The SET Domain Proteins SUVH2 and SUVH9 Are Required for Pol V Occupancy at RNA-Directed DNA Methylation Loci

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Abstract

RNA-directed DNA methylation (RdDM) is required for transcriptional silencing of transposons and other DNA repeats in Arabidopsis thaliana. Although previous research has demonstrated that the SET domain-containing SU(VAR)3–9 homologs SUVH2 and SUVH9 are involved in the RdDM pathway, the underlying mechanism remains unknown. Our results indicated that SUVH2 and/or SUVH9 not only interact with the chromatin-remodeling complex termed DDR (DMS3, DRD1, and RDM1) but also with the newly characterized complex composed of two conserved Microrchidia (MORC) family proteins, MORC1 and MORC6. The effect of suvh2suvh9 on Pol IV-dependent siRNA accumulation and DNA methylation is comparable to that of the Pol V mutant nrpe1 and the DDR complex mutant dms3, suggesting that SUVH2 and SUVH9 are functionally associated with RdDM. Our CHIP assay demonstrated that SUVH2 and SUVH9 are required for the occupancy of Pol V at RdDM loci and facilitate the production of Pol V-dependent noncoding RNAs. Moreover, SUVH2 and SUVH9 are also involved in the occupancy of DMS3 at RdDM loci. The putative catalytic active site in the SET domain of SUVH2 is dispensable for the function of SUVH2 in RdDM and H3K9 dimethylation. We propose that SUVH2 and SUVH9 bind to methylated DNA and facilitate the recruitment of Pol V to RdDM loci by associating with the DDR complex and the MORC complex.


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Introduction

DNA methylation is an important epigenetic modification that contributes to transposable element silencing, genome stability, and gene regulation in plants and animals [1–3]. In Arabidopsis thaliana, DNA methylation can be established through a well-characterized RNA-directed DNA methylation (RdDM) pathway [1,2]. The RdDM pathway is involved in the silencing of transposable elements and DNA repeats [1,2], the stability of the genome [4,5], the development of gametophytes and embryos [6–8], and the inheritance of stress-induced transcriptional silencing [9].

In the RdDM pathway, the multi-subunit DNA-dependent RNA polymerase IV (Pol IV) collaborates with RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) to produce double-stranded siRNAs, which are subsequently cleaved into 24-nt siRNAs (small interfering RNAs) by DICER-LIKE 3 (DCL3) [10–13]. The recruitment of Pol IV to chromatin requires SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1)/DNA-BINDING TRANSCRIPTION FACTOR 1 (DTF1) [13,14]. Pol IV-dependent siRNAs are loaded onto ARGO-NAUTE 4 (AGO4) in the cytoplasm and then transported into the nucleus to complete the assembly of the RdDM effector complex [15]. The multi-subunit DNA-dependent RNA polymerase V (Pol V) produces long noncoding RNAs, which act as scaffold RNAs for the assembly of the RdDM effector complex [16–18]. The DDR complex, which is composed of DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and RNA-DIRECTED DNA METHYLATION 1 (RDM1), is required for the recruitment of Pol V to RdDM loci and for the accumulation of Pol V-produced noncoding RNAs [17–23]. RNA-DIRECTED DNA METHYLATION 4 (RDM4)/DEFECTIVE IN MERISTEM SILENCING 4 (DMS4), an IWR1-like protein, is likely to act as a general transcription factor that is shared by Pol II, Pol IV, and Pol V [24–26]. KOW-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1)/SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L) can bind to Pol V-produced noncoding RNAs and is recruited to chromatin in parallel with AGO4 [27–29]. INVOLVED IN DE NOVO 2 (IDN2) and IDN2 PARALOG 1 and 2 (IDP1 and IDP2) can form a complex required for RdDM [30–32]. IDN2 physically associates with the SWI/SNF chromatin-remodeling complex, which is required for noncoding RNA-mediated transcriptional silencing [33]. DOMAIN REARRANGED METHYLTRANSFERASE 2 (DRM2) is the main DNA methyltransferase that catalyzes DNA methylation at RdDM loci [34,35]. MORC1 and MORC6, two members of the conserved Microrchidia (MORC)
Author Summary

Small RNA-induced transcriptional silencing at transposable elements and other DNA repeats is an evolutionarily conserved mechanism in plants, fungi, and animals. In Arabidopsis thaliana, an RNA-directed DNA methylation pathway is involved in transcriptional silencing. Noncoding RNAs produced by the plant-specific DNA-dependent RNA polymerase V are required for RNA-directed DNA methylation. A chromatin-remodeling complex was previously demonstrated to be required for the occupancy of DNA-dependent RNA polymerase V at RNA-directed DNA methylation loci. Our results suggest that two putative histone methyltransferases are inactive in their enzymatic activity and act as adaptor proteins to facilitate the recruitment of DNA-dependent RNA polymerase V to chromatin by associating with the chromatin-remodeling complex. In combination with previous studies, we propose that the inactive histone methyltransferases bind to methylated DNA, thereby linking DNA methylation to Pol V transcription at RNA-directed DNA methylation loci.

adénosine triphosphatase (ATPase) family, were thought to be required for decondensation of pericentromeric heterochromatin in a DNA methylation-independent manner [36]. However, another two reports indicated that MORC6/DEFECTIVE IN MERISTEM SILENCING 11 (DMS11) is involved in RNA-directed DNA methylation and transcriptional silencing [37,38]. Further study is required to clarify the functional mechanism of the MORC family proteins in transcriptional silencing.

In Arabidopsis, H3K9 methylation is catalyzed by the conserved SU(VAR)3–9 homologs (SUVHs) and related proteins (SUVRs) [2,39]. The SUVHs contain a catalytic SET domain and an N-terminal SRA (SET- or RING-associated) domain [39]. The active histone H3K9 methyltransferases SUVH4/KRYPTONITE (KYP), SUVH5, and SUVH6 are not only required for H3K9 dimethylation (H3K9me2) but also for DNA methylation [40,41]. The connection between DNA methylation and histone H3K9me2 is dependent on the SRA domain in the SUVHs that directly bind to methylated DNA [42,43]. CHROMOMETHYLASE 3 (CMT3), a plant-specific DNA methyltransferase, can directly bind to H3K9me2-containing nucleosomes and connect the histone H3K9me2 with DNA methylation at CHG sites [44]. At RdDM loci, canonical RdDM components including Pol IV, Pol V, and AGO4 are required for both DNA methylation and histone H3K9me2 [17,45,46]. Previous reports demonstrated that SUVH2 and SUVH9 are involved in RNA-directed DNA methylation and transcriptional silencing [47,48], but the underlying mechanism is unknown.

