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ORIGINAL ARTICLE

An atypical component of RNA-directed DNA methylation machinery has both DNA methylation-dependent and -independent roles in locus-specific transcriptional gene silencing

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RNA-directed DNA methylation (RdDM) is an important *de novo* DNA methylation pathway in plants. RdDM mediates the transcriptional silencing of many endogenous genomic loci, most of which are transposon related. A forward genetics screen identified DTF1 (DNA-binding transcription factor 1) as a new component for RdDM in *Arabidopsis*. Loss-of-function mutations in *DTF1* release the transcriptional silencing of RdDM target loci and reduce the accumulation of 24-nt small interfering RNAs (siRNAs) from some of the targets. Interestingly, in the *dtf1* mutant plants, the release of transcriptional gene silencing at *solo-LTR* is accompanied by decreased siRNA accumulation but not by reduced DNA methylation. These results suggest that DTF1 is an atypical component of RdDM and has both DNA methylation-dependent and -independent roles in transcriptional gene silencing. We suggest that besides DNA methylation, siRNAs may cause some other uncharacterized epigenetic modifications that lead to transcriptional gene silencing.

Keywords: DTF1; siRNA; DNA methylation; transcriptional gene silencing; RdDM
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Introduction

The RNA-directed DNA methylation (RdDM) and transcriptional gene-silencing pathway has been well characterized in plants [1-5]. RdDM has been shown to be required for various epigenetic processes, which include transgene silencing at the transcriptional level, genomic imprinting and suppression of retrotransposition of transposable elements [6-10]. 24-nt siRNAs and long noncoding RNAs are required for RdDM at target sequences [3, 6, 11]. The plant-specific DNA-dependent RNA polymerase IV is presumably responsible for synthesizing the precursors of siRNAs [3, 12]. The precursors are converted into double-stranded RNAs by the RNA-dependent RNA polymerase RDR2 [1, 13]. The double-stranded RNA is cleaved by Dicer-like 3 into 24-nt siRNAs, which are loaded into ARGONAUTE 4 (AGO4) [13, 14]. Another DNA-dependent RNA polymerase, Pol V, is required for producing non-coding, presumably scaffold RNAs from RdDM targets [11]. The chromatin-remodeling protein DRD1, structural-maintenance-of-chromosomes hinge domain-containing protein DMS3 and the novel plant-specific protein RDM1 form a tight protein complex (DDR), which is required for Pol V-dependent transcripts [15-18]. RDM1 is also associated with AGO4 and the DNA methytransferase DRM2 [18]. The largest subunit of Pol V, NRPE1 and the SPT5-like protein KTF1 have WG/GW repeats in

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their C-terminal regions, which bind AGO4 and the Pol V-dependent scaffold RNAs, and help to form RdDM effector complexes [19, 20]. The yeast IWR1-like protein RDM4/DMS4 is a transcription factor that interacts with both Pol V and Pol II. The *rdm4* mutation affects Pol V-dependent transcripts and plant development [21, 22]. DRM2 and the recently characterized DRM3 are the *de novo* DNA methytransferases in the *Arabidopsis* RdDM pathway [23-26].

siRNA-directed chromatin modification and TGS also occur in unicellular eukaryotes, such as the fission yeast [27]. Several key components of plant RdDM appear to have counterparts in fission yeast, including Argonaute AGO1, Dicer DCR1 and RNA-dependent RNA polymerase RDP1 [28]. Moreover, like the *Arabidopsis* WG/GW motif-containing protein KTF1, fission yeast has a WG/GW-motif-containing protein TAS3, which is also required for interaction with Argonaute [29, 30]. The fission yeast AGO1, TAS3 and the chromodomain protein CHP1 form an RNA-induced transcriptional silencing (RITS) complex, which is essential for heterochromatin formation at pericentromeric repeats [29]. However, there is no DNA methylation in fission yeast, instead, H3K9 dimethylation catalyzed by the H3K9 methytransferase CLR4 is required for siRNA-directed repressive heterochromatin formation in fission yeast [27, 31]. In plants, H3K9 dimethylation is catalyzed by histone H3K9 methytransferases such as KYP/SUVH4 [32]. KYP forms a self-enhancing loop with the DNA methytransferase CMT3, which catalyzes DNA methylation at CHG sites [33, 34].

At some RdDM-target loci, the transcriptional gene silencing depends on MOM1 [35, 36]. In *mom1* mutants, although the silencing of transgenes and endogenous genomic target loci is released, neither DNA methylation nor histone modification is altered at these loci [35-37]. MOM1 seems to act downstream of RdDM, although further studies are required to understand how MOM1 mediates transcriptional gene silencing independently of DNA methylation at RdDM-target loci. Interestingly, a recent study reported that MOM1 is required to prevent aberrant RNA transcriptional read-through [38]. The minimal functional domain of MOM1, CMM2, forms a coiled-coil structure, which likely interacts with other proteins [39, 40].

ROS1 encodes a 5-methylcytosine DNA glycosylase, which functions as a DNA demethylase by initiating a base excision repair pathway for active DNA demethylation [41-44]. *ros1* mutations cause transcriptional silencing of the transgenic *RD29A* promoter-driven luciferase reporter gene (*RD29A-LUC*) as well as the endogenous *RD29A* gene [41]. By screening for second site suppressors of *ros1* mutant plants, we have identified many RdDM components [45]. Our results showed that the transcriptional silencing of the *RD29A-LUC* transgene in *ros1* is dependent on RdDM.

