RNA-directed DNA methylation involves co-transcriptional small-RNA-guided slicing of polymerase V transcripts in Arabidopsis

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Small RNAs regulate chromatin modifications such as DNA methylation and gene silencing across eukaryotic genomes. In plants, RNA-directed DNA methylation (RdDM) requires 24-nucleotide small interfering RNAs (siRNAs) that bind to ARGONAUTE 4 (AGO4) and target genomic regions for silencing. RdDM also requires non-coding RNAs transcribed by RNA polymerase V (Pol V) that probably serve as scaffolds for binding of AGO4–siRNA complexes. Here, we used a modified global nuclear run-on protocol followed by deep sequencing to capture Pol V nascent transcripts genome-wide. We uncovered unique characteristics of Pol V RNAs, including a uracil (U) common at position 10. This uracil was complementary to the 5'-adenine found in many AGO4-bound 24-nucleotide siRNAs and was eliminated in a siRNA-deficient mutant as well as in the ago4/6/9 triple mutant, suggesting that the +10 U signature is due to siRNA-mediated co-transcriptional slicing of Pol V transcripts. Expression of wild-type AGO4 in ago4/6/9 mutants was able to restore slicing of Pol V transcripts, but a catalytically inactive AGO4 mutant did not correct the slicing defect. We also found that Pol V transcript slicing required SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L), an elongation factor whose function is not well understood. These results highlight the importance of Pol V transcript slicing in RNA-mediated transcriptional gene silencing, which is a conserved process in many eukaryotes.

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immunoprecipitation followed by sequencing (ChIP–seq) with an endogenous antibody against NRPE1, the largest catalytic subunit of Pol V. Combining Pol V ChIP–seq and GRO–seq in Col-0, \textit{nrpe1} and \textit{nrpd1/e1}, we identified GRO–seq reads that mapped to Pol V regions, including those at previously defined individual Pol V intergenic non-coding (IGN) transcripts \(^{11}\) (Fig. 1b). As expected, we found that GRO–seq signals generated from Pol V-occupied regions were largely eliminated in the \textit{nrpe1} mutant, whereas signals over mRNA regions in the \textit{nrpe1} mutant remained unchanged (Supplementary Fig. 1b,c), confirming that we had indeed identified Pol V-dependent nascent transcripts. In addition to the tight spatial co-localization of Pol V ChIP–seq and GRO–seq signals, we

**Fig. 1 | Capturing Pol V-dependent transcripts with GRO–seq.** \(a\), Procedure for constructing the Arabidopsis GRO–seq library, which captures nascent Pol V transcripts. 7-methylguanine (7meG)-capped transcripts generated by Pol II are excluded by selective ligation to the 5′ monophosphorylated (5′Pi) RNAs generated by Pol I, IV and V. IP, immunoprecipitation; RNAP, RNA polymerase. \(b\), Screenshot of CG, CHG and CHH methylation (mCG, mCHG and mCHH, respectively) in wild-type Col-0, Pol V ChIP–seq in Col-0, and GRO–seq in Col-0, \textit{nrpe1} and \textit{nrpd1/e1} over the previously identified Pol V locus IGN5 (ref. \(^{11}\)). For mCG, mCHG and mCHH, the y axis indicates the percentage of methylation. Plus (+) and minus (–) indicate the strandness of the GRO–seq signal. \(c\), Metaplot of the Pol V ChIP–seq signal over input, and the ratio of the GRO–seq signal in Col-0 to \textit{nrpe1} plotted over the centres of the Pol V-occupied regions defined by Pol V ChIP–seq.

**Fig. 2 | Characteristics of Pol V-dependent transcripts.** \(a\), Distribution of ratios of plus-strand GRO–seq signals over minus-strand GRO–seq signals in Col-0 over the top 500 expressed mRNAs. \(b\), Distribution of ratios of plus-strand GRO–seq signals over minus-strand GRO–seq signals in Col-0 over the top 500 Pol V-enriched regions defined by Pol V ChIP–seq. In a and b, purple lines show the kernel density estimates for each histogram. \(c\), Pol V ChIP–seq signals over inputs, and the ratio of the GRO–seq signal in Col-0 to \textit{nrpe1} plotted over Pol V-associated transposable elements (TEs) with different lengths.
also observed a positive correlation in signal intensity between the two (Supplementary Fig. 1d). However, Pol V-dependent GRO–seq signals were much more narrowly defined than signals from Pol V ChIP–seq, thereby providing a higher-resolution view of Pol V transcription (Fig. 1c). Unlike Pol II transcripts, which are primarily transcribed from one strand (Figs. 1b and 2a), Pol V-dependent transcripts were present roughly equally on both strands (Figs. 1b and 2b). RdDM has been shown to be enriched at short transposons as well as at the edges of long transposons13. Consistent with Pol V occupancy at long transposon edges14, we found that Pol V-dependent GRO–seq transcripts were also preferentially localized over those regions (Fig. 2c and Supplementary Fig. 1e).