In this study, we found that SUVH2 and SUVH9 associate with the DDR complex and the newly characterized MORC1-MORC6 complex and act as adaptor proteins to mediate Pol V occupancy at RdDM loci. The conserved catalytic active site of SUVH2 is not required for the function of SUVH2 in RdDM and histone H3K9me2. The results revealed the functional mechanism of SUVH2 and SUVH9 in the RdDM pathway.

Results

SUVH2 and SUVH9 associate with DDR components and MORC family proteins in vivo

SUVH2 and SUVH9 were previously demonstrated to be involved in RNA-directed DNA methylation [47,48], but the underlying mechanism is unclear. To understand the role of SUVH2 and SUVH9 in RdDM, we generated the native promoter-driven SUVH2-3xMyc and SUVH9-3xFlag transgenic plants. The stably expressed SUVH2-3xMyc and SUVH9-3xFlag transgenic lines were used for affinity purification of the proteins that associate with SUVH2 and SUVH9. Mass spectrometric assay revealed that SUVH2 and SUVH9 were the most abundant proteins in affinity purification of SUVH2-3xMyc and SUVH9-3xFlag, respectively (Table 1). Moreover, two RdDM components DMS3 and DRD1 were identified in affinity purification of SUVH2-3xMyc, whereas MORC6/DMS11 and DMS3 were identified in affinity purification of SUVH9-3xFlag (Table 1). The presence of RdDM components DMS3 and DRD1 in affinity purification of SUVH2-3xMyc and SUVH9-3xFlag suggests that SUVH2 and SUVH9 are involved in RdDM by physically associating with RdDM components. MORC6/DMS11 was recently demonstrated to be involved in RNA-directed DNA methylation and transcriptional silencing [37,38]. The finding of MORC6 in affinity purification of SUVH9-3xMyc suggests that MORC6 interacts with SUVH9. We carried out affinity purification of MORC6-3xFlag in MORC6-3xFlag transgenic plants and identified SUVH9 by mass spectrometric assay, confirming that SUVH9 can interact with MORC6 in vivo (Table 1). To confirm the interaction between SUVH2 and DMS3, we performed co-immunoprecipitation (co-IP) in SUVH2-3xMyc transgenic plants using either anti-Myc antibody or anti-DMS3 antibody. The results indicated that SUVH2-3xMyc and DMS3 were co-precipitated not only by anti-Myc antibody but also by anti-Myc antibody (Figure 1A; Figure S1A). The interaction between SUVH9 and MORC6 was also demonstrated by co-IP in the transgenic plants harboring both SUVH9-Flag and MORC6-Myc transgenes (Figure 1B). We therefore concluded that SUVH2 and/or SUVH9 can physically associate with the canonical RdDM components in vivo. However, we could not detect the interaction between SUVH9 and DMS3 by co-IP (Figure S1B, S1C), suggesting that the interaction is either weak or highly locus-specific.

We carried out gel filtration to determine whether SUVH2, SUVH9, and related RdDM components act as protein complexes in vivo. For gel filtration, the protein extracts from the wild-type plants and SUVH2-3xMyc, SUVH9-3xFlag, and MORC6-3xFlag transgenic plants were separately eluted and then subjected to Western blotting (Figure 1C). The elution profile of these protein extracts revealed that each protein (SUVH2-3xMyc, SUVH9-3xFlag, and MORC6-3xFlag) was eluted in the form of a high-molecular-weight protein complex rather than as a monomer. SUVH2-3xMyc, SUVH9-3xFlag, and DMS3 were co-eluted with the peak at the size between 440 KDa and 669 KDa, which is consistent with the mass spectrometric analysis indicating that both SUVH2 and SUVH9 associated with DMS3 (Figure 1C). The elution peak of MORC6-3xFlag was <440 KDa, indicating that the complex containing MORC6 is smaller than the complex containing SUVH2, SUVH9, or DMS3 (Figure 1C). The small molecular size of the MORC6 elution peak indicated that MORC6 may form a different complex that does not include SUVH2, SUVH9, and DMS3 (Figure 1C).

Mass spectrometric analysis indicated that affinity purification of MORC6-3xFlag produced a large number of peptides corresponding to MORC1 and another MORC family protein, MORC2 (AT1G36280), in MORC6-3xFlag transgenic plants (Table 1). Therefore, MORC1, MORC2, and MORC6 may form a tight complex in vivo. Affinity purification of MORC6-3xFlag generated three specific silver-stained bands on SDS-PAGE gel (Figure 1D). The three bands were separately cut from the gel and subjected to mass spectrometric analysis. The results
indicated the three bands from top to bottom correspond to the MORC6-3xFlag fusion protein, the endogenous MORC6 protein, and the endogenous MORC1 protein (Figure 1D). These results suggest that MORC6 not only forms a homodimer with another MORC6 but also forms a heterodimer with its homologs MORC1 and MORC2 in vivo. The formation of the MORC1-MORC6 heterodimer was confirmed by co-IP between MORC1-3xFlag and MORC6-3xMyc in the transgenic plants harboring both MORC1-3xFlag and MORC6-3xMyc transgenes (Figure 1E). Furthermore, the interaction between MORC6 and MORC1 or MORC2 was examined by yeast two-hybrid assay. The results demonstrated that MORC6, MORC1, and MORC6 can form a homodimer or heterodimer (Figure S2), which is consistent with the result of the gel filtration assay indicating that MORC6 acts in vivo dependent siRNAs.

### Effect of suvh2 and suvh9 on accumulation of Pol IV-dependent siRNAs

We carried out small RNA deep sequencing to compare the global effect of *suvh2*, *suvh9*, and *suvh2suvh9* relative to that of the canonical RdDM mutants *nrpd1*, *nrpe1*, and *dms3* on the accumulation of Pol IV-dependent siRNAs. We obtained at least 2.6 million uniquely genome-matched small RNA reads for the small RNA libraries of the wild type, *suvh2*, *suvh9*, *suvh2suvh9*, *nrpd1*, *nrpe1*, and *dms3* (Table S1). Pol IV-dependent 24-nt-siRNA regions were defined as the genomic regions in which the abundance of 24-nt siRNAs was significantly reduced in *nrpd1* relative to the wild type. Consistent with previous reports [49–51], we found that Pol IV-dependent siRNAs are predominantly enriched in centrometric regions where highly repetitive DNA sequences exist, and are also present in intergenic regions of each chromosome, especially when the regions contain transposable elements (Figure 3A; Table S2). Both NRPE1 and DMS3 are required for the production of Pol V-dependent noncoding RNAs and act at the downstream step of the RdDM pathway [17,18]. Our small RNA data indicated that NRPE1 and DMS3 predominantly contribute to accumulation of the Pol IV-dependent siRNAs produced from intergenic regions at two arms of each chromosome, but that both proteins have little impact on the Pol IV-dependent siRNAs produced from highly duplicated DNA repeats.

