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An SGS3-like protein functions in RNA-directed DNA methylation and transcriptional gene silencing in Arabidopsis

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Summary

RNA-directed DNA methylation (RdDM) is an important epigenetic mechanism for silencing transgenes and endogenous repetitive sequences such as transposons. The *RD29A* promoter-driven *LUCIFERASE* transgene and its corresponding endogenous *RD29A* gene are hypermethylated and silenced in the Arabidopsis DNA demethylase mutant *ros1*. By screening for second-site suppressors of *ros1*, we identified the *RDM12* locus. The *rdm12* mutation releases the silencing of the *RD29A-LUC* transgene and the endogenous *RD29A* gene by reducing the promoter DNA methylation. The *rdm12* mutation also reduces DNA methylation at endogenous RdDM target loci including transposons and other repetitive sequences. In addition, the *rdm12* mutation affects the levels of siRNAs from some of the RdDM target loci. *RDM12* encodes a protein with XS and coiled-coil domains and is similar to SGS3, which is a partner protein of RDR6 and can bind to double-stranded RNAs with a 5' overhang and is required for several posttranscriptional gene silencing pathways. Our results show that RDM12 is a component of the RdDM pathway and suggest that RdDM may involve double stranded RNAs with a 5' overhang and the partnering between RDM12 and RDR2.

Keywords

RDM12; SGS3; epigenetics; DNA methylation; siRNA

Introduction

Transcriptional as well as posttranscriptional gene silencing is frequently caused by transgenes and virus infection (Baulcombe, 2004; Vaucheret et al., 2001). Transcriptional gene silencing (TGS) also occurs at endogenous loci, particularly transposons, retrotransposons and other repetitive sequences (Chan et al., 2004; Matzke and Birchler, 2005; Huettel et al., 2006). In the TGS pathway, the transgenes and endogenous sequences are usually associated with cytosine methylation and repressive histone modifications,

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epigenetic changes that maintain the silencing and ensure genome stability (Henderson and Jacobsen, 2007; Matzke et al., 2009). RNA-directed DNA methylation (RdDM) is a mechanism for establishing TGS. The RdDM pathway in plants involves three DNA-dependent RNA polymerases, Pol II (Zheng et al., 2009), Pol IV and Pol V (Wierzbicki et al., 2008). Pol IV is hypothesized to be responsible for transcribing methylated sequences to generate abberant RNA transcripts, which are converted to double-stranded RNAs by the RNA-dependent RNA polymerase RDR2 (Xie et al., 2004; Herr et al., 2005; Pontes et al., 2006). The double-stranded RNA is cleaved into 24-nt siRNAs by the Dicer-like protein DCL3 (Xie et al., 2004). Pol II and Pol V appear to be involved in the synthesis of scaffold RNA transcripts that help recruit the RdDM effector complex (Zheng et al., 2009; Wierzbicki et al., 2008). The RdDM effector complex contains the ARGONAUTE protein AGO4 that binds to the 24 nt siRNAs, the WG/GW repeats-containing protein KTF1 that binds the scaffold transcripts and AGO4, and the de novo DNA methyltransferase DRM2 (Zilberman et al., 2003; Wierzbicki et al., 2008; He et al., 2009b; Cao and Jacobsen, 2002). The effector is guided to specific genomic targets by the base pairing between the siRNA guide and the nascent scaffold transcripts, and leads to de novo DNA methylation (Cao and Jacobsen, 2002; Li et al., 2006; Pontes et al., 2006; He et al., 2009b). The chromatin remodeling protein DRD1 and the SMC-related protein DMS3 facilitate the process and function at downstream steps in the RdDM pathway (Kanno et al., 2004; Kanno et al., 2008; Ausin et al., 2009).

