

A conserved transcriptional regulator is required for RNA-directed DNA methylation and plant development

Xin-Jian He,^{1,7} Yi-Feng Hsu,^{1,2,7} Shihua Zhu,³ Hai-Liang Liu,¹ Olga Pontes,⁴ Jianhua Zhu,⁵ Xinping Cui,⁶ Co-Shine Wang,² and Jian-Kang Zhu^{1,8}

¹Institute for Integrative Genome Biology and Department of Botany and Plant Sciences, University of California at Riverside, Riverside, California 92521, USA; ²Graduate Institute of Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan; ³College of Science and Technology, Ningbo University, Ningbo 315211, China; ⁴Biology Department, Washington University in St. Louis, St. Louis, Missouri 63130, USA; ⁵Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland 20742, USA; ⁶Department of Statistics, University of California at Riverside, Riverside, California 92521, USA

RNA-directed DNA methylation (RdDM) is a conserved mechanism for epigenetic silencing of transposons and other repetitive elements. We report that the *rdm4* (RNA-directed DNA Methylation4) mutation not only impairs RdDM, but also causes pleiotropic developmental defects in *Arabidopsis*. Both RNA polymerase II (Pol II)- and Pol V-dependent transcripts are affected in the *rdm4* mutant. *RDM4* encodes a novel protein that is conserved from yeast to humans and interacts with Pol II and Pol V in plants. Our results suggest that RDM4 functions in epigenetic regulation and plant development by serving as a transcriptional regulator for RNA Pol V and Pol II, respectively.

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DNA methylation and histone modifications are important epigenetic silencing mechanisms in eukaryotic cells (Chan et al. 2005; Matzke and Birchler 2005). In plants, DNA methylation is catalyzed by DNA methyltransferase MET1, CMT3, and DRM2 (Chan et al. 2005). MET1 and CMT3 mainly function in maintaining DNA hypermethylation at CG and CHG sites (H is A, T, or C) during DNA duplication, while the function of DRM2 is involved in de novo DNA methylation at CHH sites directed by RNA (Chan et al. 2005). RNA-directed DNA methylation (RdDM) was first discovered in plants

(Wassenegger et al. 1994), and plays important roles in transgene silencing, genome integrity, and transposon stability (Matzke et al. 2009).

In the RdDM pathway, both 24-nucleotide (nt) siRNAs and long noncoding RNA transcripts are essential for de novo DNA methylation (Wierzbicki et al. 2008). The 24-nt siRNAs are generated in a pathway involving the putative DNA-directed RNA polymerase IV (Pol IV), RDR2 (RNA-dependent RNA polymerase 2), and DCL3 (Dicer-like 3) (Matzke et al. 2009), while noncoding RNAs are produced by another putative DNA-directed RNA polymerase, Pol V (Wierzbicki et al. 2008). The 24-nt siRNAs are loaded onto ARGONAUTE proteins AGO4 and/or AGO6 (Zheng et al. 2007; Matzke et al. 2009) and target genomic regions, possibly by base-pairing with nascent Pol V transcripts from the target loci. The Pol V-dependent RNA transcripts are tethered to AGO4 by the action of KTF1, forming an effector complex containing AGO4, KTF1, Pol V-dependent transcripts, and siRNAs (He et al. 2009a). The effector complex directs DRM2 for de novo methylation of target genomic sequences. The process is facilitated by the chromatin remodeling protein DRD1 and the hinge domain-containing protein DMS3 (Matzke et al. 2009).

RNA Pol IV and Pol V have distinct largest subunits—NRPD1 and NRPE1, respectively—but share some common subunits such as NRPD/E2 and NRPD/E4 (He et al. 2009b). Some subunits of Pol IV and Pol V are also shared with Pol II (Huang et al. 2009; Ream et al. 2009). It appears that the plant-specific RNA Pol IV and Pol V evolved from an ancestral RNA polymerase to function specifically in RdDM. The transcription activity of Pol II is tightly regulated with the help of a series of general transcription factors (Kornberg 2007). Although many general transcription factors of Pol II have been studied extensively, nothing is known about the regulation of transcription by Pol IV and Pol V.

In the present study, we carried out a forward genetic screen for second site suppressors of the DNA demethylase mutant *ros1*, and identified a transcription factor, RDM4 (RNA-directed DNA Methylation4), that is required for RdDM in *Arabidopsis*. Interestingly, unlike other RdDM mutants, the *rdm4* mutant plants display pleiotropic developmental phenotypes. *RDM4* encodes a novel protein that is conserved in eukaryotic organisms. We found that RDM4 interacts with Pol II and Pol V in plants. Our results suggest that RDM4 is a regulator of Pol II and Pol V transcription, and thereby contributes to both development and RdDM.