### Table 1. List of affinity co-purified proteins of SUHV2-Myc, SUHV9-Flag, and MORC6-Flag.

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in centromeric regions (Figure 3A; Table S2), which is consistent with previous reports [50,51]. Accumulation of Pol IV-dependent siRNAs was only marginally reduced in the suvh2 and suvh9 single mutants and the reduction was significantly enhanced in the suvh2suvh9 double mutant (Figure 3A). The effect of suvh2suvh9 on accumulation of Pol IV-dependent siRNAs is comparable to that of nrpe1 and dms3 at the whole-genome level (Figure 3A), suggesting that SUVH2 and SUVH9 function redundantly at the downstream step of the RdDM pathway.

In total, we identified 5751 siRNA regions where the abundance of 24-nt siRNAs is strongly decreased (>5-fold decrease) in nrpd1 compared to the wild type (Figure 3B, 3C, 3D, 3E). In 63.8% (3670/5751) of the Pol IV-dependent siRNA regions, 24-nt siRNAs are significantly decreased in nrpe1 (Figure 3B, 3C, 3D, 3E). Thus, Pol IV-dependent siRNA regions were divided into two groups: Pol V-independent siRNA regions and Pol V-dependent siRNA regions. Of the 3670 Pol V-dependent siRNA regions, 98.6% (3618/3670) have 24-nt siRNAs that are also decreased in
dms3, whereas only 14.1% (293/2081) of Pol IV-dependent and
Pol V-independent siRNA regions are significantly decreased in
24-nt siRNAs in dms3 (Figure 3B). The highly similar effect of
nrpe1 and dms3 on 24-nt siRNAs supported the inference that DMS3
facilitates the recruitment of Pol V and mediates the production of
Pol V-dependent noncoding RNAs at a later step in the RdDM
pathway.

In the suvh2 and suvh9 single mutants, 12.6% (726/5751) and
7.5% (434/5751) of the Pol IV-dependent siRNA regions show a
significant decrease in 24-nt siRNAs, respectively (Figure 3C, 3D).
The low effect of suvh2 and suvh9 on Pol IV-dependent siRNAs
may account for the functional redundancy between SUVH2 and
SUVH9. Although the effect of the suvh2 and suvh9 single mutants
is quite low, we still find that most of these SUVH2- and SUVH9-
dependent siRNA regions (691/726 for SUVH2 and 353/434 for
SUVH9) belong to Pol IV- and Pol V-dependent siRNA regions
(Figure 3C, 3D). The results suggest that like Pol V, SUVH2 and
SUVH9 may act downstream of the primary siRNA biogenesis in
the RdDM pathway. In the suvh2suvh9 double mutant, a high
percentage of Pol IV- and Pol V-dependent siRNAs regions (88.9%, 3670)
show a decrease in 24-siRNAs, whereas only 5.2% (109/2081) of Pol IV-dependent and Pol V-dependent
siRNAs regions have decreased 24-nt siRNAs (Figure 3E). The
effect of suvh2suvh9 is comparable to that of the Pol V mutant nrpe1
and of the DDR complex mutant dms3 (Figure 3B, 3E).

Based on the small RNA deep sequencing data, we tested the
effect of suvh2 and suvh9 on well-known Pol IV-dependent siRNAs,
which include siRNAs from 180 bp centromeric repeats (180 bp
CEN), AtGP1, AtSN1, and MEA-ISR loci. As expected, all these Pol
IV-dependent siRNAs were blocked in the Pol IV mutant nrpd1,
whereas AtSN1 siRNA and MEA-ISR siRNA but not 180 bp CEN
siRNA and AtGP1 siRNA were reduced in the Pol V mutant nrpe1
and the DDR complex mutant dms3 (Figure S4). We found that
AtSN1 siRNA and MEA-ISR siRNA were weakly reduced in
suvh2 but not in suvh9. In the suvh2suvh9 double mutant, AtSN1 siRNA
and MEA-ISR siRNA were reduced to much more extent than that
in suvh2 (Figure S4). The effect of suvh2suvh9 on accumulation of
AtSN1 siRNA and MEA-ISR siRNA is similar to that of nrpe1 and
dms3 (Figure S4), suggesting that the function of SUVH2 and
SUVH9 in Pol V-dependent siRNA accumulation is partially
redundant. Neither suvh2 and suvh9 single mutants nor suvh2suvh9
double mutant reduced the Pol V-independent siRNAs, 180 bp
CEN siRNA and AtGP1 siRNA (Figure S4). These results support
the inference that SUVH2 and SUVH9 act together with Pol V
and the DDR complex at a later step in the RdDM pathway.

SUVH2 and SUVH9 are required for Pol V-dependent
noncoding RNAs and transcriptional silencing

We carried out quantitative RT-PCR to determine the effect of
suvh2 and suvh9 on transcriptional silencing at RdDM loci. The
results indicated that the RNA transcript levels of solo LTR, AtGP1, SDC, and AtX1 are markedly induced in the canonical RdDM mutants including nrpe1, drd1, dms3, and nrpe1 (Figure 4A). The RNA transcript levels of solo LTR, AtGP1, and AtX1 are either not affected or are weakly induced in either the svuh2 or svuh9 single mutant (Figure 4A). In the svuh2/svuh9 double mutant, however, the induction of the RNA transcript levels of the three loci are significantly enhanced compared to the levels in the single mutants (Figure 4A). The SDC transcript is intermittently induced in svuh9 rather than in svuh2 and the transcript is drastically enhanced in the svuh2/svuh9 double mutant (Figure 4A). These results suggest that SUVH2 and SUVH9 function redundantly in transcriptional silencing.