In several post-transcriptional gene silencing (PTGS) pathways, the double-stranded RNA is synthesized by the RNA-dependent RNA polymerase RDR6 (Mourrain et al., 2000; Vaucheret et al., 2001). The stabilization of the double-stranded RNA requires the RNA binding protein SGS3 (Mourrain et al., 2000; Fukunaga and Doudna, 2009). DCL4 is responsible for the dicing of the double-stranded RNA into 21 nt siRNAs, which are loaded onto the ARGONAUTE protein AGO1 to target complementary RNAs for cleavage (Yoshikawa et al., 2005; Morel et al., 2002; Vazquez et al., 2004). The PTGS and TGS pathways share the methyltransferase protein HEN1, which introduces a 2'-O-methyl group to the 3′-terminal nucleotide of small RNAs for increased stability (Boutet et al., 2003; Yu et al., 2005). In addition, the TGS and PTGS pathways both require ARGONAUTE proteins, Dicer-like proteins and RNA-dependent RNA polymerases, suggesting an extensive similarity between the two gene silencing pathways (Mourrain et al., 2000; Morel et al., 2002; Zilberman et al., 2003; Xie et al., 2004; Yoshikawa et al., 2005).

We have previously shown that the *RD29A* promoter-driven *LUCIFERASE* transgene is expressed at high levels in wild-type genetic backgrounds, although 24 nt siRNAs are generated from the transgene promoter (Gong et al., 2002). However, in the *ros1* mutant, the 24 nt siRNAs cause DNA hypermethylation at the *RD29A* promoter and consequent TGS of the transgene and endogenous gene (Gong et al., 2002). *ROS1* encodes a bifunctional DNA glycosylase/lyase, which functions to demethylate DNA through a base excision repair pathway (Gong et al., 2002; Agius et al., 2006). The methylation status of the *RD29A* promoter is under dynamic regulation by RdDM and the ROS1-mediated DNA demethylation pathway (Agius et al., 2006; Zhu et al., 2007). By screening for second-site suppressors of *ros1*, we identified most of the previously known RdDM components such as NRPD1, NRPE1, NRPD2, AGO4, DRD1 and HEN1 (He et al., 2009a). Moreover, we found several additional RdDM components including AGO6, the histone 2B deubiquitination enzyme UBP26, NRPD/E4, a common subunit of Pol IV/Pol V, KTF1, a WG/GW motif containing protein, and RDM4, a transcription factor for both Pol II and Pol V (He et al., 2009a; He et al., 2009b; He et al., 2009c). The results suggest that screening for *ros1* suppressor mutants is an excellent approach to identifying new components of the RdDM pathway. Here, we report another important RdDM component, RDM12. The *rdm12* mutation releases the silencing of the *RD29A-LUC* transgene and endogenous *RD29A* gene

in the *ros1* mutant background. This effect is correlated with reductions in promoter DNA methylation. Moreover, the *rdm12* mutation also substantially reduces DNA methylation and releases silencing at endogenous RdDM target loci including transposons and other repetitive DNA sequences. We show that *RDM12* encodes a protein that is similar to SGS3, which is known to be a partner of RDR6 in PTGS and can bind double stranded RNAs with a 5' overhang (Fukunaga and Doudna, 2009). Our results suggest that RDM12 may be a partner protein of RDR2 and that the RdDM pathway may involve 5' overhang-containing double stranded RNAs.

Results

Isolation of *rdm12* from *ros1* suppressor screen

A construct containing the *RD29A* promoter-driven *LUCIFERASE* reporter gene (*RD29A-LUC*) and the CaMV 35S promoter-driven *NPTII* marker gene (*CaMV 35S-NPTII*) was introduced into wild-type (C24 ecotype) Arabidopsis plants (Ishitani et al., 1997). Both transgenes are expressed in the wild type, but are silenced in the *ros1* mutant background (Gong et al., 2002). A T-DNA mutagenized library in the *ros1* background was generated, and screened for second site suppressors of *ros1* based on the luminescence phenotype (Kapoor et al., 2005; He et al., 2009a). Most RdDM components, including NRPD1, NRPE1, NRPD2, AGO4, DRD1 and HEN1, as well as RDM2/NRPD4/NRPE4, RDM3/KTF1 and RDM4 have been identified from the screen (He et al., 2009a; He et al., 2009b; He et al., 2009c).

