RNA polymerase V transcription guides ARGONAUTE4 to chromatin

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Retrotransposons and repetitive DNA elements in eukaryotes are silenced by small RNA-directed heterochromatin formation. In Arabidopsis, this process involves 24-nt siRNAs that bind to ARGONAUTE4 (AGO4) and facilitate the targeting of complementary loci via unknown mechanisms. Nuclear RNA polymerase V (Pol V) is an RNA silencing enzyme recently shown to generate noncoding transcripts at loci silenced by 24-nt siRNAs. We show that AGO4 physically interacts with these Pol V transcripts and is thereby recruited to the corresponding chromatin. We further show that DEFECTIVE IN MERISTEM SILENCING3 (DMS3), a structural relationship between these two activities is unclear.

Mutations disrupting NRPE1 (encoding the largest Pol V subunit), AGO4 or DMS3 cause similar losses of RNA-directed DNA methylation at AtSN1 retrotransposons, IGN5 (INTERGENIC REGION 5) and a retroelement solo LTR locus. Likewise, histone H3 lysine 27 monomethylation (H3K27me1), a characteristic of silenced heterochromatin, is reduced at these loci in nrpe1, ago4 and dms3 mutants compared to wild-type plants (ecotype Col-0). These results indicate that Pol V, AGO4 and DMS3 collaborate in the establishment of repressive chromatin modifications. At the solo LTR locus transcribed by RNA polymerase II (Pol II), chromatin immunoprecipitation (ChIP) shows that levels of diacetylated histone H3 (H3Ac2; acetylated on lysines 9 and 14), a mark of active chromatin, increase in the mutants (Fig. 1d), coincident with increased Pol II occupancy of the locus (Fig. 1e; compare to no-antibody controls in Fig. 1f). At IGN5 and AtSN1, which lack associated Pol II (Fig. 1e), no increase in histone H3 acetylation is observed in the mutants (Fig. 1d). AtSN1 elements are thought to be transcribed by Pol III; therefore, differences in H3 acetylation at the solo LTR and AtSN1 loci may reflect the different polymerases involved.

AGO4 and Pol V colocalize in a nucleolus-associated Cajal body. To test whether Pol V enzymatic activity is required for AGO4 binding to chromatin, we examined AGO4–chromatin associations in nrpe1 mutants that had been transformed with either a full-length,
wild-type NRPE1 transgene or an equivalent transgene bearing point mutations within the metal A motif of the active site (NRPE1 ASM transgene). The active site point mutations do not affect NRPE1 stability or its association with the second-largest subunit but eliminate Pol V transcripts and Pol V biological activity. Whereas the wild-type NRPE1 genomic transgene (NRPE1 wt) restored AGO4 interaction with the solo LTR, IGN5, AtSN1 and IGN6 loci in the nrpe1 mutant background (Fig. 3b), the active-site mutant (NRPE1 ASM) failed to do so. Immunoblotting ruled out the trivial explanation that AGO4 protein levels might be differentially affected by the nrpe1 mutation or the NRPE1 transgenes (Fig. 3c) and also demonstrated that the antibody specifically recognizes AGO4, which is absent in the ago4 mutant. Collectively, the data indicate that Pol V transcriptional activity is required to recruit AGO4 to chromatin.

Base-pairing between AGO4-associated siRNAs and nascent Pol V transcripts could be a mechanism by which Pol V transcription recruits AGO4 to target loci. To test this hypothesis, we used RNA immunoprecipitation to ask whether AGO4 associates with Pol V transcripts in vivo. In wild-type (Col-0) plants, anti-AGO4 immunoprecipitates IGN5 and IGN6 Pol V transcripts (Fig. 4a). Important controls show that Pol V transcripts are not immunoprecipitated in the ago4 or nrpe1 mutant backgrounds. Anti-AGO4

Figure 1 Pol V, AGO4 and DMS3 work nonredundantly in heterochromatin formation. (a,b) DNA methylation analysis at the AtSN1, IGN5 and solo LTR loci in nrpe1, ago4 and dms3 mutants. Genomic DNA was digested with HaeIII (a) or AluI (b) methylation-sensitive restriction endonucleases followed by PCR. Sequences lacking HaeIII sites (actin 2; a) or AluI sites (IGN3, b) served as controls to show that equivalent amounts of DNA were tested in all reactions. (c,d) ChIP analysis of H3K27me1 (c) and H3Ac2 (d) levels in nrpe1, ago4 and dms3 mutants. Histograms show means ± s.d. obtained from three independent amplifications. (e) ChIP analysis of Pol II binding to chromatin in nrpe1, ago4 and dms3 mutants. Histograms show means ± s.d. obtained from three independent amplifications. (f) Control ChIP reactions carried out in the absence of antibody reveal background signal levels.

