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An Effector of RNA-Directed DNA Methylation in *Arabidopsis* Is an ARGONAUTE 4- and RNA-Binding Protein

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SUMMARY

DNA methylation is a conserved epigenetic mark in plants and mammals. In *Arabidopsis*, DNA methylation can be triggered by small interfering RNAs (siRNAs) through an RNA-directed DNA methylation (RdDM) pathway. Here, we report the identification of an RdDM effector, KTF1. Loss-of-function mutations in *KTF1* reduce DNA methylation and release the silencing of RdDM target loci without abolishing the siRNA triggers. KTF1 has similarity to the transcription elongation factor SPT5 and contains a C-terminal extension rich in GW/WG repeats. KTF1 colocalizes with ARGONAUTE 4 (AGO4) in punctate nuclear foci and binds AGO4 and RNA transcripts. Our results suggest KTF1 as an adaptor protein that binds scaffold transcripts generated by Pol V and recruits AGO4 and AGO4-bound siRNAs to form an RdDM effector complex. The dual interaction of an effector protein with AGO and small RNA target transcripts may be a general feature of RNA-silencing effector complexes.

INTRODUCTION

RNA interference (RNAi) is a conserved gene-silencing mechanism in eukaryotic cells (Matzke and Birchler, 2005; Zaratiegui et al., 2007). In RNAi, double-stranded RNAs are processed by the RNaseIII enzyme Dicer into small interfering RNAs (siRNAs) that are then incorporated into an RNA-induced silencing complex (RISC) to direct the cleavage or translational inhibition of complementary RNA (Matzke and Birchler, 2005; Tomari and Zamore, 2005; Filipowicz, 2005). The core component of RISC is the PAZ- and PIWI-domain-containing protein, Argonaute (AGO), which binds to siRNAs and can slice complementary RNAs (Filipowicz, 2005; Qi et al., 2006). Similarly, miRNAs are also generated by Dicers and direct a miRNA RISC to cause degradation or translational inhibition of target mRNAs (Bartel, 2004). In fission yeast, siRNAs are incorporated into the RNA-induced transcriptional silencing (RITS) complex to cause heterochromatin formation (Volpe et al., 2002; Verdel et al., 2004). RITS also contains an AGO that slices transcripts complementary to the bound siRNAs (Verdel et al., 2004; Irvine et al., 2006). The conserved GW182 family of proteins is associated with miRNA RISC by binding to AGOs and is required for miRNA-mediated gene silencing (Ding and Han, 2007; Eulalio et al., 2008). In RITS, the Tas3 protein binds to AGO1 and is necessary for transcriptional gene silencing (Partridge et al., 2007; Till et al., 2007). The GW182 family of proteins and Tas3 all contain the GW/WG repeat sequence motif, which is considered an AGO hook that mediates interaction with AGOs (Ding and Han, 2007).

In plants, the overwhelming majority of small RNAs are 24 nt siRNAs corresponding to transposons and other repetitive elements (Zhang et al., 2007; Mosher et al., 2008). The 24 nt siRNAs cause epigenetic silencing by directing de novo DNA methylation through the RNA-directed DNA methylation (RdDM) pathway (Matzke and Birchler, 2005; Chan et al., 2005). In the RdDM pathway, siRNAs are generated by the action of the putative DNA-directed RNA polymerase Pol IV, RDR2 (RNA-dependent RNA polymerase 2), and DCL3 (Dicer-like 3) (Matzke et al., 2009). The siRNAs are loaded into AGO4 and AGO6 to direct DNA methylation by the de novo DNA methyltransferase DRM2 (Matzke et al., 2009). The functioning of the siRNAs also requires another putative DNA-dependent RNA polymerase, Pol V; the chromatin-remodeling protein, DRD1; and a structural-maintenance-of-chromosomes hinge domain-containing protein (Matzke et al., 2009). Pol IV and Pol V have distinct largest subunits, NRPD1 and NRPE1, respectively, but share with Pol II and/or with each other numerous additional subunits (Ream et al., 2009; Huang et al., 2009; He et al., 2009). NRPE1 contains a long C-terminal domain that is very rich in GW/WG repeats (El-Shami et al., 2007). The GW/WG repeats are required for

Pol V function and are both sufficient and necessary for interaction with AGO4 (El-Shami et al., 2007). Recently, Pol V was found to generate uncapped and nonpolyadenylated transcripts from several noncoding sequences that are targeted by RdDM (Wierzbicki et al., 2008). The evidence suggests that AGO4/6-bound siRNAs may find target DNA by binding to nascent scaffold transcripts generated by Pol V (Wierzbicki et al., 2009). The de novo DNA methyltransferase DRM2, which is presumably in the RdDM effector complex, is responsible for catalyzing cytosine methylation in CG, CHG, and CHH (H represents A, T, or C) sequence contexts (Cao and Jacobsen, 2002).