Further screening of the library led to the isolation of a novel suppressor mutant, *ros1rdm12-1*, which rescues the luminescence phenotype of *ros1*, but does not significantly affect the kanamycin sensitivity of *ros1* (Figure 1a). Northern blot analysis shows that the expression of the *RD29A-LUC* transgene and endogenous *RD29A* gene in *ros1rdm12-1* is substantially higher than that in *ros1*, although the *LUC* expression in *ros1rdm12-1* is still much weaker than that in the wild type (Figure 1b). The results show that the *rdm12* mutation partially releases the silencing of the *RD29A-LUC* transgene and corresponding endogenous *RD29A* gene, but does not release the silencing of the kanamycin resistance transgene *NPTII*. This phenotype is very similar to those of other suppressor mutants with lesions in RdDM components such as NRPD1, NRPE1, NRPD2 and DRD1 (He et al., 2009a; He et al., 2009b; He et al., 2009c).

The *rdm12* mutation reduces DNA methylation at RdDM target loci

To investigate whether the reactivation of the *RD29A-LUC* transgene and endogenous *RD29A* gene in *ros1rdm12-1* is related to changes in *RD29A* promoter DNA methylation, we examined the DNA methylation status of the *RD29A* promoter by Southern blotting. The result shows that the *RD29A* promoter DNA methylation level is partially reduced in *ros1rdm12-1* compared to that in *ros1* (Figure 2a). This partial reduction in DNA methylation in *ros1rdm12-1* is similar to what was observed in *ros1nrpd1* (He et al., 2009a). The result suggests that the *rdm12* mutation suppresses the TGS of the *RD29A-LUC* transgene and endogenous *RD29A* gene by blocking DNA hypermethylation.

The DNA methylation of endogenous RdDM targets was also examined in the *ros1rdm12-1* mutant. The genomic DNA from the indicated genotypes was digested by the DNA methylation sensitive enzyme HaeIII, followed by amplification of the *AtSN1* transposon. The result shows that *AtSN1* methylation was reduced in *ros1rdm12-1* as well as in *ros1nrpd1*, although the effect of *rdm12* is not as strong as that of *nrpd1* (Figure 2b). The DNA methylation status of the transposon *AtMU1* was tested by Southern blotting. The result shows that *AtMU1* genomic DNA is partially digested by HaeIII in *ros1rdm12-1* and

is completely digested in *ros1nrpd1*, whereas there is little digestion in the wild type or *ros1* (Figure 2c). The result suggests that *rdm12-1* reduces DNA methylation at *AtMU1*.

The effect of *rdm12* on 5S rDNA methylation was also investigated by Southern blotting. The result suggests that in *ros1rdm12-1* and *ros1nrpd1*, the 5S rDNA methylation is reduced at non-symmetric cytosine sites as detected by HaeIII digestion, as well as at symmetric cytosine sites as detected by HpaII digestion (Figure 2d). However, no differences in DNA methylation were found among wild type, *ros1*, *ros1rdm12-1* and *ros1nrpd1* at the highly repetitive 180-bp centromeric repeat, which is not an RdDM target (Figure S1).

The effect of *rdm4* on siRNA accumulation and transposon silencing

The siRNA accumulation was assessed by small RNA Northern blotting. The results show that the 24-nt siRNAs from the *RD29A* promoter are not substantially affected by the *rdm12-1* mutation (Figure 3a). For endogenous siRNAs, AtSN1 siRNA and siRNA1003 (from 5S rDNA) are partially reduced in *ros1rdm12-1* compared to those in the wild type and *ros1*, whereas the other tested siRNAs including AtMU1 siRNA, Cluster4 siRNA and siRNA02 appear not affected by the *rdm12-1* mutation (Figure 3a). However, all these siRNAs are abolished in *ros1nrpd1*. The ta-siRNA255 and microRNA171 accumulate to a similar levels in wild type, *ros1*, *ros1rdm12-1* and *ros1nrpd1* (Figure 3a). The results show that *rdm12-1* only affects some 24-nt siRNAs and does not affect miRNAs or ta-siRNAs, and suggest that RDM12 may function at a downstream step in the RdDM pathway.