Figure 2 AGO4 is not required for Pol V transcription. (a) Strand-specific RT-PCR of Pol V transcription at IGN5, IGN6 and AtSN1 in ago4 and rdr2 mutants as well as nrpe1 ago4 and rdr2 ago4 double mutants. Wild-type sibling is a wild-type sibling of the ago4 mutant identified in a segregating family. Actin RT-PCR products and ethidium bromide-stained rRNAs resolved by agarose gel electrophoresis serve as loading controls. To control for background DNA contamination, we carried out a reaction using IGN5 top strand primers but no reverse transcriptase (no RT). No-RNA (0 μg) controls are provided for all primer pairs. (b) Densitometric analysis of RT-PCR data for the ago4 mutant presented in a. The histogram provides mean band intensities relative to wild type Col-0, ± s.d. obtained from three independent experiments.
irradiation of IGN5 or IGN6 RNAs was also reduced or eliminated in rdr2 mutant plants, indicating that AGO4–Pol V transcript interactions are dependent on siRNAs. However, in the absence of siRNA biogenesis, as in the rdr2, nrpd1, nrpe1, and drd2 mutants, AGO4 protein levels drop below the limits of immunoblot detection7,8 (Fig. 4b–d). By contrast, AGO4 protein levels are unaffected in nrpe1 (Fig. 4b–d) or drd2 mutants (ref. 7). The loss of detectable Pol V transcription in the absence of siRNAs complicates the interpretation of these results. Although we favor the hypothesis that siRNA–Pol V transcript base-pairing is responsible for AGO4 association with Pol V RNAs, we cannot rule out the possibility that AGO4 binds Pol V transcripts directly, with siRNAs merely being required for AGO4 stability.

DM33 was recently identified as a gene required for RNA-directed DNA methylation that acts at an unspecified step downstream of siRNA biogenesis4. The encoded protein shares sequence similarity with the hinge-domain regions of SMC proteins, such as the core proteins of cohesin and condensin complexes19, suggesting a chromatin-related function. We found that at IGN5, IGN6 and AtSN1 loci, Pol V transcripts are substantially reduced or absent in dms3 mutant plants, as in nrpe1 (Fig. 5a) or ddr1 mutants3. Likewise, transcriptional suppression of AtSN1 and solo LTR elements is similarly disrupted in dms3 and nrpe1 mutants (Fig. 5b). ChIP using an antibody to NRPE1 revealed that, in the dms3 mutant, Pol V–chromatin associations are reduced to background levels, resembling the actin and nrpe1 mutant controls (Fig. 5c). Collectively, these data (Fig. 5) indicate that DM3 is required for Pol V transcription, as shown previously for the chromatin remodeler DRD1 (ref. 3). The loss of detectable Pol V–chromatin association in dms3 or ddr1 mutants suggests that these chromatin proteins participate in the assembly of Pol V transcription complexes.

Figure 3 Pol V transcription is necessary for AGO4–chromatin interactions. (a) ChIP data showing AGO4 binding to chromatin at solo LTR, IGN5, AtSN1 and IGN6 loci in ago4, nrpe1 and ddr2 mutants. DNA purified from input chromatin samples, chromatin subjected to the immunoprecipitation procedure in the absence of antibody (no Ab) and chromatin immunoprecipitated using anti-AGO4 (αAGO4) was amplified by PCR using locus-specific primers. Primers amplifying the Actin2 locus served as a loading control. (b) ChIP data showing AGO4 binding to chromatin at solo LTR, IGN5, AtSN1 and IGN6 loci in nrpe1 mutant transformed with either a wild-type NRPE1 transgene (NRPE1 wt) or an NRPE1 active site mutant transgene (NRPE1 ASM). (c) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), ago4, nrpe1, or nrpe1 transformed with either a wild-type NRPE1 transgene (NRPE1 wt) or an NRPE1 active site mutant transgene (NRPE1 ASM). Ponceau S staining revealed equal loading of lanes; 100% and 50% sample loadings indicate that the assay is semiquantitative.