Results and Discussion

The rdm4 mutation suppresses transcriptional gene silencing (TGS)

A subset of the RdDM target loci is under dynamic control by DNA methylation and active demethylation (Zhu et al. 2007). In our genetic system, these targets include an active *LUCIFERASE* transgene driven by the cold and salt stress-responsive *RD29A* promoter (*RD29A-LUC*) and the corresponding endogenous *RD29A* gene, which are silenced when the DNA demethylase gene *ROS1* loses its function (Gong et al. 2002). This TGS is caused by DNA hypermethylation, and requires

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⁷These authors contributed equally to this work.

⁸Corresponding author.

E-MAIL jian-kang.zhu@ucr.edu; FAX (951) 827-7115.

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heterochromatic 24-nt siRNAs from the *RD29A* promoter (Zheng et al. 2007). The CaMV 35S promoter-driven *NPTII* transgene (*35S-NPTII*) linked to the *RD29A-LUC* transgene is also silenced in *ros1* plants (Gong et al. 2002). A T-DNA mutagenized population in the *ros1-1* background was screened to identify new components in the RdDM pathway, based on the luminescence phenotype of *RD29A-LUC*. This screen recovered not only mutants in known RdDM pathway components—such as *NRPD1*, *NRPE1*, *NRPD2/NRPE2*, *AGO4*, *HEN1*, *DRD1*, and *HDA6* (He et al. 2009b)—but also several new components. One of the new components is designated as *RDM4*. The *rdm4* mutation released the silencing of the *RD29A-LUC* transgene in the *ros1* background, resulting in enhanced luminescence after cold or salt treatment of plants (Fig. 1A,B). However, the mutation did not release the silencing of *35S-NPTII* in *ros1* (Fig. 1C). When the *ros1rdm4* mutant was crossed to *ros1*, the F1 plants emitted as little luminescence as the *ros1* plants, suggesting that the *rdm4* mutation was recessive. Analysis of the resulting selfed F2 population confirmed the recessive and single-gene nature of the mutation (data not shown).

While the expression of endogenous *RD29A*, *RD29A-LUC*, and *35S-NPTII* was blocked by the *ros1* mutation as reported previously (Gong et al. 2002), the *rdm4* mutation partially restored the transcript levels of endogenous *RD29A* and transgene *RD29A-LUC*, but not of *35S-NPTII* (Fig. 1C). Therefore, the *rdm4* mutation releases the silencing of *RD29A-LUC* but not *35S-NPTII*. This effect is very similar to that of *nRPD1*, *nrpe1*, *nRPD2*, *drd1*, and *ago6* mutations (Zheng et al. 2007; He et al. 2009b). Because the TGS of the *35S-NPTII* transgene is mediated by an siRNA-independent pathway (He et al. 2009b), these results suggest that, like

NRPD1, *NRPE1*, *NRPD2*, *DRD1*, and *AGO6*, *RDM4* functions specifically in the siRNA-dependent pathway of TGS.

The rdm4 mutation reduces DNA methylation at RdDM target loci

To test whether the release of TGS in *ros1rdm4* mutant plants correlates with DNA hypomethylation, we determined the DNA methylation status of a 361-base-pair (bp) region of the *RD29A* promoter in both the *RD29A-LUC* transgene and endogenous *RD29A* by Southern hybridization and bisulfite sequencing. Consistent with previous results, high levels of DNA methylation were observed in *ros1* in all three cytosine contexts (CG, CHG, and CHH; H stands for A, T, or C) at the promoter of both the *RD29A-LUC* transgene and the endogenous *RD29A* gene, whereas low levels of DNA methylation were observed in the wild-type plants (Fig. 2A,B). As in *ros1nrpd1*, the DNA methylation was reduced in *ros1rdm4* at both the transgene and endogenous *RD29A* promoters in all cytosine contexts. In both the transgene and endogenous *RD29A* promoters, the reduction in cytosine methylation was dramatic at CHH, modest at CHG, and marginal at CG sites (Fig. 2A,B). For example, at the *RD29A-LUC* transgene promoter, CHH methylation was 15.2% in *ros1*, 4.4% in *ros1rdm4*, and 2.8% in *ros1nrpd1*, while the CG methylation is 75.3%, 64.3%, and 58.3%, respectively, for the different genotypes. The inhibitory effect of *rdm4* on DNA methylation was further supported by Southern hybridization. Genomic DNA from wild type, *ros1*, *ros1rdm4*, and *ros1nrpd1* was digested with a methylation-sensitive restriction enzyme, *Bst*UI (CGCG), followed by Southern hybridization using the *RD29A* coding region as a probe to assess methylation of the endogenous *RD29A* gene. The result was consistent with the bisulfite sequencing data and showed that the DNA methylation of the endogenous *RD29A* promoter in *ros1rdm4* and *ros1nrpd1* is partially suppressed compared with *ros1* (Fig. 2C). Therefore, like *nRPD1*, *rdm4* suppresses TGS of *RD29A-LUC* and endogenous *RD29A* in *ros1* by blocking DNA hypermethylation at the *RD29A* promoter.