Small RNA analysis indicated that the effect of svuh2/svuh9 on Pol V-dependent siRNAs is similar to that of the Pol V mutant, nrpe1 (Figure 3A, 3E). Moreover, both SUVH2 and SUVH9 can interact with DMS3 (Table 1; Figure 1A, 2A, 2B), which acts as a subunit of the DDR complex and is required for Pol V chromatin association at RdDM loci [22,23]. Therefore, it is possible that SUVH2 and SUVH9 are involved in the accumulation of Pol V-dependent noncoding RNAs. The effect of svuh2 and svuh9 on the Pol V-dependent transcript AtSYV1B was previously noted [48]. We determined the effect of svuh2, svuh9, and svuh2/svuh9 on the Pol V-dependent noncoding RNAs at IGN5B, IGN23, and IGN25 by RT-PCR (Figure 4B; Figure S5). The results showed that the RNA transcript levels of the three tested loci were markedly reduced in svuh2, drd1, and dms3 (Figure 4B; Figure S5), suggesting that the RNA transcripts of these loci are dependent on Pol V. The Pol V-dependent transcripts IGN5B, IGN23, and IGN25 were either not affected or marginally reduced in the svuh2 and svuh9 single mutants but were markedly reduced in the svuh2/svuh9 double mutant (Figure 4B; Figure S5), which demonstrated that SUVH2 and SUVH9 are required for the production of Pol V-dependent noncoding RNAs at RdDM loci. SUVH2 and SUVH9 are functionally redundant in Pol V-dependent RNA accumulation.

Previous reports suggest that disruption of the RdDM machinery leads to derepression not only of transposable elements but also of their flanking genes [2,32,33]. We performed RNA deep sequencing to compare the effect of svuh2/svuh9 vs. nrpe1 and dms3 on gene expression at the whole-genome level. For each RNA library, we obtained at least 14.7 million RNA reads that were mapped to the Arabidopsis genome (Table S3). The Heat map results indicated that the genome-wide effect of svuh2/svuh9 on gene expression is generally similar to that of nrpe1 and dms3 (Figure 4C). The genes upregulated in the RdDM mutants nrpe1 and dms3 are thought to be RdDM loci (Table S2). We found that 361, 383, and 303 genes that are significantly log2 (Mutant/WT) ≥ 1, P < 0.01) upregulated in nrpe1, dms3, and svuh2/svuh9, respectively (Table S4; Figure 4D), suggesting that the three mutants have a similar effect on gene expression at the whole-genome level. Of the 383 genes upregulated in dms3, 49.6% (190/383) are also upregulated in nrpe1 (Figure 4D), supporting the inference that DMS3 acts together with NRPE1 in the RdDM pathway. Of the 303 genes upregulated in svuh2/svuh9, nearly half
SUVH2 and SUVH9 are upregulated in either nrpe1 or dms3 (Figure 4D). The data are consistent with the finding that SUVH2 and SUVH9 associate with DMS3 and mediate transcriptional silencing through the RdDM pathway.

SUVH2 and SUVH9 are involved in the association of Pol V and DMS3 with chromatin at RdDM loci

Given that Pol V-dependent noncoding RNAs are markedly decreased in the suvh2suvh9 double mutant, we determined whether suvh2suvh9 reduces Pol V chromatin association at RdDM loci. The NRPE1-Flag transgene was introduced and expressed in WT, dms3, and suvh2suvh9 equivalently (Figure S6A). By CHIP assay using anti-Flag antibody, we found that NRPE1 was enriched at the RdDM loci including solo LTR, IGN5, IGN22, IGN25, and IGN26, and that the enrichment was decreased in the DDR complex mutant dms3 (Figure 5A), which is consistent with previous reports [17,18]. Interestingly, our results indicated that the enrichment of NRPE1 on chromatin at these RdDM loci was
also significantly decreased in *suh2suh9* (Figure 5A), suggesting that SUVH2 and SUVH9 are required for Pol V chromatin association. The results demonstrate that SUVH2 and SUVH9 not only physically associate with the DDR complex but also act together with the complex to facilitate Pol V occupancy at RdDM loci.

The DDR complex was previously demonstrated to be co-purified with Pol V [22], whereas no Pol V subunits were identified in SUVH2 and SUVH9 co-purified proteins (Table 1). The results suggest that the role of SUVH2 and SUVH9 in Pol V occupancy is probably indirectly through their action on the DDR complex. Thus, we determined whether *suh2suh9* affects the association of the DDR complex with chromatin at RdDM loci. We carried out DMS3 CHIP assay in the wild type as well as in *suh2suh9* by using anti-DMS3 antibody. The specificity of the antibody was confirmed by Western blotting assay (Figure S6B). The CHIP assay demonstrated that DMS3 occupancy at RdDM loci, including *IGN25*, *IGN22*, and *IGN25* is significantly reduced in *suh2suh9* relative to the wild type (Figure 5B), suggesting that SUVH2 and SUVH9 enable the association of the DDR complex with chromatin, thereby facilitating the recruitment of Pol V to RdDM loci.

SUVH2 and SUVH9 are required for DNA methylation and H3K9me2 at RdDM loci

The effect of *suh2* and *suh9* on DNA methylation at RdDM loci was evaluated previously [47,52]. At the genome-wide level, *suh2* reduces DNA methylation at a large number of RdDM loci, whereas *suh9* affects only a very small portion of RdDM loci [52,53]. We determined the effect of *suh2* and *suh9* on DNA methylation by chop-PCR (Figure 6A). The results indicated that DNA methylation on *AtSN1*, *IGN5*, *IGN25*, and solo LTR was either not reduced or only marginally reduced in the *suh2* and *suh9* single mutants but the reduction was greatly enhanced in the *suh2suh9* double mutant (Figure 6A). The reduction of DNA methylation in *suh2suh9* was comparable to that in the canonical RdDM mutants *nrpe1*, *dms3*, and *dms1* (Figure 6A). The results demonstrate that SUVH2 and SUVH9 are functionally redundant in DNA methylation at RdDM loci.

Previous studies revealed that disruption of canonical RdDM components can lead to reduction of repressive histone H3K9me2 at RdDM loci [17,45,46]. To determine whether H3K9me2 at these loci is related to the putative histone methyltransferases SUVH2 and SUVH9, we performed chromatin immunoprecipitation (CHIP) assay using anti-H3K9me2 antibody. The results demonstrated that SUVH2 and SUVH9 are functionally redundant at RdDM loci [17,45,46]. To determine whether H3K9me2 at RdDM loci is related to the putative histone methyltransferases SUVH2 and SUVH9, we preformed chromatin immunoprecipitation (CHIP) assay using anti-H3K9me2 antibody. The results demonstrated that SUVH2 and SUVH9 are functionally redundant at RdDM loci.