In this study, we identified a novel component of RdDM, DTF1, by screening for suppressors of *ros1*. *dtf1* mutations release the silencing of *RD29A-LUC* transgene as well as the endogenous *RD29A* in *ros1*. Moreover, DTF1 is required for silencing of endogenous transposable elements that are targeted by RdDM. The *dtf1* mutations cause reduced siRNA accumulation and DNA methylation at some of the RdDM targets. Surprisingly, the role of DTF1 in transcriptional silencing at some RdDM targets is not dependent on DNA methylation. These results suggest that DTF1 is an atypical RdDM component, and functions in transcriptional silencing through both DNA methylation-dependent and -independent pathways.

Results

A putative DNA-binding transcription factor, DTF1, is required for transcriptional silencing of the RD29A-LUC transgene

Our previous results showed that the *RD29A* promoter-driven luciferase transgene is expressed in the wild-type C24 plants under stress conditions, whereas loss-of-function mutations in *ROS1* result in transcriptional silencing of the *RD29A-LUC* transgene as well as the corresponding endogenous *RD29A* [41]. RdDM is required for transcriptional silencing of transgenic *RD29A-LUC* and endogenous *RD29A* in *ros1* [45]. Nearly all of the canonical RdDM components have been identified by screening for suppressors of *ros1* in our previous [45] and current studies (unpublished data). These results suggest that ROS1-mediated DNA demethylation and RdDM function antagonistically in the control of DNA methylation and transcriptional gene silencing.

Our previous *ros1* suppressor screens were carried out using an *Agrobacteria*-transformed population of *ros1* [45, 46]. In order to identify new components of the RdDM pathway, we constructed an EMS-mutagenized library in the *ros1* mutant background that has the silenced *RD29A-LUC* transgene. We found most of the known RdDM components by screening for *ros1* suppressors from the library (unpublished data). In addition, we recovered a new mutant (referred to as *dtf1*) where the silencing of the *RD29A-LUC* transgene in the *ros1* background is released.

The expression of *RD29A-LUC* transgene and *35S* promoter-driven *NPTII* (*35S-NPTII*) gene is indicated by bioluminescence intensity and kanamycin resistance, respective-

ly [41, 46]. In the wild-type C24, *RD29A-LUC* was highly expressed under stress conditions, and strong bioluminescence was emitted (Figure 1A). The *35S-NPTII* transgene was normally expressed in the wild type, which is resistant to kanamycin (Figure 1A and 1B). *ros1* mutations cause silencing of *RD29A-LUC*, corresponding endogenous *RD29A* and *35S-NPTII* (Figure 1B) [41]. In the *ros1dtf1-1* double mutant, the silencing of *RD29A-LUC* and corresponding endogenous *RD29A* was partially released, whereas the silencing was released in *ros1nrpe1* to a greater extent (Figure 1A and 1B). However, neither the *dtf1-1* nor *nrpe1* mutation suppressed silencing of the *35S-NPTII* transgene, which is known to be independent of RdDM (Figure 1B) [45]. The results suggest that DTF1 play an important role in transgene silencing in a manner similar to the canonical RdDM component NRPE1.

The *ros1dtf1-1* mutant was crossed to a homozygous *ros1* mutant (Salk_045303) in the Col-0 background, and the resulting F2 population was used for map-based cloning. The *dtf1* mutation was mapped to a ~180 kb region on chromosome 1 (Figure 2A). Next-generation high-throughput DNA sequencing was carried out to sequence the whole genome of the mutant. In the ~180 kb mapping interval, we found a G to A mutation in the fourth exon of At1G15215, which creates a premature stop codon (Figure 2B). The mutation was confirmed by Sanger sequencing. The results suggest that At1g15215 might be the *DTF1* gene. At1g15215 encodes a putative sequence-specific DNA-binding transcription factor (hence the name DTF1, for DNA-binding transcription factor 1).

To confirm that the mutation in At1g15215 is responsible for the silencing defect in *ros1dtf1-1*, the genomic fragment of At1g15215 was cloned from wild-type plants and transformed into *ros1dtf1-1* for complementation test. Compared to *ros1dtf1-1*, the transgenic plants emitted much less luminescence (Figure 2C), suggesting that the silencing of *RD29A-LUC* transgene is restored by the wild-type transgene. In addition, we tested and found that *AtSN1* methylation in *ros1dtf1* was low, but the methylation in the transgenic plants was restored (Figure 2C). Furthermore, we obtained a homozygous T-DNA insertion mutant (Salk_074540C), and found that the T-DNA mutant also showed a reduction in DNA methylation at *AtSN1* (Figure 2D). These results demonstrate that At1G15215 is *DTF1*, and is required for the silencing of *RD29A-LUC* transgene.