We assayed the DNA methylation status of the centromeric region using the methylation-sensitive restriction enzyme *Hae*III (for CHH methylation) and the isoschizomers *Hpa*II (for CG and CHG methylation) and *Msp*I (for CG methylation) followed by Southern hybridization. The results showed no differences in DNA methylation of the highly repetitive 180-bp centromeric repeat among wild-type, *ros1*, *ros1rdm4*, and *ros1nrpd1* mutant plants (Supplemental Fig. S1). The DNA methylation of 5S rDNA was also detected by Southern hybridization. In contrast to the heavy methylation of 5S rDNA in the wild type and *ros1*, methylation in all cytosine contexts was clearly reduced in *ros1rdm4*, as it was in *ros1nrpd1* (Fig. 2D). Southern hybridization also revealed a reduction of DNA methylation at *AtMU1* in *ros1rdm4* and *ros1nrpd1*, compared with the wild type and *ros1* (Fig. 2E). Furthermore, *AtSN1* methylation was examined. After digestion with the methylation-sensitive enzyme *Hae*III, *AtSN1* could be amplified in the wild type and *ros1*, but not in *ros1rdm4*, *ros1nrpd1*, and *rdm4* (Fig. 2F), indicating that *AtSN1* methylation was blocked by the *rdm4* mutation.

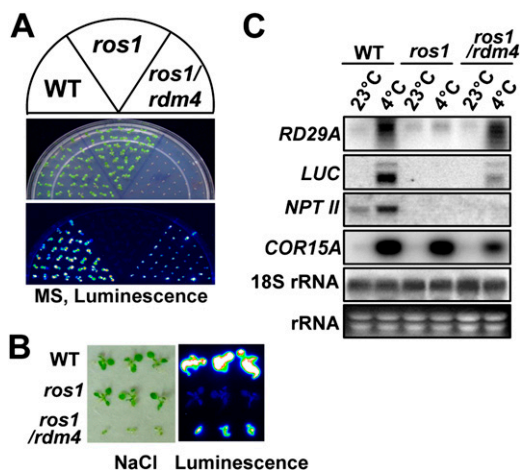


Figure 1. TGS phenotypes of the *ros1rdm4* mutant plants. (A,B) Expression of the *RD29A-LUC* transgene by luminescence emission. (A) Wild type, *ros1*, and *ros1rdm4* were grown on MS plates and imaged after cold treatment (24 h, 4°C). (B) The indicated plants were treated with 200 mM NaCl for 3 h, followed by luminescence imaging. (C) The transcript levels of endogenous *RD29A*, and *RD29A-LUC* and *35S-NPTII* transgenes in wild type, *ros1*, and *ros1rdm4* were determined by RNA blot analysis. 18S rRNA hybridization and ethidium bromide-stained rRNA bands were used as RNA loading controls, and *COR15A* was used as a cold treatment control.

