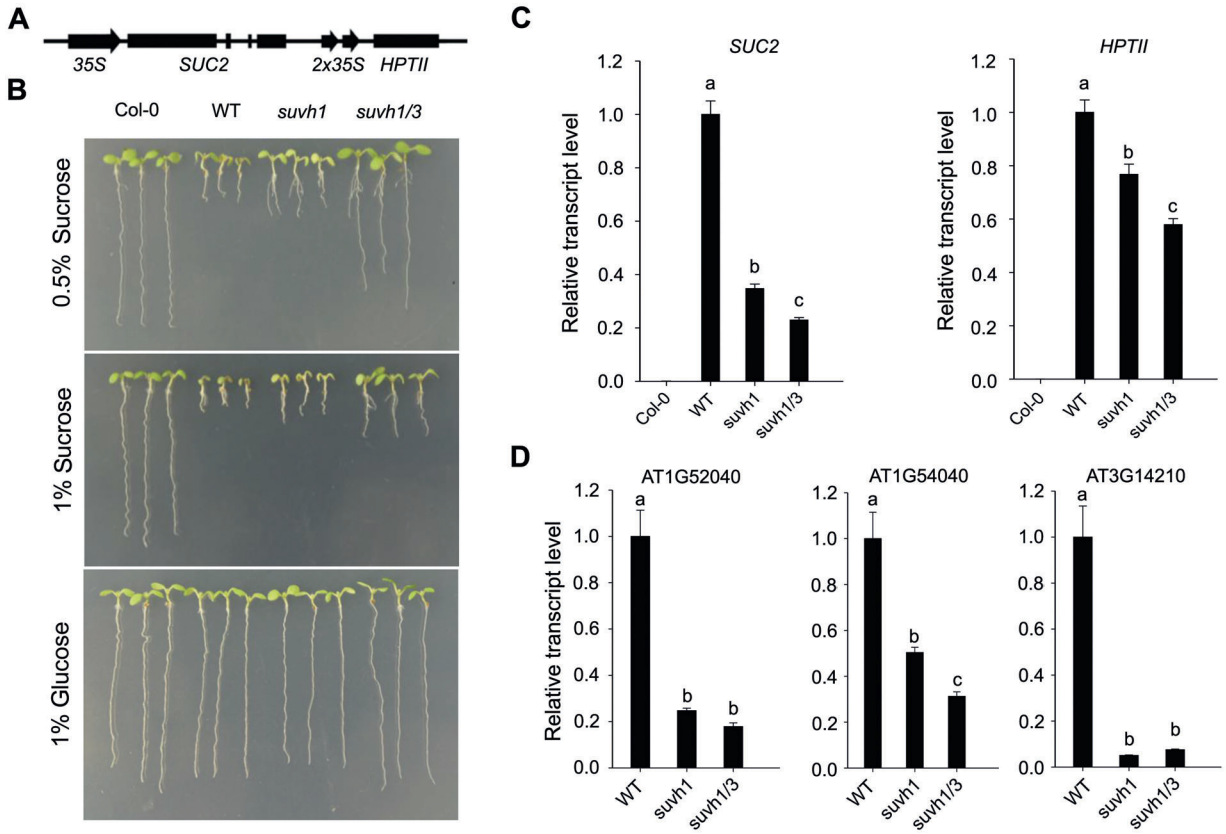

Figure 1. SUVH1 and SUVH3 function redundantly to prevent gene silencing

(**A**) Schematic diagram of the transgenes in *35S-SUC2* background. (**B**) Comparison of seedling phenotype of Columbia-0 (Col-0), *35S-SUC2* wild type (WT), *suvh1* and *suvh1 suvh3* mutants grown on Murashige and Skoog (MS) medium supplemented with 0.5% sucrose, 1% sucrose or 1% glucose. Expression of the *SUC2* and *HPTII* transgenes (**C**) and three endogenous genes (**D**) were examined in *suvh1* single and *suvh1 suvh3* double mutants grown on MS medium supplemented with 1% glucose with quantitative real-time polymerase chain reaction. Values are mean ± *SE* of three biological replicates and normalized to transcript level in *35S-SUC2* wild type. Means with different letters are significantly different at $P < 0.01$.