![A](https://example.com/image1.png)

**A.** NRPE1-Flag CHIP

**B.** DMS3 CHIP

**Figure 5. Effect of *suh2* and *suh9* on the occupancy of Pol V and the DDR complex at RdDM loci.** (A) The effect of *suh2suh9* on Pol V occupancy at RdDM loci was determined by CHIP assay with anti-Flag antibody. The enrichment of the largest subunit of Pol V, NRPE1, was measured at IGN5, solo LTR, IGN22, IGN25, and *IGN26*. The abundance of NRPE1 on the actin gene was used as an internal negative control. Enrichment of NRPE1-Flag on RdDM loci and the tubulin gene was normalized by the abundance of NRPE1-Flag on the actin gene. (B) The effect of *suh2suh9* on the occupancy of the DDR complex at RdDM loci was determined by CHIP assay with anti-DMS3 antibody. Enrichment of the DDR complex component DMS3 was determined in the wild type and *suh2suh9*. Relative enrichment of DMS3 is shown.

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The catalytic active site of SUVH2 is dispensable for its function in RdDM and H3K9me2

The SET domain is responsible for histone methyltransferase activity and is conserved not only in plants but also in animals and fungi [39]. However, the SET domain-containing proteins SUVH2 and SUVH9 have no detectable histone methyltransferase activity by *in vitro* assay and the overall histone H3K9me2 level is not affected in the *suh2suh9* double mutant [47]. It is necessary to determine whether the possible histone methyltransferase activity is required for the function of SUVH2 and SUVH9 in RdDM and H3K9me2 in *vivo*. We mutated two conserved residues (SUVH2-H596K and SUVH2-P600A) in the catalytic active site of the SET domain in SUVH2 and transformed the mutated SUVH2 sequences into *suh2suh9* (Figure 7A). The transgenic plants harboring the stably expressed wild-type or mutant SUVH2 transgene were used for complementation assay. Previous studies suggest that the two residues SUVH2-H596 and SUVH2-P600 are highly conserved and are essential for the catalytic activity of the SET domain [54,55]. If histone methyltransferase activity is required for the role of SUVH2 in RdDM and H3K9me2, disruption of the conserved catalytic active site in SUVH2 is expected to block its function in *vivo*. DNA methylation assay demonstrated that the DNA methylation defect in *IGN5* and *IGN25* caused by *suh2* was complemented by the two mutated SUVH2 transgene sequences as well as by the wild-type SUVH2 transgene (Figure 7B). Moreover, H3K9me2 CHIP assay indicated that the two mutations in SUVH2 also have no effect on the in *vivo*
SUVH2 and SUVH9 Mediate Pol V Occupancy

**Figure 6. Effect of suvh2 and suvh9 on DNA methylation and histone H3K9me2 at RdDM targets.** (A) The effect of suvh2, suvh9, and suvh2suvh9 on DNA methylation as indicated by chop-PCR. DNA methylation was determined for AtSN1, IGN5, IGN25, and solo LTR. Genomic DNA from the wild type, nrpe1, dms3, suvh2, suvh9, and suvh2suvh9 was cleaved by the DNA methylation-sensitive restriction enzyme HaeIII or AluI and subjected to semiquantitative PCR. Genomic DNA from the wild type, nrpe1, dms3, suvh2, suvh9, and suvh2suvh9 was cleaved by the DNA methylation-sensitive restriction enzyme HaeIII or AluI and subjected to semiquantitative PCR. The actin gene, which lacks HaeIII recognition site, was amplified as an internal control. The solo LTR locus was amplified using uncut genomic DNA as template for a control. (B) Effect of suvh2suvh9 on histone H3K9me2 was measured by CHIP assay with anti-H3K9me2 antibody. The RdDM mutants nrpe1 and dms3 were included as controls, in which the H3K9me2 levels at indicated RdDM loci were decreased. The actin gene, which lacks detectable H3K9me2, was used as a negative control. doi:10.1371/journal.pgen.1003948.g006

SUVH2 and SUVH9 function redundantly at a downstream step of the RdDM pathway [47,48]. Previous reports suggest that disruption of the downstream RdDM components including NRPE1 and DMS3 indirectly affects accumulation of Pol IV-dependent 24-nt siRNAs [50,51]. Our small RNA deep sequencing data indicate that the effect of suvh2suvh9 on 24-nt siRNA accumulation is comparable to that of nrpe1 and dms3 (Figure 3A, 3B, 3E, Figure S4), supporting the functional association of SUVH2 and SUVH9 with the downstream RdDM components NRPE1 and DMS3. We demonstrate that SUVH2 and SUVH9 can be physically associate with the components of the DDR complex and mediate Pol V occupancy at RdDM loci (Table 1; Figure 1, 2, 3A, 3E). In the RdDM pathway, Pol V produces long noncoding RNAs that act as scaffold RNAs for the assembly of the RdDM effector complex [17]. The DDR complex is involved in the recruitment of Pol V to chromatin and facilitates the production of Pol V-dependent noncoding RNAs [18,22,23]. The association of SUVH2 and SUVH9 with the DDR complex is consistent with the function of SUVH2 and SUVH9 in Pol V occupancy at RdDM loci. The SRA domain of SUVH2 and SUVH9 can bind to methylated DNA [47], but the role of the methylated DNA-binding ability in RdDM remains elusive. Given the finding that SUVH2 and SUVH9 can physically associate with the DDR complex (Table 1; Figure 1, 2), the binding of SUVH2 and SUVH9 to methylated DNA is probably required for the occupancy of the DDR complex on chromatin. Our DMS3 CHIP assay indicates that the occupancy of DMS3 at RdDM sites is significantly reduced in suvh2suvh9 relative to the wild type (Figure 5B). Thus, reduced Pol V occupancy at RdDM sites in suvh2suvh9 is likely due to reduced occupancy of DMS3. We propose that the binding of SUVH2 and SUVH9 to methylated DNA mediates the occupancy of the DDR complex at RdDM loci, thereby recruiting Pol V to the loci (Figure 8). Noncoding RNAs produced by Pol V interact with 24-nt siRNAs bound by AGO4 and recruit the de novo DNA methyltransferase DRM2 to mediate DNA methylation [17,18,21]. Thus, SUVH2 and SUVH9 act as a linker between DNA methylation and Pol V transcription in the RdDM pathway (Figure 8).