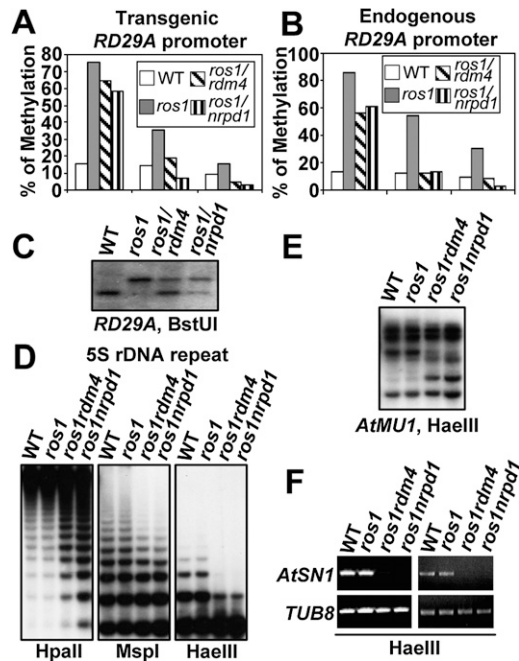


Figure 2. Effect of *rdm4* on DNA methylation. The percentage of cytosine methylation at transgene (A) and endogenous (B) *RD29A* promoters was determined by bisulfite sequencing. The percentage of cytosine methylation on CG, CHG, and CHH (H stands for A, T, or C) sites is shown. (C) The *rdm4* mutation reduces DNA methylation at the endogenous *RD29A* promoter as determined by Southern hybridization. Genomic DNA was digested with the methylation-sensitive restriction enzyme BstUI. (D) The *rdm4* mutation reduces DNA methylation at 5S rDNA repeat. (E) Genomic DNA from indicated genotypes was digested with the methylation-sensitive restriction enzyme HaeIII, followed by Southern hybridization with the *AtMU1* probe. (F) Effect of *rdm4* on DNA methylation of *AtSN1*. PCR amplification of *AtSN1* was performed after the genomic DNA was digested with HaeIII. *TUB8* DNA was amplified as an internal control.

The effect of the *rdm4* mutation on the expression, siRNAs, and Pol V-dependent transcripts of RdDM target loci

The reduced DNA methylation at the transposons and other RdDM target loci prompted us to examine the expression levels of these loci. We determined the transcript levels of *AtSN1*, *AtGP1*, and *AtMU1* by semiquantitative RT-PCR. The results indicated that the transcript levels of *AtSN1*, *AtGP1*, and *AtMU1* were substantially increased in *ros1rdm4*, *rdm4*, and *ros1nrpd1*, compared with those in the wild type and *ros1* (Fig. 3A). The data suggest that, like in *ros1nrpd1*, the loss of DNA methylation in *ros1rdm4* and *rdm4* results in increased expression of the RdDM target loci. Previous studies showed that the *ROS1* transcript level is down-regulated in RdDM pathway mutants and other mutants with reduced genomic DNA methylation (Huettel et al. 2006; Mathieu et al. 2007). Consistent with the effect of *rdm4* on DNA methylation, the *ROS1* transcript level was also reduced in *ros1rdm4* and *rdm4*, as well as in *ros1nrpd1*, compared with the wild type and *ros1* (Fig. 3A).

In the *ros1* mutant background, 24-nt siRNAs from the transgene *RD29A* promoter are the trigger of TGS of *RD29A-LUC* and endogenous *RD29A* (Gong et al. 2002; Zheng et al. 2007). A small RNA blot analysis showed

that 24-nt *RD29A* promoter siRNAs were reduced in *ros1rdm4*, as they were in *ros1nrpd1* (Fig. 3B). In contrast, the siRNAs were completely blocked in *ros1nrpd1* (Fig. 3B). We also tested the accumulation of siRNAs from other RdDM target loci, including *AtSN1*, 5S rDNA/siRNA1003, *AtGP1*, *AtMU1*, Cluster 4, and siRNA02. Similar to the *RD29A* promoter siRNAs, the accumulation of 24-nt siRNAs from *AtSN1*, 5S rDNA/siRNA1003, *AtGP1*, *AtMU1*, and Cluster 4 was reduced but not abolished in *ros1rdm4* (Fig. 3B). siRNA02 was not affected in *ros1rdm4* (Fig. 3B). The siRNA pattern in *ros1rdm4* is very similar to that in *ros1nrpd1*, whereas in *ros1nrpd1* the heterochromatic siRNAs were eliminated. The accumulation of microRNA171 (miRNA171) and *trans*-acting siRNA255 (ta-siRNA255) was also tested, and the results suggest that the *rdm4* mutation has little effect on miRNAs and ta-siRNAs (Fig. 3B). Based on these results, we conclude that RDM4 is required for the full accumulation of heterochromatic siRNAs and, like NRPE1 of Pol V, it may also be required for the functioning of the siRNAs through the RdDM pathway.