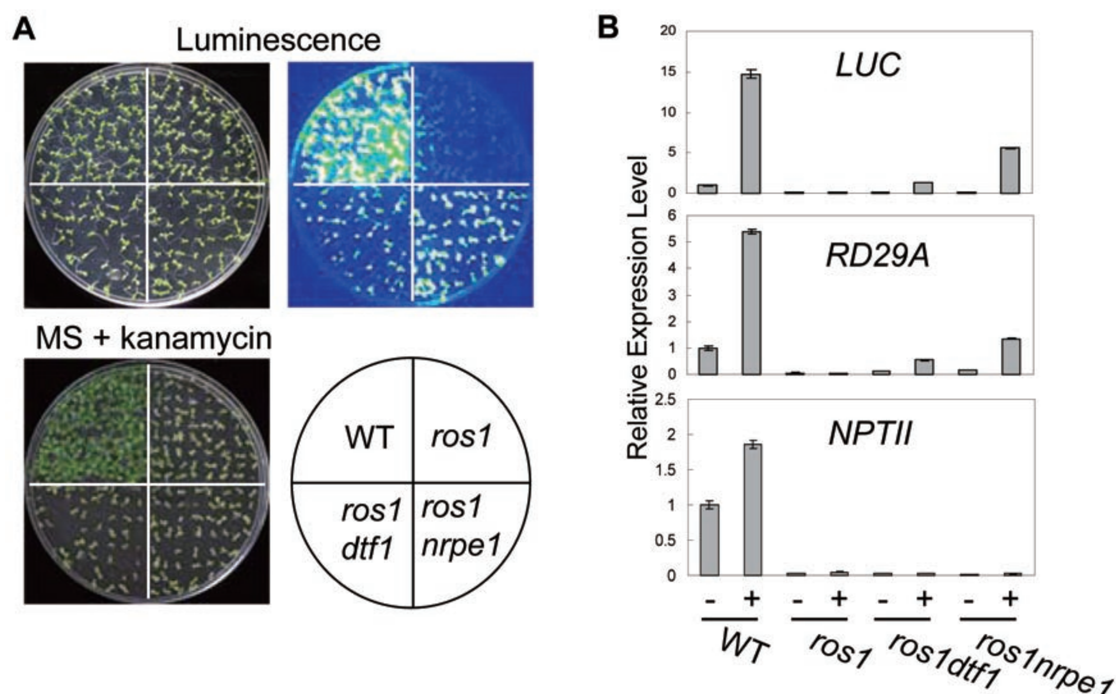

Figure 1 The *dtf1* mutation releases the silencing of *RD29A-LUC* and endogenous *RD29A*. **(A)** The *dtf1* mutation releases the silencing of *RD29A-LUC*. The transgenic wild-type, *ros1*, *ros1dtf1-1* and *ros1nrpe1* were grown on MS medium for 2 weeks followed by treatment at 4 °C for 2 days. The treated plants were sprayed with luciferin for luminescence imaging. The same plants were grown on MS medium supplemented with 100 µg/ml kanamycin for 3 weeks and photographed. **(B)** Effect of *dtf1* on expression of transgenic *RD29A-LUC*, endogenous *RD29A* and *35S-NPTII*, as detected by real-time PCR. *TUB4* was used as an internal control for normalization.