The effect of *rdm12* on transposon expression was investigated by semi-quantitative RT-PCR. The results show that the *AtSN1* transcript is increased in *ros1rdm12-1* and *ros1nrpd1*, compared to that in the wild type and *ros1* (Figure 3b). However, the *TSI* transcript is not affected by *rdm12-1* or *nrpd1*. As reported previously (Huettel et al. 2006; Mathieu et al. 2007; He et al., 2009c), mutations in RdDM components reduce the transcript level of *ROS1*. Here, we found that similar to *nrpd1*, the *rdm12-1* mutation also decreases the *ROS1* transcript level, although the effect of *rdm12-1* is not as strong as that of *nrpd1* (Figure 3b).

RDM12 encodes an SGS3-like protein

Since the *ros1rdm12-1* mutant was isolated from a T-DNA insertion library, we tried and succeeded in finding an Arabidopsis genomic sequence flanking the T-DNA by TAIL-PCR. However, the T-DNA insertion was found not to co-segregate with the luminescence phenotype of *ros1rdm12-1* (data not shown). Thus, we attempted to identify the *RDM12* gene by map-based cloning. We generated a mapping population by crossing *ros1rdm12-1* in the C24 ecotype to *ros1-4* in the Col-0 ecotype. In the segregating selfed F2 population, *ros1rdm12* mutants were selected based on the luminescence phenotype. We mapped the *rdm12-1* mutation to a short region on Chromsome 3, to within the BAC clone T8P19 (Figure S2). Candidate genes in this region were sequenced and a 49-bp deletion was found in the second exon of At3G48670 in *ros1rdm12-1* (Figure 4a). The At3G48670 transcript is abolished in *ros1rdm12-1* (Figure 4b).

To confirm that At3G48670 is the *RDM12* gene, a T-DNA insertion allele of At3G48670 (FLAG_550B05, named as *rdm12-2*) (Figure 4a) was obtained from the ABRC stock center. The At3G48670 transcript is also abolished in the *rdm12-2* allele (Figure 4b). This allele shows a reduced DNA methylation at *AtSN1*, similar to the effect of *rdm12-1* (Figure 4c). Moreover, the wild-type At3G48670 genomic sequence was cloned and introduced into *ros1rdm12-1* for a complementation assay (Figure 4d). The result shows that in all six randomly selected T1 transformants the silencing of *RD29A-LUC* and *AtSN1* hypermethylation were restored (Figure 4d). Taken together, the results show that At3G48670 is the *RDM12* gene.

RDM12 is predicted to encode a protein of 648 amino acids. The protein contains a zinc finger domain, an XS domain and a coiled-coil domain, which are conserved in SGS3, a protein required for the post-transcriptional gene silencing (PTGS) pathway (Figure 5a, 5b). SGS3 is capable of binding double stranded RNAs with a 5' overhang, and the XS domain appears to be responsible for this RNA binding activity (Fukunaga and Doudna, 2009). RDM12 is highly similar to two other predicted proteins that are encoded by At3G12550 and At4G01780, respectively (Figure S3). In addition to the XS and coiled-coil domains, RDM12 and the two paralogs contain the XH domain that is not present in SGS3. The function of the XH domain is currently not known.

Discussion

In our *RD29A-LUC* transgene system, 24 nt siRNAs are generated from the *RD29A* promoter, but cannot cause the hypermethylation and silencing of the promoter due to the DNA demethylation activity of ROS1 (Gong et al., 2002; Agius et al., 2006). Only in DNA demethylation mutants like *ros1*, the 24 nt siRNAs can lead to DNA hypermethylation of the *RD29A* promoter and consequently the *RD29A-LUC* transgene and the corresponding endogenous *RD29A* gene are silenced (Gong et al., 2002; Agius et al., 2006). The DNA methylation of a number of endogenous loci is also dynamically regulated by the opposing activities of RdDM and active DNA demethylation (Zhu et al., 2007; Penterman et al., 2007). This dynamic regulation of the *RD29A-LUC* transgene provides an excellent genetic system for identifying both active DNA demethylation pathway components as well as components of the RdDM pathway (Gong et al., 2002; Zheng et al., 2008; He et al., 2009a). In this study, we discovered RDM12 by employing the *RD29A-LUC* system in *ros1*. The phenotypes of the *rdm12* mutants are similar to those of mutants defective in the known components of the RdDM pathway. Our results suggest that RDM12 is an important new component of the RdDM pathway. The DNA methylation and TGS phenotypes of the *rdm12* mutants are relatively weak compared to those of many other RdDM pathway mutants. This is probably due to a partial redundancy between RDM12 and closely related proteins. There are two other proteins (At3G12550 and At4G01780) that are highly similar to RDM12, and these paralogs may be partially redundant with RDM12.