Figure 4 AGO4 physically interacts with Pol V transcripts. (a) RNA immunoprecipitation using anti-AGO4 (αAGO4). Immunoprecipitated RNA isolated from the indicated mutants was digested with DNaseI and amplified by RT-PCR. Total RNA controls show that the Pol V transcripts are present in equivalent amounts in all mutants tested except nrpe1. Ethidium bromide–stained rRNAs (bottom left) show that equal amounts of RNA were tested. The no reverse transcriptase (no RT) control was done with IGN5 bottom-strand primers. No-RNA controls were carried out for all primer pairs tested. RT-PCR amplification of actin RNA serves as a loading control. (b) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0) plants or ago4 mutant. Asterisks denote nonspecific bands. (c) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), rdr2, dc3, dcI234 or nrpe1 mutants. Asterisks denote nonspecific bands. (d) Immunoblot detection of AGO4 in protein extracts of wild-type (Col-0), nrpd1 (Pol IV), nrpe1 (Pol V), nrpd2/nrpe2 (shared subunit of Pol IV and Pol V) or rdr2 mutants. Asterisks denote nonspecific bands.
Our results suggest that siRNAs and Pol V transcripts are produced by independent pathways that intersect to bring about heterochromatin formation and gene silencing (Fig. 6). In one pathway, Pol IV, RDR2 and DCL3 collaborate to produce 24-nt siRNAs that associate with AGO4 (ref. 1). Independent of this pathway, DRD1 and DMS3 facilitate noncoding Pol V transcription at target loci. AGO4's interaction with Pol V transcripts, and the fact that AGO4 association with chromatin requires the Pol V active site, suggests that siRNA–AGO4 interactions with nascent Pol II transcripts19. Our findings suggest that plants have evolved a unique RNA polymerase, Pol V, whose specialized role seems to be the generation of noncoding RNAs that can serve as scaffolds for Aragonaute recruitment.

**METHODS**

**Plant strains.** *Arabidopsis thaliana nrpe1 (nrpd1b-11)* was described previously5. The *dms3-4* mutant (SALK_125019C) of locus At3g49250 was obtained from the *Arabidopsis* Biological Resource Center. The *dcl2, dcl3, dcl4* triple mutant (*dcl2,3,4*) was provided by T. Blevins (Washington University, St. Louis). The *ago4-1* mutant ( Ler ecotype background) was provided by S. Jacobsen (University of California, Los Angeles) and was introgressed into the Col-0 background by three rounds of backcrossing.

**Antibodies.** Anti-Pol II (anti-NRPB2) was described previously20. Anti-H3K27me1 (ref. 21) was provided by T. Jenuwein (Max Planck Institute of Immunobiology). Antibody against diacetyl-H3 (K9 and K14) was obtained from Millipore (cat. #06599, lot #JBC1349702). Rabbit anti-AGO4 was raised against a C-terminal portion of the protein (amino acids 573–924) expressed in bacteria.

**RNA and DNA analysis.** RNA isolation, RT-PCR and real-time quantitative PCR were carried out as described3 except that real-time quantitative PCR analysis of the *IGN5* locus was done using the following oligonucleotide primers: A195, 5′-ACATGAAGAAAGCCCAAACCA-3′; A196,
5′-GGCCGAATACAGCAAGTCCT-3′. Densitometric analysis of DNA resolved by agarose gel electrophoresis was performed using ImageJ.

ChIP and RNA IP. ChIP and RNA IP were carried out as described except that for ChIP with anti-AGO4, RNase A was added during immunoprecipitation, washes with TE buffer were omitted, immune complexes were eluted with 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS for 10 min at room temperature and a second elution at 65 °C was performed. Crosslinking was reversed at 65 °C for 1 h in the presence of 40 µg Proteinase K (Invitrogen). DNA was purified by extraction with phenol-chloroform and ethanol precipitation. DNA recovery was assayed by PCR using 1.5 u Platinum Taq (Invitrogen).

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AUTHOR CONTRIBUTIONS

T.S.R. generated anti-AGO4; J.R.H. and T.S.R. assayed NRPE1–AGO4 interactions; J.R.H. produced Figure 4d; A.T.W. performed all remaining experiments. A.T.W. and C.S.P. wrote the manuscript.

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