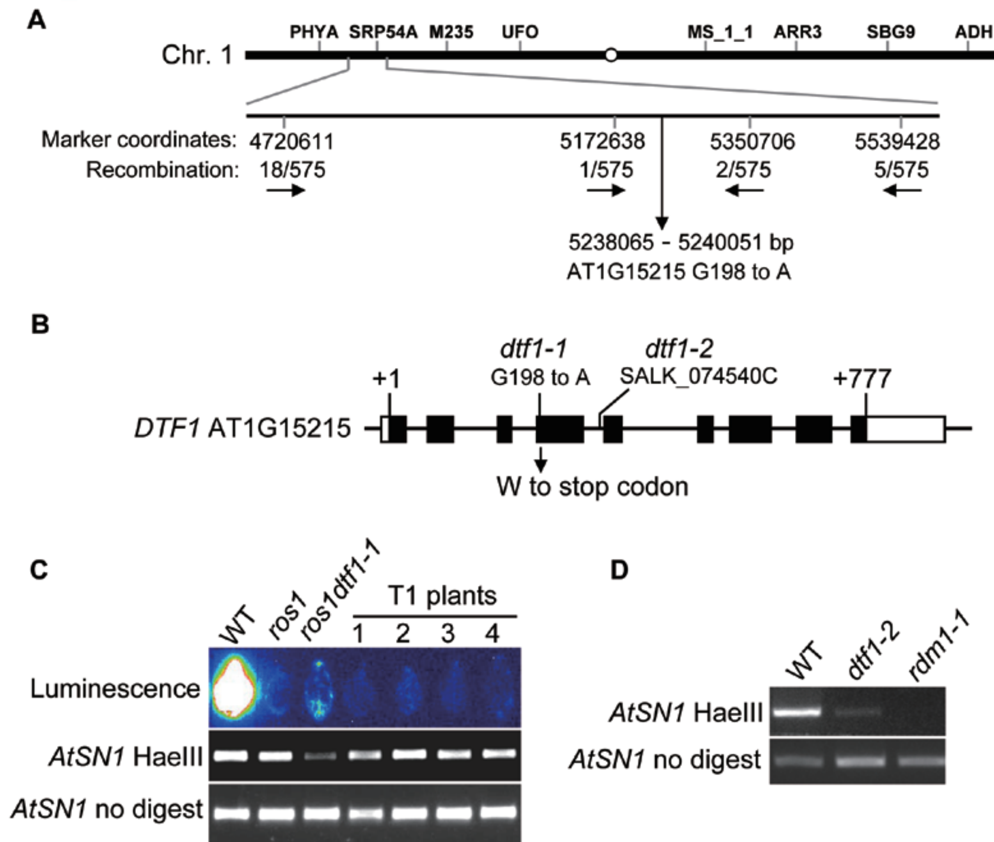

Figure 2 Map-based cloning and characterization of *DTF1*. **(A)** Map-based cloning of *DTF1*. The gene was located to the indicated ~180 kb region of chromosome 1 by genetic mapping. A G-to-A mutation in the fourth exon of At1G15215 creates a premature stop codon. **(B)** Diagram of *DTF1* and the *dtf1-1* and *dtf1-2* mutations. The diagram shows the *DTF1* gene with the indicated positions of exons (boxes), introns (line) and open reading frame (solid boxes). The *dtf1-1* and *dtf1-2* mutations are labeled. **(C)** Complementation assay in *ros1dtf1-1*. The leaves from wild type, *ros1*, *ros1dtf1-1*, and four individual *DTF1* transgenic T1 plants in *ros1dtf1-1* were collected for luminescence imaging after treatment with 2% NaCl for 4 hours. The *AtSN1* methylation was tested by chop-PCR. **(D)** The *dtf1-2* mutation reduces *AtSN1* DNA methylation. Genomic DNA from the indicated genotypes was digested with *Hae*III, and used for amplification of *AtSN1*. The *rdm1* mutant was used as a control.

DTF1 encodes a putative DNA-binding transcription factor that contains a homeodomain in its N-terminal region. The homeodomain is known as the DNA-binding domain in some transcription factors. In addition to DTF1, there is a DTF1-like protein, At1G18380, encoded in the *Arabidopsis* genome. DTF1 and the DTF1-like protein are highly similar, except that the DTF1-like protein has a C-terminal extension (Supplementary information, Figure S1A). However, T-DNA insertion mutations in At1g18380 have no apparent effect on DNA methylation and transcriptional gene silencing (data not shown), suggesting that At1G18380 may have functions other than in transcriptional gene silencing.

DTF1 encodes a plant-specific gene, which is conserved in angiosperms, gymnosperms and bryophytes (Supplementary information, Figure S1B and S1C), but not in fungi and animals. DTF1 is conserved in the N-terminal homeodomain, which may function in DNA binding. In addition, the uncharacterized domain in the C-terminal half is also highly conserved.

DTF1 is required for the silencing of some endogenous RdDM targets and accumulation of 24-nt siRNAs

The above results showed that DTF1 is required for transgene silencing. Using semi-quantitative RT-PCR, we tested the effect of the *dtf1-1* mutation on the silencing of endogenous RdDM targets. The results show that the *dtf1-1* mutation released silencing of a subset of RdDM targets (Figure 3A). *solo-LTR* and *AtGP1* were previously identified as RdDM targets, which show a low level of expression in wild-type and *ros1* plants [45, 47]. The *dtf1-1* mutation released the silencing of *solo-*

LTR and *AtGP1*, while it weakly released the silencing of *AtSN1A* (Figure 3A). As a control, the *nrpe1* mutation caused more substantial expression of *AtSN1 A*. The results suggest that DTF1 is required for the silencing of a subset of RdDM targets.

Pol V-dependent noncoding RNA transcripts are required for RdDM and transcriptional gene silencing [11]. We tested and found that the *dtf1-1* mutation did not affect Pol V-dependent transcripts, while loss of the largest subunit of Pol V, NRPE1, blocked accumulation of the Pol V-dependent transcripts, including *AtSN1 B* and *IGN5 B* (Figure 3A). Previous studies showed that mutations in canonical RdDM components suppress *ROS1* expression [21, 48, 49]. We found that the *dtf1-1* mutation also suppressed the expression of *ROS1* (Figure 3A).

Accumulation of 24-nt siRNAs was detected in *ros1dtf1-1* as well as in the wild type, *ros1*, and *ros1nrpe1*. 24-nt siRNAs generated from the transgenic *RD29A* promoter are required for *RD29A-LUC* silencing in *ros1* [45]. RNA blot analysis showed that the level of *RD29A* promoter siRNAs was reduced in *ros1dtf1-1* and *ros1nrpe1* (Figure 3B). The result suggests that the role of DTF1 in *RD29A-LUC* trangene silencing is related to siRNA-directed DNA methylation and transcriptional silencing. The accumulation of 24-nt siRNAs was also reduced in *ros1dtf1-1* at some endogenous RdDM targets, including *AtSN1*, *solo-LTR* and 5S rDNA, whereas the siRNAs from some other genomic loci (*AtGP1*, *AtMU1*, *Cluster4*, and *siRNA02*) were not affected by the *dtf1* mutation (Figure 3B). The pattern of siRNA accumulation in *ros1dtf1-1* was very similar to that in *ros1nrpe1*, although the *dtf1* mutation affected siRNA accumulation to a greater extent than *nrpe1* (Figure 3B). Neither trans-acting siRNA255 nor microRNA171 was affected in *ros1dtf1-1* and *ros1nrpe1* (Figure 3B), supporting that DTF1 specifically functions in 24-nt siRNA-directed transcriptional gene silencing.