RDM12 and the two paralogs are similar to SGS3 in that all contain the conserved XS and coiled-coil domains. SGS3 is an important component of PTGS pathways, and is required for the accumulation of viral siRNAs, trans-acting siRNAs and nat-siRNAs (Mourrain et al., 2000; Yoshikawa et al., 2004; Borsani et al., 2005). It contains a zinc finger domain, an XS domain and a coiled-coil domain (Bateman, 2002). Of these, the XS domain is probably involved in binding double stranded RNAs with a 5' overhang (Fukunaga and Doudna, 2009) and the coiled-coil domain is likely involved in protein dimerization (Elmayan et al., 2009). SGS3 interacts with and co-localizes with RDR6 in the cytoplasm (Kumakura et al., 2009). Like SGS3, RDR6 is also a critical PTGS component and is required for the production of viral siRNAs, trans-acting siRNAs and nat-siRNAs (Mourrain et al., 2000; Yoshikawa et al., 2004; Borsani et al., 2005). The binding of SGS3 to double stranded RNAs might lead to the stabilization of RDR6-produced double-stranded RNAs and facilitate subsequent PTGS steps.

Unlike SGS3, RDM12 is not required for ta-siRNAs. However, like SGS3, RDM12 and its paralogs may also be able to bind double-stranded RNAs with a 5' overhang. The XH domain in RDM12 may confer additional activities to this protein. The double stranded RNAs are presumably produced by RDR2. In addition, the base-pairing between guide siRNAs and complementary nascent scaffold RNA transcripts produced by Pol II or Pol V could also generate double stranded RNAs with a 5' overhang. The binding of RDM12 may help stabilize this base-pairing interaction. As a component of the nuclear RdDM pathway,

RDM12 is presumably a nuclear protein and may interact with the nucleus-localized RDR2. Just as SGS3 is a partner protein of RDR6 in PTGS, we suggest that RDM12 may be a partner protein of RDR2 in TGS. During the preparation of this manuscript, Ausin et al (2009) reported the *IDN2* gene that is required for de novo DNA methylation. IDN2 is identical to RDM12. The identification of RDM12/IDN2 as a component of RdDM required for de novo DNA methylation from two completely independent genetic screens further underlies the importance of RDM12/IDN2 in the RdDM pathway. Future studies will reveal whether and how RDM12/IDN2 may partner with RDR2 to function in RdDM.

Materials and methods

Plant growth, mutant screening and cloning

The wild-type C24 and *ros1* mutant plants carry the homozygous stress-inducible *RD29A-LUC* transgene (He et al., 2009a). A T-DNA mutagenized library in the *ros1* mutant background were generated (Kapoor et al., 2005). Plants were grown in a controlled room at 23°C with 16 h of light and 8 h of darkness. The library screening was as described previously (He et al., 2009a). The identified *ros1rdm12-1* mutant in the C24 ecotype was crossed to the *ros1-4* mutant in the Col-0 ecotype (Salk_045303) to generate a F2 mapping population. About 800 F2 progenies with a high luminescence phenotype were selected for mapping. For complementation assay, The *RDM12* genomic sequence were amplified, and cloned into the binary vector pCAMBIA1303. The *RDM12* construct was introduced to *ros1rdm12-1* using the *Agrobacterium tumefaciens* strain GV3101.