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 *ros1-4* 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 *ros1-4* 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 *suv1 suvh3 suvh7 suvh8* quadruple mutant and 3,542 in the *ros1-4* mutant. We also identified 713, 660 and 404 hypomethylated genomic regions in *suvh1 suvh3*, *suv1 suvh3 suvh7 suvh8*

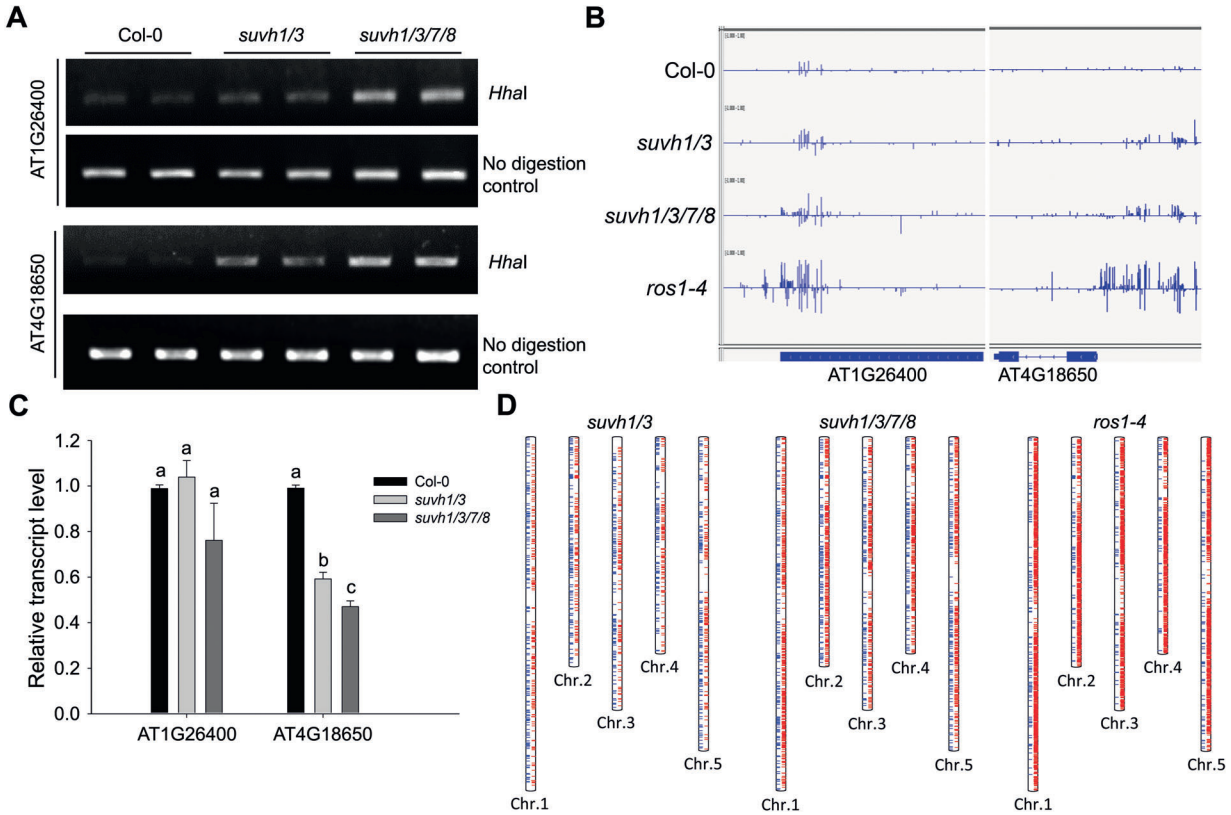

Figure 2. SUVH1, SUVH3, SUVH7 and SUVH8 prevent DNA hypermethylation

(**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.