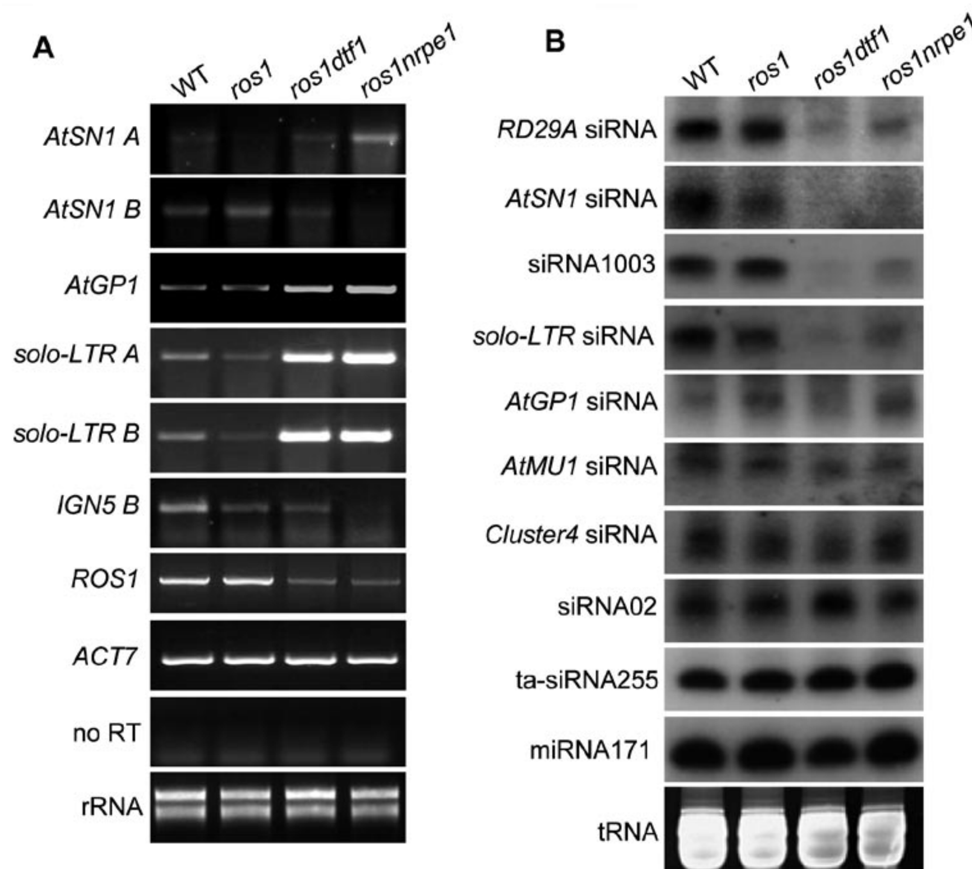

Figure 3 Effect of *dtf1* mutation on transcriptional gene silencing and small RNA accumulation. **(A)** Effect of *dtf1* on the transcript levels of endogenous RdDM targets was determined by semi-quantitative RT-PCR. *ACT7* was used as an internal control. No RT amplification indicates that there was no DNA contamination in RNA samples. **(B)** Effect of *dtf1* on accumulation of small RNAs. The levels of 24-nt siRNAs, 21-nt ta-siRNA255 and miRNA171 were tested in the indicated genotypes. The ethidium bromide-stained small RNA gel was used as a loading control.