Active DNA demethylation mediated by the ROS1 family of DNA glycosylases is important for counteracting the activity of RdDM to prevent or attenuate hypermethylation and transcriptional silencing of transgene repeats, certain endogenous genes, transposons, and other repetitive sequences (Gong et al., 2002; Zhu et al., 2007; Penterman et al., 2007; Lister et al., 2008; He et al., 2009). In the DNA demethylase mutant *ros1*, these sequences show enhanced transcriptional silencing (Gong et al., 2002; Zhu et al., 2007; Lister et al., 2008). To identify RdDM pathway components, we carried out a genetic screen for second-site suppressors of the *ros1* mutant. Here, we report two allelic *ros1* suppressor mutants, *rdm3-1* and *rdm3-2*. The *rdm3* mutations release the silencing of an *RD29A* promoter-driven *luciferase* (*LUC*) transgene and the endogenous *RD29A* gene in *ros1* mutant plants. In the *rdm3* mutants, DNA methylation is reduced at RdDM target loci such as *5S rDNA*, *MEA-ISR*, *AtSN1*, *AtGP1*, and *AtMU1*. The *rdm3* mutations do not affect the levels of siRNAs corresponding to these loci, suggesting that RDM3 may function with Pol V in the effector step of RNA-directed DNA methylation. Like *ago4* mutations, however, *rdm3* does not block production of Pol V transcripts. *RDM3* encodes a protein that was annotated as KTF1 (KOW domain-containing transcription factor 1). KTF1 has similarity to SPT5, a conserved transcription elongation factor for RNA polymerase II (Wada et al., 1998; Winston, 2001). We found that KTF1 and AGO4 interact in vitro and in vivo, and the two proteins are colocalized in discrete nucleoplasmic foci. These results suggest that KTF1 may physically link Pol V transcription with AGO4-mediated transcript cleavage and epigenetic regulation. KTF1 contains a C-terminal region rich in WG repeats, and these repeats are sufficient for interaction with AGO4. Importantly, an RNA-binding site was identified in the C-terminal region of KTF1. We hypothesize that WG repeats have coevolved with an RNA-binding site in AGO-interacting proteins to facilitate the formation of a tight protein-transcript-siRNA effector complex for gene silencing.

RESULTS

The *rdm3* Mutations Suppress Transcriptional Gene Silencing in the *ros1* Mutant

Loss-of-function mutations in the DNA demethylase ROS1 cause transcriptional gene silencing (TGS) of the stress-responsive *RD29A* promoter-driven *luciferase* (*RD29A-LUC*) transgene, the endogenous *RD29A* gene, and the *CaMV 35S* promoter-driven *NPTII* (*35S-NPTII*) kanamycin resistance transgene that is physically linked to the *RD29A-LUC* transgene (Gong et al., 2002). The silencing of the *35S-NPTII* and *RD29A-LUC* transgenes is indicated by plant sensitivity to kanamycin and loss of luminescence, respectively. To identify components mediating TGS in *ros1*, we screened a T-DNA-mutagenized population in the *ros1* background, based on reactivation of luminescence from *RD29A-LUC* (He et al., 2009). Two allelic mutants, *rdm3-1* and *rdm3-2* (for RNA-directed DNA methylation 3), were characterized in this study.

Figure 1A shows the luminescence phenotypes of the wild-type, *ros1*, *ros1rdm3-1*, and *ros1rdm3-2*. Like the wild-type, both *ros1rdm3-1* and *ros1rdm3-2* emitted strong luminescence after cold treatment, whereas *ros1* emitted little or no luminescence. The result shows that the silencing of *RD29A-LUC* in *ros1* was suppressed by the *rdm3* mutations. However, the kanamycin sensitivity of *ros1rdm3-1* and *ros1rdm3-2* was similar to that of *ros1*, which indicated that the silencing of *35S-NPTII* in *ros1* was not suppressed by the *rdm3* mutations. The *ros1rdm3-1* and *ros1rdm3-2* double mutants were crossed to *ros1* (Figure 1A). The F1 plants emitted as little luminescence as the *ros1* plants, but the F2 progenies segregated ~3:1 for *ros1*:*ros1rdm3* luminescence phenotypes, suggesting that the *rdm3-1* and *rdm3-2* mutations were recessive and that each mutation was in a single nuclear gene (data not shown).

We crossed *ros1rdm3-1* plants to the wild-type and identified the *rdm3-1* single mutants. Interestingly, *rdm3-1* plants emitted stronger luminescence than the wild-type (Figures S1A and S1B available online). The result is consistent with the presence of a low level of TGS of the *RD29A-LUC* transgene in the wild-type (Gong et al., 2002; Agius et al., 2006) and suggests that RDM3 is required for this TGS.

As reported previously (Gong et al., 2002), the mRNA levels of the endogenous *RD29A*, *RD29A-LUC*, and *NPTII* transgenes were dramatically reduced by the *ros1* mutation. In *ros1rdm3-1*, the mRNA levels of both the endogenous *RD29A* and the *RD29A-LUC* transgene were substantially higher than those in *ros1* (Figure 1B). In contrast, the mRNA level of the *NPTII* transgene in *ros1rdm3-1* mutant was undetectable, as it was in *ros1*, which is consistent with the kanamycin-sensitive phenotype of the *ros1rdm3-1* and *ros1* plants (Figure 1B). These results demonstrate that the *rdm3* mutations suppress the TGS of the endogenous *RD29A* gene and the *RD29A-LUC* transgene, but not the *NPTII* transgene in the *ros1* mutant.

The *rdm3* Mutation Reduces DNA Methylation at the *RD29A* Promoter and Other RdDM Targets

To test whether the suppression of *RD29A-LUC* transgene silencing in the *ros1rdm3-1* mutant correlates with loss of DNA hypermethylation, we analyzed the DNA methylation status of the *RD29A* promoter by bisulfite sequencing. The results show that both the endogenous and transgenic *RD29A* promoters were heavily methylated at cytosine residues in all sequence contexts (CG, CHG, and CHH; H represents A, T, or C) in the *ros1* mutant, but the methylation was reduced in the *ros1rdm3-1* mutant (Figures 2A and 2B). The reductions were comparable to those in *ros1nrpd1* and were particularly evident at CHG and CHH sites (Figures 2A and 2B). For example, at the transgenic *RD29A* promoter, the asymmetric CHH methylation was 8.9% in the wild-type, 15.2% in *ros1*, 4.5% in *ros1rdm3-1*, and 2.8% in *ros1nrpd1* (Figure 2A). The methylation change at the