Although SUVH2 and SUVH9 have been demonstrated to be required for H3K9me2 at RdDM sites (Figure 6B), SUVH2 and SUVH9 have no detectable histone methyltransferase activity by in vitro histone methyltransferase assay [47]. In Arabidopsis, another three SUVH proteins SUHV4/KYP, SUHV5, and SUHV6 have histone H3K9 methyltransferase activity and are required for maintenance of histone H3K9 methylation at the whole-genome level [2,40,41]. It is possible that H3K9me2 at RdDM sites is catalyzed by these active histone H3K9 methyltransferases. Our results indicate that disruption of the putative catalytic active site of SUVH2 has no effect on the function of SUVH2 in RdDM and H3K9me2 (Figure 7A, 7B, 7C), supporting the inference that SUVH2 and SUVH9 act as adaptor proteins that are involved in Pol V recruitment but not as active histone methyltransferases. H3K9me2 of RdDM loci requires not only SUVH2 and SUVH9 but also other RdDM components including NRPD1, NRPE1, and AGO4 [17,45,46], suggesting that SUVH2 and SUVH9 are implicated in H3K9me2 through the RdDM pathway.

SUVH2 and/or SUVH9 can associate not only with the DDR complex but also with the MORC family proteins (Table 1; Figure 1, 2). A recent report suggests that involvement of MORC1 and MORC6/DMS11 in transcriptional silencing is not related to DNA methylation [36], whereas another two reports indicate that MORC6/DMS11 is a new component of the RdDM machinery [37,38]. Our results demonstrate that MORC6/DMS11 and its homologs MORC1 and MORC2 associate with SUVH9 (Table 1; Figure 1B, 1C, 2D), which is required for Pol V occupancy in the RdDM pathway (Figure 5A). MORC6/DMS11 was previously demonstrated to be involved in the production of Pol V-dependent
**SUVH2 and SUVH9 Mediate Pol V Occupancy**

Family proteins act together and mediate Pol V occupancy at RdDM loci, thereby facilitating the production of Pol V-dependent noncoding RNAs (Figure 8). Moreover, the MORC family proteins can also mediate the change of histone superstructure and lead to transcriptional silencing without involvement of DNA methylation [36]. Our mass spectrometric assay has identified CHBS/SWEID, a subunit of the SWI/SNF-type chromatin-remodeling complex, as a MORC6-associating protein in affinity purification of MORC6-3xFlag (Table 1). The SWI/SNF-type chromatin-remodeling complex was recently demonstrated to act in Pol V-dependent noncoding RNA-mediated transcriptional silencing by establishing positioned nucleosomes on specific genomic loci [33]. Therefore, in addition to the role of the MORC family proteins in the RdDM pathway, they may also act with the SWI/SNF-type chromatin-remodeling complex to contribute to RNA-mediated transcriptional silencing through a DNA methylation-independent manner.

Our results demonstrate that MORC1, MORC2, and MORC6 can form a homodimer or a heterodimer in vivo (Table 1; Figure 1D, 1E; Figure S2). A recent report suggests that the GHLK ATPase domain-containing MORC6/DMS11 can physically interact with the SMC hinge domain-containing protein DMS3 and form a functional analog of an authentic SMC protein, which functions in a dimer and binds complementary DNA strands during sister chromatid cohesion, chromatin condensation, and DNA repair [37, 56]. Given that DMS3 can form a homodimer as described previously [20], a MORC dimer and a DMS3 dimer may form a functional SMC analog in the RdDM pathway, which may help produce Pol V-dependent noncoding RNAs. However, no DMS3 peptide is identified by affinity purification of MORC6-3xFlag (Table 1), which does not support the tight in vivo interaction between the MORC dimer and the DMS3 dimer.

Overall, our results indicate that SUVH2 and SUVH9 are physically and functionally associated with the DDR complex and the MORC family proteins and facilitate Pol V occupancy at RdDM loci, concluding that SUVH2 and SUVH9 are canonical components of the RdDM machinery. SUVH2 and SUVH9 may bind to methylated DNA at RdDM loci and interact with the DDR complex at the loci, thereby recruiting Pol V to produce noncoding RNAs. Moreover, we demonstrate that the putative histone methyltransferase activity of SUVH2 is dispensable for the function of SUVH2 in RdDM and H3K9me2 at RdDM loci. Involvement of SUVH2 in H3K9me2 at RdDM loci is via its action in the RdDM pathway. Further study is required to elucidate how SUVH2, SUVH9, the DDR complex, and the MORC family proteins are coordinated to mediate Pol V occupancy at RdDM loci.

**Materials and Methods**

Plant materials, cloning, and generation of transgenic plants

The Arabidopsis materials included the wild-type Col-0, such2 (Gabi_kat_516A07), such9 (SALK_048033), such2such9 [48], nrp1-l1 (SALK_128428), nrp1-l1 (SALK_029919c), dms3 (Salk_125019c), ddr1-6 [19], ddr1-6, morc1 (Salk_006160), and morc6 (GK-599B06-023140). The seedlings were grown on MS medium plates under long-day conditions (16 h day time and 8 h night time) at 22°C. Two-week-old seedlings were directly used for the study or transplanted into soil when adult plants or flower tissues were needed. The Flag- and/or Myc-tagged SUVH2, SUVH9, MORC1, and MORC6 genomic sequences were constructed into the modified

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**Figure 7. Mutation of the conserved SET domain in SUVH2 has no effect on DNA methylation and H3K9me2 at RdDM loci**

(A) Alignment of the catalytic active site in the Arabidopsis SET domain proteins SUVH2, SUVH9, SUVH4, SUVH5, and SUVH6, and their homologs including G9a and SUV39H1 in human, Ctf4 in fission yeast, and Dim-5 in Neurospora homologs including G9a and SUV39H1 in human, Clr4 in fission yeast, and Dim-5 in Neurospora.

(B) Site-directed mutagenesis was carried out to introduce the SUVH2-P600 mutation in the SET domain of SUVH2 (strain IGN5, IGN23, and G9a). The conserved residues SUVH2-H596 and SUVH2-H596 are marked with arrows. (B) Site-directed mutagenesis was carried out to introduce the SUVH2-P600 mutation in the SET domain of SUVH2 (strain IGN5, IGN23, and G9a). The conserved residues SUVH2-H596 and SUVH2-H596 are marked with arrows.