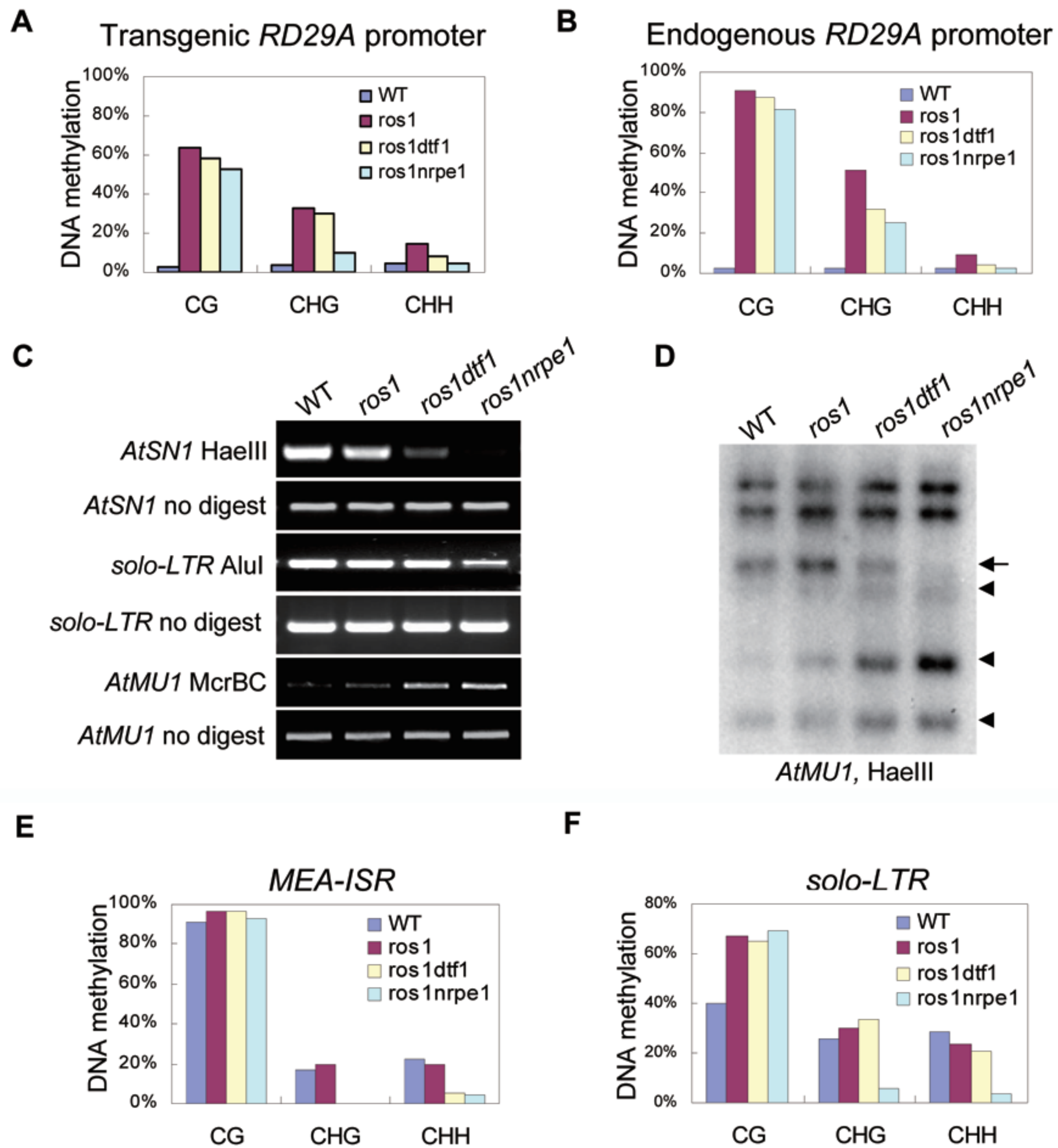

Figure 4 Effect of *dtf1* on DNA methylation. **(A**, **B)** The effect of *dtf1* on the promoter DNA methylation of *RD29A-LUC* **(A)** and endogenous *RD29A* **(B)** was determined by bisulfite sequencing. The percentage of methylated cytosine in different cytosine contexts is shown. **(C)** The DNA methylation level of *AtSN1*, *solo-LTR* and *AtMU1* was determined by chop-PCR. The DNA methylation-sensitive restriction enzymes *Hae*III and *Alu*I, and DNA mehylation-insensitive enzyme McrBC were used for DNA methylation analysis as indicated. **(D)** The effect of *dtf1* on *AtMU1* DNA methylation was detected by Southern blot analysis. Genomic DNA from the indicated genotypes was digested by *Hae*III followed by Southern hybridization. **(E** and **F)** The DNA methylation status of MEA-ISR and solo-LTR was determined by bisulfite sequencing in the indicated genotypes.

DTF1 is required for DNA methylation at some endogenous RdDM targets

To test whether the suppression of transcriptional gene silencing in *ros1dtf1-1* is correlated with a reduction in DNA methylation, we analyzed the DNA methylation status at the *RD29A-LUC* transgene, endogenous *RD29A* and several other endogenous RdDM targets. Consistent with our previous results [41], the DNA methylation level of both transgenic and endogenous *RD29A* promoters was very low in wild type, and the methylation in all cytosine contexts (CG, CHG, CHH) was substantially increased in *ros1* (Figure 4A and 4B).

The DNA methylation level was well correlated with the silencing of *RD29A-LUC* transgene and endogenous *RD29A* gene. We found that the *RD29A* promoter DNA methylation at non-CG sites was reduced in *ros1dtf1-1*, as in *ros1nrpe1* (Figure 4A and 4B). The results further suggest that DTF1 functions in RdDM. Consistent with the weak reactivation of *AtSN1* in *dtf1* mutants (Figure 3A), the *AtSN1* DNA methylation level was partially reduced by the *dtf1* mutation (Figure 4C). The DNA methylation of the endogenous RdDM target *AtMU1* was reduced in *ros1dtf1-1* as well as in *ros1nrpe1* (Figure 4C and 4D), although siRNA accumulation at the locus was not affected by *dtf1* or *nrpe1* (Figure 3B). These results suggest that like NRPE1, DTF1 functions downstream of siRNA generation to mediate DNA methylation and transcriptional gene silencing. As in *ros1nrpe1*, non-CG DNA methylation at *MEA-ISR* was reduced in *ros1dtf1-1* (Figure 4E). Taken together, the results suggest that DTF1 is a new component of RdDM, and it functions in the DNA methylation and transcriptional silencing of a subset of RdDM targets, including *AtMU1* and *MEA-ISR*.

As the silencing of *solo-LTR* is substantially released in *ros1dtf1-1*, we tested whether the DNA methylation of *solo-LTR* is reduced in the mutant by bisulfite sequencing. The results show that the DNA methylation of *solo-LTR* was clearly reduced at CHG and CHH sites in *ros1nrpe1*, but not in *ros1dtf1-1* (Figure 4F). This is in contrast to the silencing of *solo-LTR*, which was released to similar levels in *ros1dtf1-1* and *ros1nrpe1* (Figure 3A). Futhermore, the DNA methylation of 5S rDNA repeats was also not affected by the *dtf1* mutations in *ros1dtf1-1* as well as in *dtf1-2*, whereas the DNA methylation was significantly reduced in the canonical RdDM mutants *nrpd1* and *nrpe1* (Figure 5A and 5B). However, although the DNA methylation of both *solo-LTR* and 5S rDNA was not altered by *dtf1*, the accumulation of *solo-LTR* siRNAs and siRNA1003 (from 5S rDNA) was substantially reduced by *dtf1*. These results suggest that the function of DTF1 in siRNA-mediated transcriptional gene silencing at *solo-LTR* and 5S rDNA is independent of DNA methylation.