Pol V was shown recently to generate transcripts from several RdDM target loci, and the nascent transcripts are proposed to function as scaffolds to recruit the AGO4-containing RdDM effector complex to the target loci (Wierzbicki et al. 2008). We found that Pol V-dependent transcripts from *AtSN1* and *IGN5* were reduced in *ros1rdm4* compared with those in the wild type and *ros1* (Fig. 3C). As expected, the transcripts were blocked in *ros1nrpd1*, but not much affected in *ros1nrpd1* (Fig. 3C).

The effect of the *rdm4* mutation on plant development and the expression of Pol II-transcribed genes

The *ros1rdm4* mutant plants are considerably smaller than the *ros1* or wild-type plants (Fig. 4A). The mutant

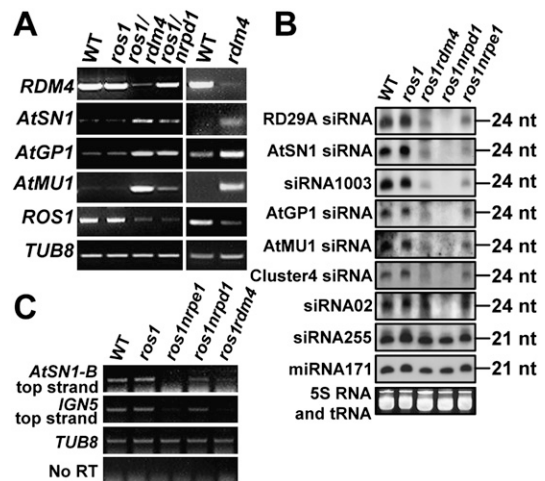


Figure 3. Effect of the *rdm4* mutation on RNA transcript levels and siRNA accumulation. (A) The RNA transcript levels of the indicated loci were determined by semiquantitative RT-PCR. *TUB8* was used as an internal control. (B) Detection of small RNAs in the indicated genotypes by Northern blot analysis. The ethidium bromide-stained gel corresponding to 5S rDNA and tRNA was included as a loading control. The positions of size markers are indicated (24 nt or 21 nt). (C) RT-PCR detection of Pol V-dependent transcripts from *AtSN1-B* and *IGN5*. *TUB8* was used as an internal control, and the RNA samples without reverse transcription (No RT) were used as negative control, indicating no DNA contamination.

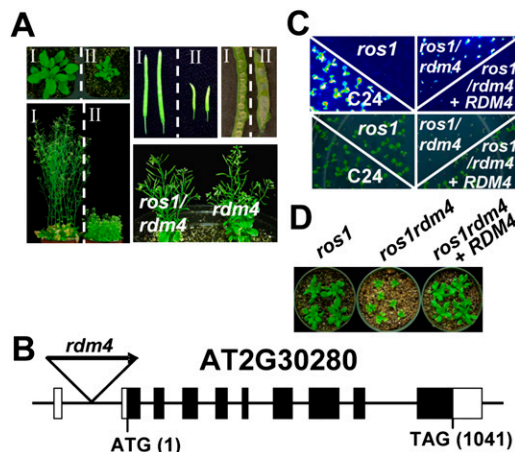


Figure 4. Developmental phenotypes of *rdm4* mutant plants and mutant complementation. (A) The pleiotropic developmental phenotypes of *ros1rdm4* and *rdm4* mutant plants. (Panels I) *ros1* plants. (Panels II) *ros1rdm4* plants. (B) Diagram of the *RDM4* gene showing the position of exons (boxes), introns (lines), and site of T-DNA insertion. (C) The *RDM4* genomic construct complements the luminescence phenotype of *ros1rdm4* in a representative T2 transgenic line. (D) The *RDM4* transgene complements the developmental defects of *ros1rdm4*. The complemented plants shown are T1 transgenic lines.

plants produce shorter siliques and exhibit partial sterility (Fig. 4A). The mutant seeds show a severe dormancy and germinate later than *ros1* (Supplemental Fig. S2). Most of the *ros1* seeds germinated at the third or fourth day after imbibition, but the *ros1rdm4* seeds did not all germinate until the 11th day (Supplemental Fig. S2). The developmental phenotypes of *ros1rdm4* are heritable and recessive, and the phenotypes are also observed in the *rdm4* single mutant (Fig. 4A). In a segregating backcrossed F2 population, the morphological phenotypes cosegregated with the luminescence phenotype.