(C) Enrichment of H3K9me2 was assayed using a ChIP assay. For DNA methylation assay, genomic DNA was digested by the DNA methylation-sensitive restriction enzyme HaeIII followed by PCR. The DNA methylation levels at RdDM loci IGN5 and IGN23 were determined. (C) Enrichment of H3K9me2 was assayed using a ChIP assay. For DNA methylation assay, genomic DNA was digested by the DNA methylation-sensitive restriction enzyme HaeIII followed by PCR. The DNA methylation levels at RdDM loci IGN5 and IGN23 were determined.
The whole genome was split into 500-bp bins, and the appearance of 24-nt reads in each bin were counted. The read perfect matched reads were extracted for subsequent analysis. Small RNA deep sequencing Small RNA was extracted from two-week-old seedlings as described previously [57] and subjected to Illumina sequencing. Raw reads from small RNA libraries were processed to remove adapter sequences and were classified according to the barcodes. Small RNAs with 18 to 27 nt were mapped to the Arabidopsis genome (TAIR10) using the Bowtie program [58], and only perfectly matched reads were extracted for subsequent analysis.

Co-immunoprecipitation and gel filtration
SUVH2-3xMyc and SUVH9-3xFlag transgenic plants were used to determine whether SUVH2 and SUVH9 interact with DRM3 by co-immunoprecipitation. SUVH9-3xFlag transgenic plants were crossed to MORC6-3xMyc transgenic plants, and the offspring plants harboring both transgenes were used to determine whether SUVH9 interact with MORC6. For co-IP, protein extracts were incubated with the agaroase-conjugated antibodies in Lysis buffer at 4°C for 2–3 h and precipitated by centrifugation. The precipitant was washed four times and then boiled for 5 min in 1×SDS sample buffer. After centrifugation, the supernatant was run on an SDS-PAGE gel, followed by Western blotting.

For gel filtration, total protein extracts from wild-type or transgenic plants were loaded onto a Superose 6 10/300 GL column (GE Healthcare, 17-5172-01) and the eluted solution was harvested once per 500 µl. The eluted fractions were run on a 10–12% SDS-PAGE gel and then subjected to Western blotting. Standard proteins were used to determine the sizes of the eluted fractions.

Yeast two-hybrid assay
The cDNA sequences were cloned into pGADT7 and pGBK7 vectors in frame to the C-termini of GAL4-AD and GAL4-BD, respectively. The DNA primers for cloning were listed in Table S5. The yeast strain PJ694a was co-transformed with pGADT7 and pGBK7 constructs and grown on the synthetic dropout medium minus Trp and Leu (SD-TL). The positive yeast colonies were streaked on both SD-TL and SD-TLH (the synthetic dropout medium minus Trp, Leu, and His) for growth assay. Growth of transformed positive yeast strains on SD-TLH indicates the interaction between GAL-AD fusion protein and GAL4-BD fusion protein in corresponding yeast strains. 20 mM 3-AT was used to inhibit the background growth of transformed strains on SD-TLH medium.

Small RNA deep sequencing
Small RNA was extracted from two-week-old seedlings as described previously [57] and subjected to Illumina sequencing. Raw reads from small RNA libraries were processed to remove adapter sequences and were classified according to the barcodes. Small RNAs with 18 to 27 nt were mapped to the Arabidopsis genome (TAIR10) using the Bowtie program [38], and only perfectly matched reads were extracted for subsequent analysis. The whole Arabidopsis genome was split into 300-bp bins, and the appearances of 24-nt reads in each bin were counted. The read

Affinity purification and mass spectrometric analysis
A 3-g quantity of flower tissue from SUVH2-3xMyc, SUVH9-3xFlag, or MORC6-3xFlag transgenic plants was harvested for affinity purification by anti-Flag antibody. Flower tissue was ground in liquid nitrogen and suspended in Lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, and 1 protease inhibitor cocktail tablet/50 ml [Roche]). The final supernatant was incubated with Anti-Flag M1 agaroase (Sigma, A 4596) in Lysis buffer at 4°C for 2.5 h and then precipitated by centrifugation. The precipitant was washed four times, and the agaroase-bound proteins were eluted with 3xFlag peptide (Sigma, F 4799).

The eluted proteins were run on a 10–12% SDS-PAGE gel and then subjected to silver staining with the ProteoSilver Silver Stain Kit (Sigma, PROT-SIL1). Total eluted proteins or specific target bands were cut from gels and extracted. Mass spectrometric analysis was performed as described previously [30]. Briefly, the peptides were eluted on a capillary column (50 µm x 10 cm) packed with 5-µm spherical C18 reversed-phase material (YMC, Kyoyo, Japan). The eluted peptides were loaded onto a LTQ mass spectrometer (Thermo Fisher Scientific) for analysis. The peptide sequence data were searched against the IPI (International Protein Index) Arabidopsis proteome database on the Mascot server (Matrix Science Ltd., London, UK).

binary pCAMBIA1305 vector or pRI909 vector. The DNA primers used for constructing were listed in Table S5. In these constructs, the genes were driven by their own native promoters, and the tags were fused to the C-terminal of the corresponding proteins. The constructs were transformed to wild-type plants by agrobacteria infection. T1 transgenic plants were grown on MS medium plates supplemented with 20 mg/ml hygromycin or 50 mg/ml kanamycin. The resistant seedlings were transplanted into soil and grown for the study. For complementation assay, the construct harboring the native promoter-driven SUVH2-3xMyc sequence was transformed into suvh2suh9 mutant Arabidopsis. The wild-type, SUVH9-3xFlag transgenic plants were used to determine whether SUVH2 and SUVH9 interact with DRM2 for DNA methylation. The methylated DNA is further bound by SUVH2 and SUVH9, resulting in a self-reinforcing loop that facilitates maintenance of DNA methylation at RdDM loci. Affinity purification and mass spectrometric analysis
A 3-g quantity of flower tissue from SUVH2-3xMyc, SUVH9-3xFlag, or MORC6-3xFlag transgenic plants was harvested for affinity purification by anti-Flag antibody. Flower tissue was ground in liquid nitrogen and suspended in Lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, and 1 protease inhibitor cocktail tablet/50 ml [Roche]). The final supernatant was incubated with Anti-Flag M1 agaroase (Sigma, A 4596) in Lysis buffer at 4°C for 2.5 h and then precipitated by centrifugation. The precipitant was washed four times, and the agaroase-bound proteins were eluted with 3xFlag peptide (Sigma, F 4799).