RNA analysis

Arabidopsis seedlings were grown on MS plates at 23°C for two weeks, and harvested after cold treatment (4°C, 1 d) or no treatment. Total RNA was extracted from the plants of each genotype using Trizol (Invitrogen). Twenty microgram of RNA for each sample was separated on 1.2% denaturing agarose gels, and transferred onto Hybond-N+ membranes (Amersham) for Northern hybridization. Small RNA was extracted from floral tissues. Small RNA Northern blotting was as described previously (He et al., 2009a). The DNA oligos used for DNA probe preparation are listed in Supplemental Table S1.

Five microgram of total RNA from the indicated genotypes was reverse transcribed to synthesize the first-strand cDNA with Superscript III System (Invitrogen). The cDNA templates were used for semi-quantitative RT-PCR or real-time PCR. For real-time PCR, the amplified DNA was labeled by Sybon (BioRad). The TUB8 was used as an internal control. The primer sequences are listed in Supplemental Table S1.

DNA methylation assays

For DNA methylation assays, the genomic DNA from each genotype was digested with the DNA methylation sensitive enzyme BstU1, HpaII or HaeIII. The digested DNA was used for Chop-PCR and Southern hybridization. For Southern hybridization, 2 ug of digested DNA was separated on 1.2% argorose gels at 40 V overnight, and transferred to Hybond-N+ membrance. The primer sequences used for chop-PCR and probe preparation are listed in Supplemental Table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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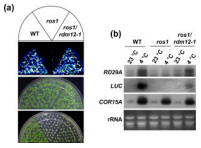

Figure 1. The *rdm12* mutation releases the silencing of *RD29A-LUC* transgene in *ros1*

(a) The two-week-old plants on MS plate were imaged after cold treatment at 4 °C for 24 h. The plants were sprayed with luciferin for luminescence imaging. The plants were grown on MS medium supplemental with 50 μg μl^{-1} kanamycin for two weeks and photographed. (b) The expression of *LUC*, *RD29A* and *COR15A* was detected by Northern blotting. The ethidium bromide-stained rRNA was used as a loading control.

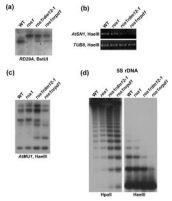

Figure 2. The *rdm12* mutation reduces DNA methylation at RdDM target loci

(a) Genomic DNA from the indicated genotypes was digested with the DNA methylation sensitive enzyme BstUI, followed by Southern blotting with the *RD29A* coding sequence as a probe. (b) The genomic DNA was digested with the DNA methylation sensitive enzyme HaeIII, and *AtSN1* was amplified. *TUB8* was amplified as an internal control. (c) The HaeIII-digested genomic DNA was used for Southern hybridization using the *AtMU1* probe. (d) The genomic DNA was digested with HpaII and HaeIII, and followed by Southern hybridization using the 5S rDNA probe.

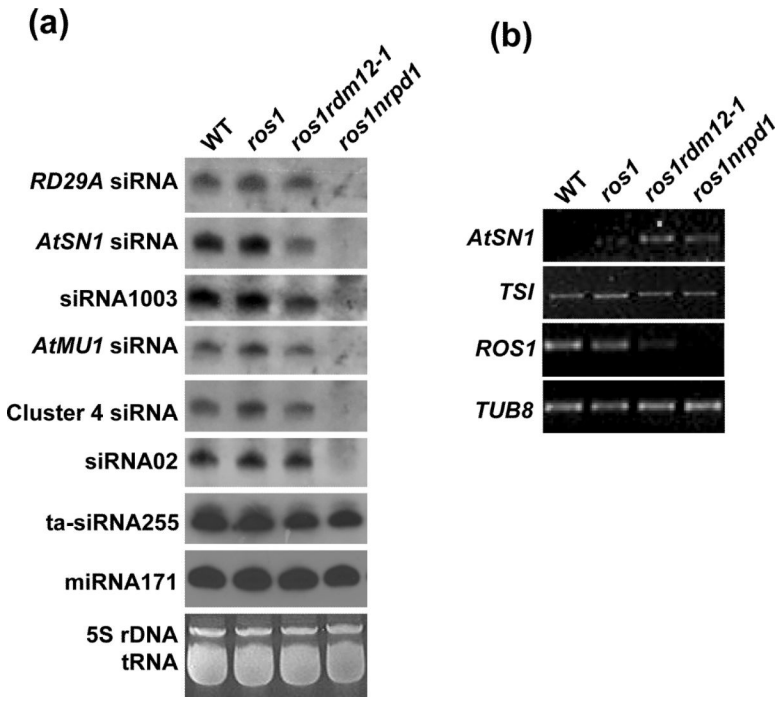

Figure 3. The effect of *rdm12* on the accumulation of siRNAs and RNA transcripts