Related to the pleiotropic developmental phenotypes of *rdm4*, there are changes in the expression of protein-coding genes in the mutant. For example, we noticed that the stress-induced *COR15A* gene, which was used as a cold stress treatment control for assays on *RD29A* expression, showed a reduced transcript level in *ros1rdm4* (Fig. 1C). Microarray analysis showed that, in addition to *COR15A*, several other abiotic stress-responsive genes—such as *KIN1*, *KIN2*, *RD26*, and *RD29B*—have reduced expression in *ros1rdm4* compared with *ros1* (Supplemental Tables S1, S2). In contrast, many pathogen resistance-related genes—such as *PR1*, *PR4*, *PAD4*—and several WRKY transcription factors show increased expression in *ros1rdm4* compared with *ros1*, suggesting that the systemic acquired resistance pathway may be activated in the *rdm4* mutant (Supplemental Tables S1, S3). The reduced expression of abiotic stress-responsive genes in *ros1rdm4* was confirmed by Northern blot analysis and real-time PCR (Supplemental Fig. S3A,B). The cold-induced expression, as well as basal expression, of *COR15A* and *KIN2* was reduced in *ros1rdm4* compared with that in the wild type and *ros1* (Supplemental Fig. S3A,B). Real-time PCR analysis showed that, in contrast to *rdm4*, the *nprpd1* and *nprpe1* mutations did not cause a reduction in *COR15A* or *KIN2* expression (Supplemental Fig. S3B). These results suggest

that, in addition to its function in RdDM, *RDM4* has a role in plant development and in regulating stress-responsive genes, which are presumed to be transcribed by RNA Pol II.

RDM4 encodes a novel protein conserved from yeast to humans

In the *ros1rdm4* mutant, a single T-DNA insertion was found in the intron of the 5'-untranslated region (UTR) of *AT2G30280* (Fig. 4B). In *ros1rdm4* and *rdm4*, the *RDM4* transcript was substantially reduced but not abolished (Fig. 3A), so the *rdm4* mutation is a partial loss-of-function allele. To confirm that *RDM4* is the correct gene, a construct harboring the full genomic sequence of *RDM4* was generated and introduced into *ros1rdm4* mutant plants. In seven independent T1 transgenic plants, the wild-type *RDM4* transgene rescued the luminescence phenotype of the *ros1rdm4* mutant (Supplemental Fig. S4). The DNA methylation status of *AtSN1* was tested in wild-type, *ros1*, *ros1rdm4*, and the seven transgenic T1 plants. The results suggest that the *RDM4* transgene also complemented the DNA methylation phenotype of *ros1rdm4* (Supplemental Fig. S4). Complementation of the luminescence phenotype was confirmed in T2 transgenic lines (Fig. 4C). Furthermore, the transgene fully rescued the developmental phenotype of the *ros1rdm4* mutant (Fig. 4D) and corrected its defects in stress-responsive genes (Supplemental Fig. S3A). Therefore, we conclude that *At2G30280* is the *RDM4* gene that functions in development and RdDM.

RDM4 encodes a protein of 346 amino acids that has sequence similarity to the yeast IWR1 (interacts with RNA Pol II) and homologous proteins in *Drosophila* and humans (Supplemental Fig. S5A,B). The homologous proteins in *Drosophila* and humans have not been characterized. The function of yeast IWR1 is also unclear, but the protein appears to interact with RNA Pol II and Pol II general transcription factors (Gavin et al. 2006; Krogan et al. 2006). Affinity-capture mass spectrometry experiments identified many Pol II subunits (e.g., RPB2, RPB3, RPB4, RPB5, RPB7, RPB8, RPB9, RPB10, RPB11, RPO21, and RPO26) and several TFIIIF subunits (e.g., TFG1 and TFG2) that formed a complex with IWR1. These interactions suggest a role for yeast IWR1 in regulating RNA Pol II transcription. Proteins highly similar to *RDM4* can be found in other plant species, such as grapes, rice, and maize (Supplemental Fig. S6A). Phylogenetic analysis shows that *RDM4* and its orthologs in grapes, rice, and maize are grouped into a plant-specific clade, which is divided into two subclades: one from dicotyledonous plants, and the other from monocotyledonous plants (Supplemental Fig. S6B). The human IWR1-like protein appears more closely related to the plant *RDM4* than to the yeast IWR1 and fly IWR1-like protein (Supplemental Figs. S5A,B, S6B).