The eluted proteins were run on a 10–12% SDS-PAGE gel and then subjected to silver staining with the ProteoSilver Silver Stain Kit (Sigma, PROT-SIL1). Total eluted proteins or specific target bands were cut from gels and extracted. Mass spectrometric analysis was performed as described previously [30]. Briefly, the peptides were eluted on a capillary column (50 µm x 10 cm) packed with 5-µm spherical C18 reversed-phase material (YMC, Kyoyo, Japan). The eluted peptides were loaded onto a LTQ mass spectrometer (Thermo Fisher Scientific) for analysis. The peptide sequence data were searched against the IPI (International Protein Index) Arabidopsis proteome database on the Mascot server (Matrix Science Ltd., London, UK).
counts were then normalized by the library size. Reads per ten million (RPTM) were calculated for the adjusted small RNA abundance in each bin. The bins with low reads (<200 RPTM in all samples) were removed, and the regions were referred to as Pol IV-dependent siRNA regions if the RPTM value was five-time greater in the wild type than in the Pol IV mutant npd1. We also determine the RPTM value in Pol IV-dependent siRNA regions using 500-Kb windows to plot the profile of Pol IV-dependent siRNAs across chromosomes.

RNA deep sequencing-based gene expression analysis

The mRNA extracted from each genotype was used for Illumina library construction. The libraries were subjected to single-end Illumina sequencing. For data analysis, 45-bp sequences were mapped to the *Arabidopsis* genome (TAIR10) using TopHat [59]. Only reads mapped uniquely to the genome with a maximum of two mismatches were used for further analysis. Cufflinks [60] was used to quantify changes in gene expression. Genes with a significant differential expression (P<0.05) was chosen to plot Heat map using Gplots package in R.

Analyses of RNA transcripts by RT-PCR

Total RNA was extracted by the routine method with Trizol as described previously [57]. For RT-PCR, total RNA was treated with DNase to remove residual DNA contamination in each RNA sample. RT-PCR was performed with the TAKARA one-step RT-PCR kit. Either sequence-specific primers or oligo-dT were used for reverse transcription. The RNA transcript levels were determined by semi-quantitative RT-PCR or quantitative RT-PCR. All RT-PCR results were biologically repeated at least two times. The primers used for RT-PCR were listed in Table S3.

DNA methylation analysis

DNA methylation was tested by chop-PCR. Genomic DNA was cleaved with the methylation-sensitive restriction enzyme HaeIII or AluI, followed by PCR. The abundance of PCR products indicates the DNA methylation levels of the amplified loci. The actin gene sequence, which lacks an HaeIII recognition site, was amplified as an internal control. The DNA methylation analyses were repeated at least two times.

CHIP assay

The levels of histone H3K9me2, NRPE1-Flag and DMS3 on chromatin were determined by CHIP assay as described previously [17]. Two-week-old seedlings were soaked in 0.5% formaldehyde under vacuum for cross-linking. After chromatin was extracted and sonicated, it was immunoprecipitated with agarose-conjugated antibodies. Anti-Flag M2 Affinity Gel (A2220, Sigma) is directly used for immunoprecipitation, whereas anti-H3K9me2 antibody (ab1220, Abcam) and anti-DMS3 antibody [30] are conjugated with Protein A agarose followed by immunoprecipitation. Precipitated DNA as well as input DNA were subjected to quantitative PCR. The actin gene that has no NRPE1 and H3K9me2 enrichment was used as an internal control. Enrichment of NRPE1-Flag, H3K9me2 and DMS3 on chromatin was normalized by that on the actin gene. The primers used for quantitative PCR were listed in Table S3. The indicated results were obtained from three independent replicates.

Supporting Information

Figure S1 Detection of the interaction between the histone methyltransferases and the RdDM component DMS3 by co-IP.

Table S1 Summary of small RNA deep sequencing data.

Table S2 Numbers of Pol IV-dependent siRNA reads in 500-bp windows of the *Arabidopsis* genome in each ecotype.

Figure S2 The interaction of MORC6 with MORC1 and MORC2 was determined by yeast two-hybrid assay. MORC1, MORC2, and MORC6 were separately cloned into pGADT7 and/or pGBK7 vectors and transformed into the yeast strain PJ694a for yeast two-hybrid assay.

Figure S3 Detection of the interaction between truncated SUVH2 and SUVH9 sequences and RdDM components by yeast two-hybrid assay. Three individual yeast strains harboring the indicated GAL4-AD and GAL4-BD fusion constructs were streaked on the yeast synthetic dropout medium minus Trp, Leu, and His (SD-TLH) but supplemented with 3-AT and on the SD medium minus Trp and Leu (SD-TL). (A) Diagram of the truncated SUVH2 and SUVH9 sequences used in yeast two-hybrid assay. (B) The interaction between truncated SUVH2 sequences and the RdDM components DMS3 and MORC1. (C) The interaction between truncated SUVH9 sequences and the RdDM components DMS3, MORC6, and MORC1.

Figure S4 Accumulation of Pol IV-dependent 24-nt siRNAs as determined by small RNA deep sequencing. Pol IV-dependent 24-nt siRNA reads from 180 bp CEN, A6S1, A6S2, and MEA-ISR loci were counted based on small RNA deep sequencing data. The numbers of ta-siRNA255 reads in each corresponding library were used as controls to normalize the Pol IV-dependent siRNA reads. Shown are relative accumulation levels of indicated siRNAs in the wild type as well as in each mutant.

Figure S5 The Pol V-dependent RNA transcripts IGN5B, IGN23, and IGN25 were examined by quantitative RT-PCR. The mutants npd1, dsl1, and dms3, which were previously demonstrated to be required for Pol V-dependent RNA transcripts, were included as controls. The npd1 mutant that has no effect on Pol V-dependent RNA transcripts was also used as a control.

Figure S6 Determination of the NRPE1-3xFlag and DMS3 expression levels by Western blotting. (A) The NRPE1-3xFlag transgene was introduced into the wild type, dms3, and suh2suh9, respectively. The NRPE1-3xFlag expression was determined in transgenic plants as well as in the wild-type control by using anti-Flag antibody. The Ponceau S-stained rubisco protein is shown as a loading control. (B) The DMS3 expression level was determined in the wild type, dms3, and suh2suh9 by using anti-DMS3 antibody.

Table S1 Summary of small RNA deep sequencing data.

Table S2 Numbers of Pol IV-dependent siRNA reads in 500-bp windows of the *Arabidopsis* genome in each ecotype.
Table S3  Summary of RNA deep sequencing data.  
(XLS)

Table S4  List of differentially expressed genes in npet1, dmc3, and such2 such9 detected by RNA deep sequencing.  
(XLS)

Table S5  List of DNA oligonucleotides used in this study.  
(XLS)

References

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Author Contributions
Conceived and designed the experiments: ZWI, XJH. Performed the experiments: ZWI, CRS CJZ, NZX SWZ LL SC. Analyzed the data: ZWI, CRS HWH TC XJH. Wrote the paper: ZWI, XJH.