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

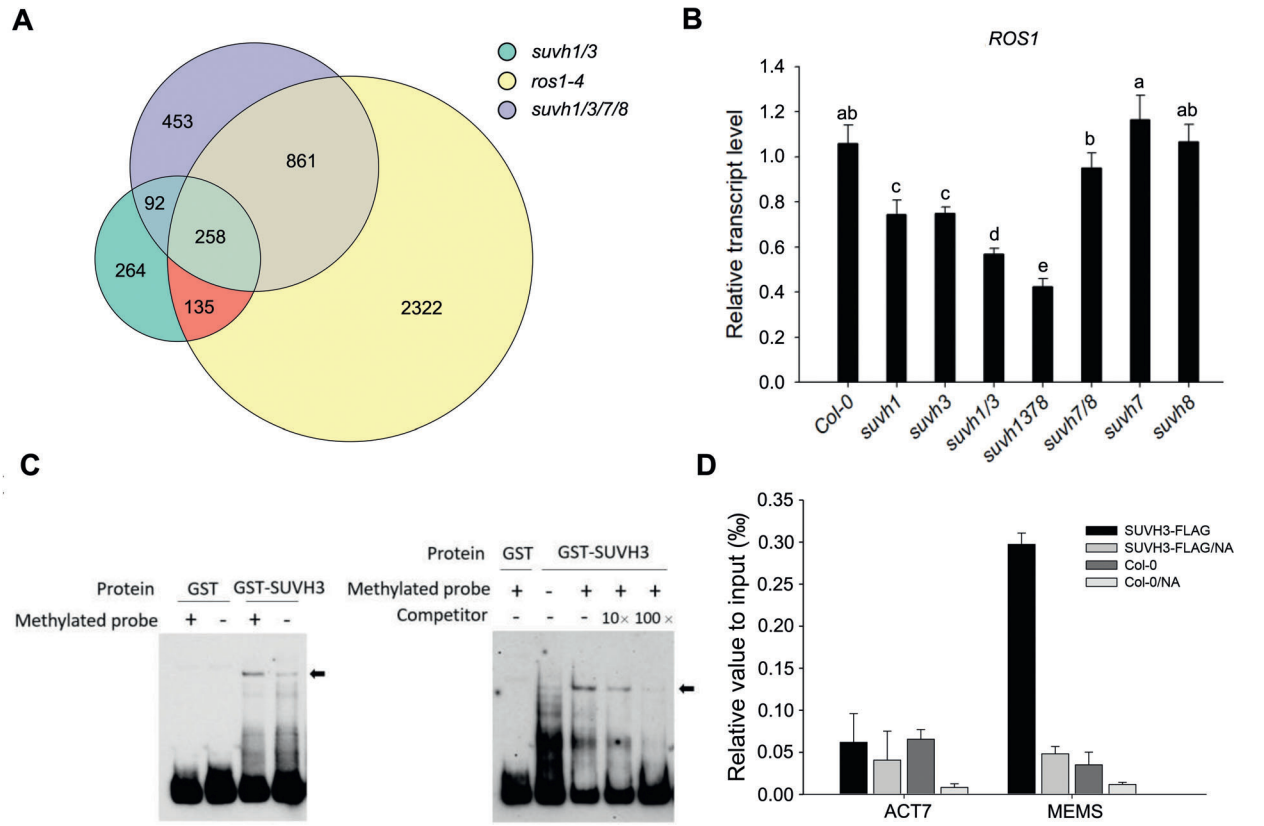

Figure 3. SUVH bind to *ROS1* promoter to regulate its expression

(**A**) Venn diagram showing the number of overlapping loci among the *suvh1 suvh3* double, *suvh1 suvh3 suvh7 suvh8* quadruple and *ros1-4* mutants. (**B**) *ROS1* gene expression was examined in *suvh* single, double and quadruple mutants with quantitative real-time polymerase chain reaction (PCR). Values are mean ± *SE* of three biological replicates and normalized to transcript level in Columbia-0 (Col-0) wild type. Means with different letters are significantly different at $P < 0.01$. (**C**) Electrophoretic mobility shift assay (EMSA) of SUVH3 binding to methylated methylation monitoring sequence (MEMS). (**D**) Association of SUVH3 protein with *ROS1* promoter as revealed by chromatin immunoprecipitation (ChIP) – quantitative PCR in *SUVH3-3xFlag* transgenic plants with Col-0 as a control. no Ab: no antibody.