(a) The small RNA from floral tissiues was used for the Northern blot assay. The ethidium bromide-stained 5S rRNA and tRNA is shown as a loading control. (b) The total RNA from indicated genotypes was used for semi-quantitative RT-PCR to assess the transcript levels of *AtSN1* and *TSI*. *TUB8* was used as an internal control.

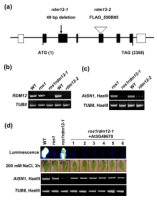

Figure 4. Identification of the *RDM12* gene and mutant complementation assay

(a) Diagram of the *RDM12* genomic sequence. Shown are the positions of exons (solid boxes) and introns, the deletion site in *rdm12-1*, and the T-DNA insertion site in *rdm12-2*. (b) Detection of the *RDM12* transcript by RT-PCR in the wild type (C24), *ros1*, *ros1rdm12-1*, and *rdm12-2* and its wild type control (Ws). (c) Genomic DNA from *ros1*, *ros1rdm12-1*, wild-type (Ws), and *rdm12-2* was digested with the methylation-sensitive enzyme HaeIII and used for amplification of *AtSN1*. (d) Complementation assay in *ros1rdm12-1*. The leaves from wild type, *ros1*, *ros1rdm12-1* and six independent *RDM12* transgenic T1 lines in the *ros1rdm12-1* background were used for luminescence imaging after treatment with 200 mM NaCl for 3 h, and for assaying *AtSN1* methylation.

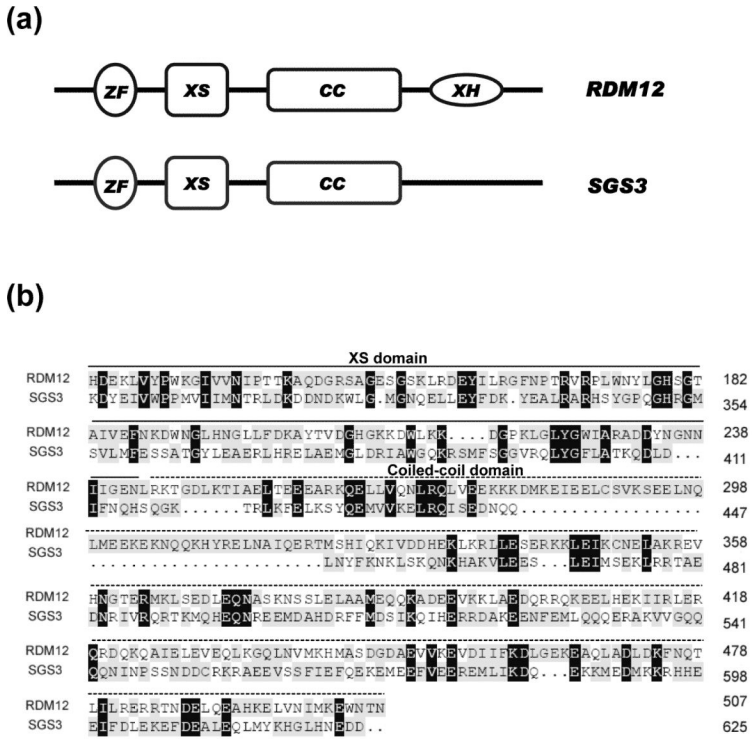

Figure 5. Characterization of the RDM12 protein

(a) Diagram of the RDM12 and SGS3 proteins. The XS domain and a coiled-coil domain are conserved in RDM12 and SGS3. (b) Sequence alignment of RDM12 and SGS3 in the XS domain and coiled-coil domain.