Discussion

In the canonical RdDM pathway, all previously characterized RdDM components are required for both the

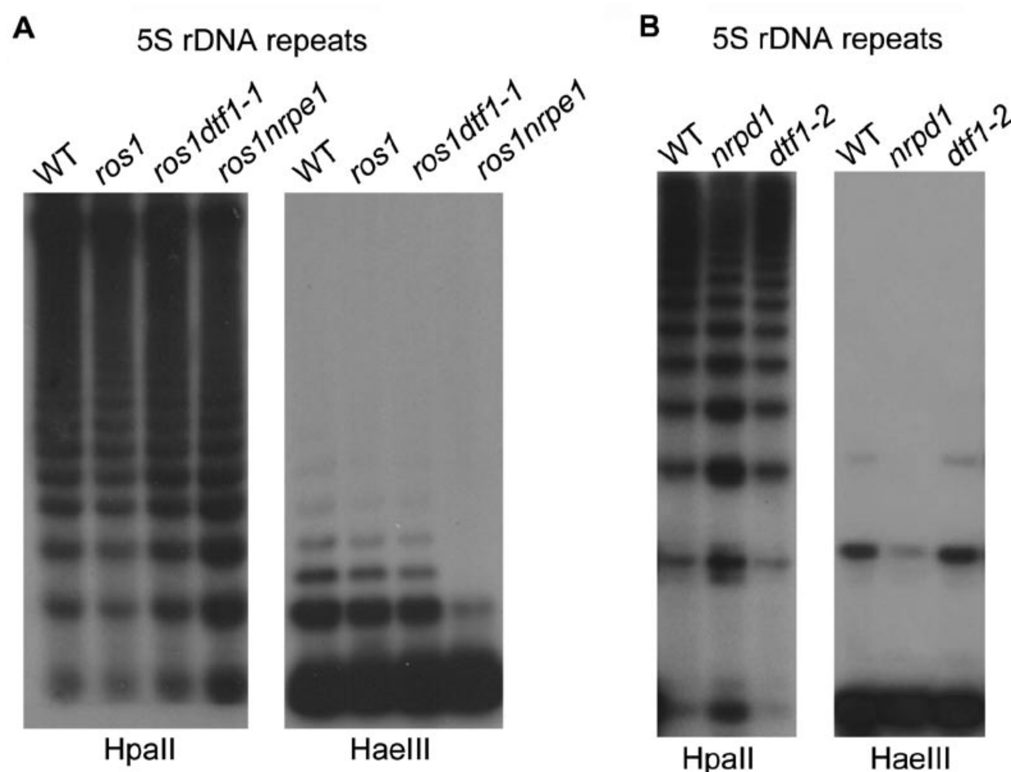

Figure 5 Effect of *dtf1* mutations on 5S rDNA methylation. Genomic DNA from the indicated genotypes was digested with *Hpa*II (for CG and CHG methylation) and *Hae*III (for CHH methylation), and used for Southern blot analysis. The effect of *dtf1* mutations on the DNA methylation of 5S rDNA repeats was tested in *ros1dtf1-1* **(A)** and *dtf1-2* **(B)**, respectively.

establishment of DNA methylation and for transcriptional gene silencing at target loci. *solo-LTR* is one of the RdDM targets, and was first discovered by the Matzke group [47]. In *nrpd1*, *nrpe1*, *rdr2* and *drd1*, the DNA methylation of *solo-LTR* is substantially reduced, resulting in the release of transcriptional gene silencing at *solo-LTR* in these mutants [11, 47, 50]. However, different from the canonical RdDM components, we found that DTF1 is dispensable for DNA methylation at *solo-LTR*, although it is required for siRNA accumulation and transcriptional gene silencing at this locus. It is worth noticing that *solo-LTR* is a rather unusual RdDM target in that the repressive histone mark H3K27 monomethylation (H3K27me1) is substantially reduced at *solo-LTR* by mutations of the canonical RdDM components, whereas the mark is not changed in the mutants at the other tested RdDM targets [47]. The dependence of H3K27me1 on RdDM at *solo-LTR* may reflect a special chromatin structure or DNA sequence specificity of the locus, which may be absent at the other tested RdDM target loci. The recently characterized *Arabidopsis* H3K27 monmethyltransferases, ATXR5 and ATXR6, are involved in transcriptional silencing of transposable elements and other repetitive DNA sequences [51]. The silencing status of some RdDM targets such as *solo-LTR* may require both DNA methylaion and H3K27me1 catalyzed by ATXR5/ATXR6. In this study, we found that the *dtf1* mutation suppresses silencing of *solo-LTR* without affecting DNA methylation, suggesting that at this locus DTF1 may function downstream of DNA methylation. As a homeodomain-containing transcription factor, DTF1 may recognize the special chromatin structure or DNA sequence of *solo-LTR* to help siRNAs guide H3K27 monomethylation and transcriptional silencing. At the other RdDM targets tested, DTF1 is required for both DNA methylation and silencing, just like the canonical RdDM components. One possibility is that DTF1 may be involved in siRNA-directed heterochromatic histone modifications, such as H3K9 methylation and H3K27 methylation, which may occur upstream (e.g., H3K9 methylation) or downstream (e.g., H3K27 methylation) of DNA methylation in the RdDM pathway. Regardless of the exact mechanism of function, our results suggest that DTF1 is an atypical RdDM component that mediates transcriptional gene silencing in both DNA methylation-dependent and -independent manners.