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

A

SUVH3 Purifcation

Gene ID	Protein	Score	Coverage	Unique Peptides
AT1G73100	SUVH3	187	61	30
AT2G01710	DnaJ-domain protein	12	14	4
AT1G62970	DnaJ-domain protein	3	2	1
AT5G64360	DnaJ-domain protein	1	1	1

B

MEMS DNA Affinity trapping

gene ID	protein	Score	Coverage	Unique Peptides
AT1G73100	SUVH3	127	44	28
AT2G01710	DnaJ-domain protein	25	31	7
AT5G04940	SUVH1	15	5	3
AT1G62970	DnaJ-domain protein	10	9	4
AT5G64360	DnaJ-domain protein	6	8	2

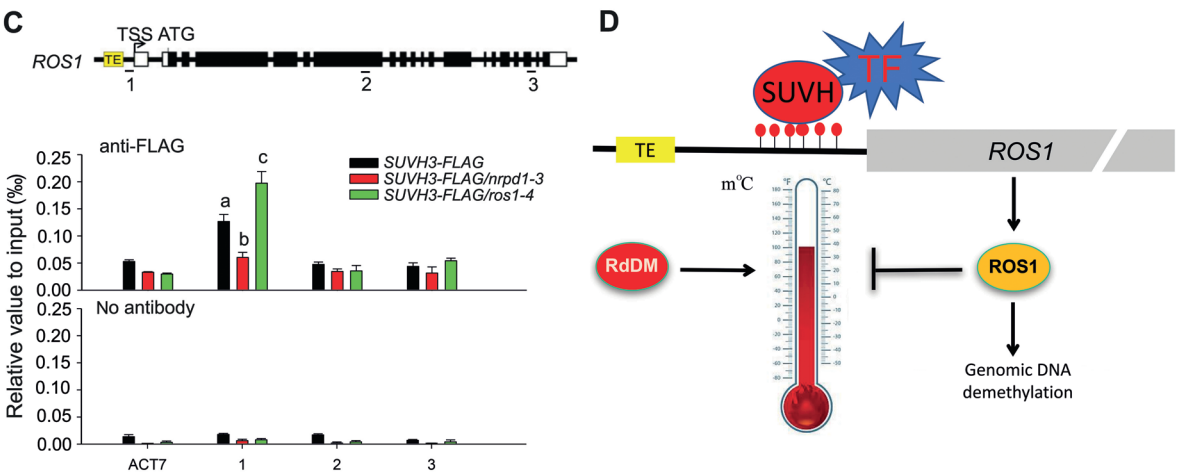

Figure 4. RNA-directed DNA methylation (RdDM) and DNA demethylation pathways regulate *ROS1* expression through the SUVH complex

(**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.

nrpd1-3 and *ros1-4* mutants and found no changes compared to those in the wild type (Figure S7A, B). These results suggest that both the RdDM and active DNA demethylation pathways can regulate SUVH3 binding to MEMS.

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/m^2/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 *Hha*I (NEB) or 0.2 μL H_2O (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 d^{5m}CTP 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′-CTAGGAGATTTGTAGAAAAG-3′ and MEMS-R, 5′-CACTATTTTTCATAACCGTTTGTT-3′. To get the methylated fragment, dCTP was substituted to d^{5m}CTP 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

Chinnusamy V, Gong Z, Zhu JK (2008) Abscisic acid-mediated epigenetic processes in plant development and stress responses. **J Integr Plant Biol** 50: 1187–1195

Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. **Plant J** 16: 735–743

Duan CG, Wang X, Tang K, Zhang H, Mangrauthia SK, Lei M, Hsu CC, Hou YJ, Wang C, Li Y, Tao WA, Zhu JK (2015) MET18 Connects the Cytosolic Iron-Sulfur Cluster Assembly Pathway to Active DNA Demethylation in *Arabidopsis*. **PLoS Genet** 11: e1005559

Duan CG, Wang X, Xie S, Pan L, Miki D, Tang K, Hsu CC, Lei M, Zhong Y, Hou YJ, Wang Z, Zhang Z, Mangrauthia SK, Xu H, Zhang H, Dilkes B, Tao WA, Zhu JK (2017) A pair of transposon-derived proteins function in a histone acetyltransferase complex for active DNA demethylation. **Cell Res** 27: 226–240