RDM4 interacts with RNA Pol II and Pol V in plants

The *RDM4* protein level in *ros1rdm4* was substantially reduced compared with that in *ros1* (Fig. 5A). The *RDM4* protein level was not affected in *ros1nprpd1* or *ros1nprpe1* (Fig. 5A). Anti-Flag antibodies pulled down *RDM4* from extracts of NRPE1-Flag-expressing plants but not NRPD1-Flag-expressing plants (Fig. 5B), suggesting that *RDM4* interacts with Pol V in vivo. Anti-*RDM4* antibodies

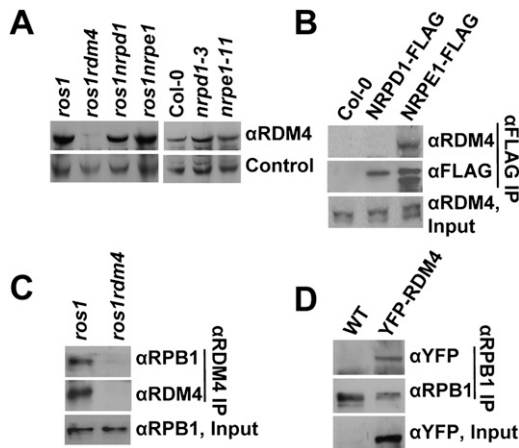


Figure 5. RDM4 interacts with NRPE1 and RPB1. (A) The RDM4 protein level was assessed by Western blot analysis in the indicated genotypes. A nonspecific band (Control) is shown as loading control. (B) Coimmunoprecipitation between RDM4 and NRPE1-Flag. The protein extracts from the indicated genotypes were precipitated by anti-Flag antibody-conjugated beads, and the precipitates were detected by anti-RDM4 and anti-Flag antibodies. (C) Coimmunoprecipitation between RDM4 and RPB1. The protein extracts from *ros1* and *ros1rdm4* were precipitated by anti-RDM4 antibody-conjugated beads, and the precipitates were detected by anti-RPB1 and anti-RDM4 antibodies. (D) The protein extracts from wild-type and *YFP-RDM4* transgenic plants were precipitated by anti-RPB1 antibody-conjugated beads, and the precipitates were detected by anti-YFP and anti-RPB1 antibodies.

pulled down RPB1 of Pol II in *ros1* but not in *ros1rdm4* (Fig. 5C). The result suggests an *in vivo* interaction between RDM4 and Pol II. This is further supported by the observation that antibodies specific for RPB1 could coimmunoprecipitate RDM4-YFP from RDM4-YFP-expressing plants, but not from wild-type control plants (Fig. 5D).

The largest and second largest subunits of RNA Pol IV and Pol V are key components of the RdDM pathway (Matzke et al. 2009). We recently found another subunit, RDM2/NRPD4, that is shared by Pol IV and Pol V, and that is also necessary for the RdDM pathway (He et al. 2009b). Like Pol II (Cramer 2002), Pol IV and Pol V are multisubunit complexes (Huang et al. 2009; Ream et al. 2009). In addition to requiring various polymerase subunits, the functioning of Pol II as well as of Pol I and Pol III requires several positive and negative transcriptional regulators (Wade and Struhl 2008). It is not known whether the functioning of Pol IV and Pol V in RdDM also requires transcriptional regulators and whether such transcriptional regulators are specific for Pol IV and/or Pol V or are shared with Pol II. RDM4 is possibly the first characterized transcriptional regulator of both Pol II and Pol V. The interaction of yeast IWR1 with Pol II and the general transcription factor TFIIF suggests that IWR1 is a regulator of Pol II transcription (Gavin et al. 2006; Krogan et al. 2006). IWR1 is conserved in yeast, plants, and animals (Supplemental Figs. S5, S6). Our findings of a coimmunoprecipitation between RDM4 and RPB1 and the effect of *rdm4* mutation on plant development and on several Pol II-transcribed genes, strongly support that RDM4 is a regulator of Pol II transcription in plants. Our results also demonstrate that RDM4 is required for RdDM and for the accumulation of Pol V transcripts,

thereby suggesting a function of RDM4 in regulating Pol V transcription. The role of RDM4 in RdDM is unlikely to be an indirect effect of its function in Pol II transcription, since the transcript level of none of the RdDM components is affected by the *rdm4* mutation (data not shown). Consistent with a direct role of RDM4 in RdDM, we found that RDM4 interacts with Pol V, a key component of the RdDM pathway.