Our small RNA blot analysis showed that DTF1 is required for accumulation of 24-nt siRNAs at some RdDM targets but has no effect on the others. The siRNA pattern in *ros1dtf1-1* appears the same as that in *ros1nrpe1*, suggesting that like Pol V, DTF1 may not be involved in the initial biogenesis of siRNAs, but may affect the accumulation of some siRNAs indirectly by influencing the heterochromatic marks of the loci. It is interesting that *dtf1* affects siRNA accumulation to a greater extent than *nrpe1*, although the effect of *dtf1* on DNA methylation is less or even undetectable. The disparaging effects of the two mutations (*dtf1* and *nrpe1*) on siRNA accumulation and DNA methylation hint at unknown complexities and mechanisms of RdDM. During the preparation of this manuscript, Law *et al*. [52] reported that SHH1 is required for DNA methylation, and associates with Pol IV based on affinity purification of NRPD1. SHH1, which is the same protein as DTF1, was suggested to function in siRNA biogenesis as a transcription factor for Pol IV. In the Law *et al*. study, RDR2, CLSY1 and RDM4 were also shown to be associated with NRPD1. RDR2 is abundant in the affinity-purified NRPD1-containing complex, and likely functions together with Pol IV to produce siRNAs. The levels of CLSY1, RDM4 and SHH1 in the purified NRPD1-containing complex were very low [52]. Moreover, RDM4 is known to be a Pol V-interacting protein required for the generation of Pol V-dependent transcripts [21]. These observations suggest that CLSY1, RDM4 and SHH1 are unlikely to be core components of the NRPD1-containing complex. Our results show clearly that unlike Pol IV, DTF1 is only required for the accumulation of siRNAs at a subset of RdDM targets. Importantly, we discovered that *dtf1* reduces siRNA accumulation and releases silencing at *solo-LTR* without affecting DNA methylation. In addition, we found that *dtf1* reduces siRNA accumulation from 5S rDNA repeats but has no apparent effect on DNA methylation of the rDNA. These surprising observations suggest that besides causing *de novo* DNA methylation, siRNAs may also guide the establishment of other silencing epigenetic marks such as heterochromatic histone modifications. DTF1 may be involved in this later function of siRNAs in the RdDM pathway. Previous studies have found that some Pol IV-dependent 24-nt siRNAs do not cause DNA methylation [53]. It would be of great interest to determine the DNA methylation-independent chromatin functions of the siRNAs and the role of DTF1 in these functions.

Materials and Methods

Plant materials, mutant screening and cloning

The wild-type C24 and *ros1* mutant carry a homozygous *RD29A* promoter-driven luciferase transgene [54]. The *ros1* mutant with *RD29-LUC* transgene was mutagenized with ethyl methanesulfonate. Suppressors of *ros1* were screened by luminescence imaging after the mutagenized M2 plants were cold-treated for 2 days at 4 °C. The selected plants were transplanted into soil, and the luminescence phenotype was confirmed in adult plants as previously described [45]. Mutants of interest were crossed to

the *ros1* mutant in the Col-0 backcrossed for map-based cloning. High-throughput sequencing (Illumina) was carried out to detect the mutations in the mapped genomic interval. For complementation assay, the full-length *DTF1* genomic sequence was amplified, and cloned into the binary vector pCAMBIA1300. The *DTF1* construct was transformed into *ros1dtf1-1* by the *Agrobacterium* strain GV3101. The transgenic T1 plants were tested for phenotypic complementation.

Analysis of RNA transcript level and small RNA accumulation

Total RNA was isolated from 2-week-old seedlings on MS plates as described previously [45]. For RT-PCR, the sequences of primers are listed in Supplementary information, Table S1. After contaminating DNA was removed by DNase, total RNA was used for RT-PCR. For RT-PCR of protein-encoding genes, oligo-dT was used as the reverse primer to synthesize the first-stranded cDNA. RT-PCR of transposable elements and other DNA repeat sequences was carried out by a previously described one-step RT-PCR protocol [11]. Briefly, the PCR condition is 95 °C for 5 min followed by 28-40 amplification cycles (95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min). The constitutively expressed ACT7 was used as an internal control. For small RNA analysis, small RNA was extracted as described previously [45], and was separated on a 15% polyacrylamide gel at 200 V for 3 h. The small RNA was electrotransfered to Hybond-N^+ membranes (Amersham). The probes for small RNA hybridization are [γ-^{32}P]ATP-labeled DNA oligonucleotides or [α-^{32}P]dCTP-labeled amplified DNA. The small RNA hybridization was carried out in PerfectHyb buffer (Sigma) at 38 °C. The DNA primer and probe sequences are listed in Supplementary information, Table S1.

DNA methylation assay

The DNA methylation level was detected by chop-PCR, Southern blot analysis, or bisulfite sequencing. For chop-PCR, the genomic DNA was digested with DNA methylation-sensitive (*Hae*III, *Alu*I, and *Msp*I) and -insensitive (*McrBC*) restriction enzymes, and used for amplification of target DNA sequences. For Southern blot analysis, 5 μg of genomic DNA was digested with indicated DNA methylation-sensitive enzymes, and resolved on 1.2% agarose gel at 40 V overnight, followed by a routine Southern blotting method. For bisulfite sequencing, 2 μg of genomic DNA was converted and purified with EpiTect Bisulfite Kit (Qiagen). The treated DNA was used for amplification and cloning. At least 10 individual clones were sequenced for each sample. The primers are described in Supplementary information, Table S1.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)