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. **Plant J** 45: 616–629

Haag JR, Pikaard CS (2011) Multisubunit RNA polymerases IV and V: Purveyors of non-coding RNA for plant gene silencing. **Nat Rev Mol Cell Biol** 12: 483–492

Harris CJ, Scheibe M, Wongpalee SP, Liu W, Cornett EM, Vaughan RM, Li X, Chen W, Xue Y, Zhong Z, Yen L, Barshop WD, Rayatpisheh S, Gallego-Bartolome J, Groth M, Wang Z, Wohlschlegel JA, Du J, Rothbart SB, Butter F, Jacobsen SE (2018) A DNA methylation reader complex that enhances gene transcription. **Science** 362: 1182–1186

He XJ, Chen T, Zhu JK (2011) Regulation and function of DNA methylation in plants and animals. **Cell Res** 21: 442–465

Jing Y, Sun H, Yuan W, Wang Y, Li Q, Liu Y, Li Y, Qian W (2016) SUVH2 and SUVH9 couple two essential steps for transcriptional gene silencing in *Arabidopsis*. **Mol Plant** 9: 1156–1167

Johnson LM, Du J, Hale CJ, Bischof S, Feng S, Chodavarapu RK, Zhong X, Marson G, Pellegrini M, Segal DJ, Patel DJ, Jacobsen SE (2014) SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. **Nature** 507: 124–128

Lang Z, Lei M, Wang X, Tang K, Miki D, Zhang H, Mangrauthia SK, Liu W, Nie W, Ma G, Yan J, Duan CG, Hsu CC, Wang C, Tao WA, Gong Z, Zhu JK (2015) The methyl-CpG-binding protein MBD7 facilitates active DNA demethylation to limit DNA hyper-methylation and transcriptional gene silencing. **Mol Cell** 57: 971–983

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. **Nat Rev Genet** 11: 204–220

Lei M, La H, Lu K, Wang P, Miki D, Ren Z, Duan CG, Wang X, Tang K, Zeng L, Yang L, Zhang H, Nie W, Liu P, Zhou J, Liu R, Zhong Y, Liu D, Zhu JK (2014) *Arabidopsis* EDM2 promotes IBM1 distal polyadenylation and regulates genome DNA methylation patterns. **Proc Natl Acad Sci USA** 111: 527–532

Lei M, Zhang H, Julian R, Tang K, Xie S, Zhu JK (2015) Regulatory link between DNA methylation and active demethylation in *Arabidopsis*. **Proc Natl Acad Sci USA** 112: 3553–3557

Li L, Wu W, Zhao Y, Zheng B (2017) A reciprocal inhibition between ARID1 and MET1 in male and female gametes in *Arabidopsis*. **J Integr Plant Biol** 59: 657–668

Li S, Liu L, Gao L, Zhao Y, Kim YJ, Chen X (2016) SUVH1, a Su(var)3-9 family member, promotes the expression of genes targeted by DNA methylation. **Nucleic Acids Res** 44: 608–620

Li X, Harris CJ, Zhong Z, Chen W, Liu R, Jia B, Wang Z, Li S, Jacobsen SE, Du J (2018) Mechanistic insights into plant SUVH family H3K9 methyltransferases and their binding to context-biased non-CG DNA methylation. **Proc Natl Acad Sci USA** 115: E8793–E8802

Lindroth AM, Cao X, Jackson JP, Zilberman D, McCallum CM, Henikoff S, Jacobsen SE (2001) Requirement of CHROMOMETHYLASE3 for maintenance of CpXpG methylation. **Science** 292: 2077–2080

Liu ZW, Shao CR, Zhang CJ, Zhou JX, Zhang SW, Li L, Chen S, Huang HW, Cai T, He XJ (2014) The SET domain proteins SUVH2 and SUVH9 are required for Pol V occupancy at RNA-directed DNA methylation loci. **PLoS Genet** 10: e1003948

Liu ZW, Zhou JX, Huang HW, Li YQ, Shao CR, Li L, Cai T, Chen S, He XJ (2016) Two components of the RNA-Directed DNA methylation pathway associate with MORC6 and silence loci targeted by MORC6 in *Arabidopsis*. **PLoS Genet** 12: e1006026