Concluding remarks

Our results suggest that RDM4 is a transcriptional regulator shared by Pol II and Pol V. Unlike the Pol V-specific *nrpe1* mutant or many other RdDM mutants reported to date, which have little or no developmental phenotypes, the *rdm4* mutant has strong developmental defects. The pleiotropic phenotypes of *rdm4* mutant plants likely reflect a role of RDM4 in Pol II function. Microarray analysis revealed several hundred Pol II-type genes having reduced or increased expression in the *rdm4* mutant (Supplemental Table S1), although it is not known which of these genes are directly affected by *rdm4*. The possible activation of systemic acquired resistance, as suggested by the elevated expression of many pathogen resistance-related genes in *rdm4*, may contribute to the slow growth of the mutant plants. Some genes may be particularly sensitive to perturbations of Pol II transcription. Alternatively, RDM4 may specifically regulate the transcription of only a subset of Pol II target genes. In conclusion, our results show that RDM4 functions in both plant development and epigenetic regulation, and suggest that RDM4 may regulate coding genes through Pol II and noncoding loci through Pol V in the RdDM pathway.

Materials and methods

Plant growth, mutant screening, cloning, and others

A homozygous *RD29A-LUC* transgene was in the wild-type C24 and *ros1* mutant plants in this study (Ishitani et al. 1997; Gong et al. 2002). A T-DNA mutagenized *ros1-1* population was generated as described previously (He et al. 2009b). A 3-d cold stratification was used to promote the uniformity of seed germination. Plants were grown with 16 h of light and 8 h of darkness at 23°C. Based on the expression of the transgenic *RD29A* promoter-driven luciferase gene by luminescence imaging (Ishitani et al. 1997), putative suppressors of *ros1* were identified and transferred to soil. To eliminate false positives, a young leaf taken from each of these seedlings was applied for luminescence imaging after treatment with 200 mM NaCl for 3 h. Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al. 1995) was carried out to determine the T-DNA flanking sequence of the mutants. The full genomic sequence of *RDM4* was amplified and cloned into a Gateway vector PMDC164 for *Arabidopsis* complementation assay. Because *ros1rdm4* is partially sterile, the F1 plants from the cross of *ros1rdm4* and *ros1* were used for transformation of the *RDM4* complementation construct. Transgenic plants containing the construct in the homozygous *ros1rdm4* background were identified in the T1 generation of the transformants. Expression of YFP-RDM4 fusion protein was driven by the CaMV 35S promoter. The Fitch-Margoliash method was applied to analyze the protein sequences of yeast IWR1 and IWR1-like proteins in *Drosophila*, humans, and plants.

RNA analysis

Northern blot analysis of mRNAs and small RNAs was carried out as described in He et al. (2009b). The sequences of DNA oligos used for probes or probe amplification are listed in Supplemental Table S4.

Semiquantitative RT-PCR was carried out to determine the transcript levels of the RdDM targets, including *AtSN1*, *AtGPI1*, and *AtMUI1*, as

described in He et al. (2009b). The procedure of RT-PCR amplification of Pol V-dependent transcripts was described by Wierzbicki et al. (2008). Primers used in RT-PCR analysis are listed in Supplemental Table S4.

Microarray analysis was carried out as described previously (Lee et al. 2005) using Affymetrix *Arabidopsis* ATH1 GeneChip. Two biological replicates were used for each genotype. The preprocessing statistical method RMA (Irizarry et al. 2003) was applied to raw .CEL files for background adjustment and quantile normalization. The log-transformed values were then analyzed using the Bioconductor package siggenes (Tusher et al. 2001). A list of genes with statistically significant changes in expression between the genotypes was generated.

DNA methylation assay

Bisulfite sequencing, Southern hybridization, and PCR-based DNA methylation assays were carried out as described previously (He et al. 2009a,b).

Coimmunoprecipitation and Western blotting

Three grams of seedlings from wild-type, NRPD1-Flag, and NRPE1-Flag (driven by their respective endogenous gene promoters) transgenic plants (Pontes et al. 2006) and the other indicated plants were grounded and suspended in 10 mL of protein extraction buffer. After centrifugation, equal amounts of protein extracts were incubated with the indicated antibody-conjugated beads, followed by washing with protein extraction buffer three times. The precipitated proteins were eluted from the beads with SDS-PAGE sample buffer. The eluted samples were resolved by 12% SDS-PAGE for Western blotting. The full-length *RDM4* ORF was cloned into the bacterial expression vector pET-28a for *RDM4* protein production. Anti-*RDM4* antibodies were produced by injecting rabbits with purified histone-tagged full-length *RDM4* recombinant. The *RDM4* antibody was affinity-purified with *RDM4* protein-conjugated resin (YenZym Antibodies).

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