Matzke MA, Kanno T, Matzke AJ (2015) RNA-directed DNA methylation: The evolution of a complex epigenetic pathway in flowering plants. **Annu Rev Plant Biol** 66: 243–267

Matzke MA, Mosher RA (2014) RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. **Nat Rev Genet** 15: 394–408

Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempst P, Reinberg D, Bird A (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. **Nat Genet** 23: 58–61

Qian W, Miki D, Lei M, Zhu X, Zhang H, Liu Y, Li Y, Lang Z, Wang J, Tang K, Liu R, Zhu JK (2014) Regulation of active DNA demethylation by an alpha-crystallin domain protein in *Arabidopsis*. **Mol Cell** 55: 361–371

Qian W, Miki D, Zhang H, Liu Y, Zhang X, Tang K, Kan Y, La H, Li X, Li S, Zhu X, Shi X, Zhang K, Pontes O, Chen X, Liu R, Gong Z, Zhu JK (2012) A histone acetyltransferase regulates active DNA demethylation in *Arabidopsis*. **Science** 336: 1445–1448

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

Shaikhali J, de Dios Barajas-Lopez J, Otvos K, Kremnev D, Garcia AS, Srivastava V, Wingsle G, Bako L, Strand A (2012)

The CRYPTOCHROME1-dependent response to excess light is mediated through the transcriptional activators ZINC FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM LIKE1 and ZML2 in *Arabidopsis*. **Plant Cell** 24: 3009–3025

Wang C, Dong X, Jin D, Zhao Y, Xie S, Li X, He X, Lang Z, Lai J, Zhu JK, Gong Z (2015) Methyl-CpG-binding domain protein MBD7 is required for active DNA demethylation in *Arabidopsis*. **Plant Physiol** 167: 905–914

Wei X, Song X, Wei L, Tang S, Sun J, Hu P, Cao X (2017) An epiallele of rice AK1 affects photosynthetic capacity. **J Integr Plant Biol** 59: 158–163

Wierzbicki AT, Haag JR, Pikaard CS (2008) Noncoding transcription by RNA polymerase Pol IVb/Pol V mediates transcriptional silencing of overlapping and adjacent genes. **Cell** 135: 635–648

Williams BP, Pignatta D, Henikoff S, Gehring M (2015) Methylation-sensitive expression of a DNA demethylase gene serves as an epigenetic rheostat. **PLoS Genet** 11: e1005142

Zhang H, Lang Z, Zhu JK (2018) Dynamics and function of DNA methylation in plants. **Nat Rev Mol Cell Biol** 19: 489–506

Zhang Z, Mao Y, Ha S, Liu W, Botella JR, Zhu JK (2016) A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*. **Plant Cell Rep** 35: 1519–1533

Zhao QQ, Lin RN, Li L, Chen S, He XJ (2019) A methylated-DNA-binding complex required for plant development mediates transcriptional activation of promoter methylated genes. **J Integr Plant Biol** 61: 120–139

Zhu JK (2009) Active DNA demethylation mediated by DNA glycosylases. **Annu Rev Genet** 43: 143–166

SUPPORTING INFORMATION

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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 chop-PCR. Genomic DNA was digested by the DNA methylation-sensitive restriction enzymes *Hha*I. 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-0 wild type. Means with different letters are significantly different at $P < 0.01$.

Figure S5. Detection of purified recombinant protein GST-SUVH3

Collected protein was running in SDS-PAGE gel, and following checked by Coomassie Brilliant Blue (CBB) staining (**A**) or Western Blot using Anti-GST (**B**).

Figure S6. Depressed *ROS1* gene expression in *suvh1/3* was rescued by native promoter driven *SUVH3-FLAG*

The seedlings of Col-0, *suvh1*, *suvh1/3* and three homozygous transgene lines which transform *SUVH3*$_{pro:SUVH3\text{-}FLAG}$ 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-0 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-0, *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-0 wild type. Means with different letters are significantly different at $P < 0.01$. (**B**) SUVH3 protein levels in Col-0, *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