To investigate the relationship between Pol IV activity and Pol V transcription production, we performed Pol V ChIP–seq and GRO–seq in the nrpd1 mutant, which specifically eliminates Pol IV activity. Although many Pol V transcripts were eliminated in the nrpd1 mutant (Supplementary Fig. 2a), most remained (Supplementary Fig. 2b). Based on whether the Pol V ChIP–seq signal remained in nrpd1, we classified Pol V regions into Pol IV/Pol V-co-dependent regions (1,903 sites) or Pol IV-independent Pol V regions (2,365 sites) (Supplementary Table 2). As expected, both the GRO–seq signal and the Pol V ChIP–seq signal were largely eliminated in nrpd1 mutants at Pol IV/Pol V co-dependent sites, whereas the signals at Pol IV-independent sites largely remained (Supplementary Fig. 2c,d).

The reason that some Pol V transcripts are dependent on Pol IV activity is probably because the RdDM pathway is a self-reinforcing loop15. For example, although Pol V is required for DNA methylation and silencing, Pol V recruitment to chromatin requires pre-existing DNA methylation via the methyl DNA-binding proteins SU(VAR)3-9 homologues SUVH2 and SUVH9 (ref. 16). Thus, we hypothesized that the reason that Pol IV is required for Pol V activity at only some genomic sites is because it has a larger role in DNA methylation maintenance at this subset of sites. To test this, we analysed cytosine methylation levels and 24-nt siRNAs abundance at both the Pol IV/Pol V-co-dependent and the Pol IV-independent sites. If Pol IV actively maintains DNA methylation at specific genomic sites to enable Pol V recruitment and transcription, then loss of Pol IV should have a more dramatic effect on the methylation levels at these sites. Indeed, Pol IV/Pol V-co-dependent sites showed significantly higher 24-nt siRNA levels and substantial reductions in all types of cytosine methylation in the nrpd1 mutants, whereas Pol IV-independent sites showed lower levels of 24-nt siRNAs and less reduction in DNA methylation (Supplementary Fig. 2e,f). This is probably because the other DNA methylation maintenance pathways involving METHYLTRANSFERASE1 (MET1), CHROMOMETHYLASE 3 (CMT3) and CMT2 are active at these loci and compensate for the loss of methylation in the Pol IV mutant. In summary, these results show that, even though Pol IV and Pol V work closely together in the RdDM pathway, Pol V can transcribe independently of Pol IV at many sites in the genome. Previous studies of Pol IV transcripts have shown them to be exceedingly rare in wild-type strains because of their efficient processing into siRNAs by Dicer enzymes17–19. However, it remains possible that trace levels of Pol IV transcripts could be present in our GRO–seq libraries. Thus, to uniquely focus on the characteristics of Pol V transcripts without any complication of the presence of small amounts of Pol IV transcripts, we focused our remaining analysis on Pol IV-independent Pol V regions.

Pol V transcripts show evidence of small-RNA-dependent slicing. Because our GRO–seq method did not include the fragmentation step typical of traditional GRO–seq14, it was possible to estimate the length of Pol V nascent transcripts and assess their 5’ nucleotide composition. We observed a range of read lengths from 30-nt to 90-nt long with a peak at around 50 nt, and detected very few reads longer than about 120 nt (Fig. 3a). Nascent Pol V transcripts observed in nrpd1 GRO–seq showed a similar size distribution (Supplementary Fig. 3a). GRO–seq involves an in vitro nuclear run-on step in which the reaction is limited by time and nucleotide concentration, meaning that the run-on is unlikely to proceed to the natural 3’ end of the transcript. Thus, the average length of Pol V transcripts measured here is probably an underestimate of the true length of Pol V transcripts in vivo. Using Pol V RIP–seq, a study estimated the median Pol V transcript length to be around 200 nt. However, as a fragmentation step was included in their RIP protocol, this was also an estimation21. Nevertheless, Pol V transcripts are clearly at least 50-nt long on average; this is significantly longer than Pol IV transcripts, which have been estimated to be around 30–40-nt long21.
Eukaryotic and bacterial RNA polymerases preferentially initiate transcription at purines (A or G), commonly with a pyrimidine (C or T) present at the -1 position with respect to the transcription start site. However, instead of this expected enrichment at Pol V transcript 5' ends, we observed a strong preference for uracil (U; on average 53.41%) at nucleotide +10 position across six Col-0 biological replicates (Fig. 3b and Supplementary Fig. 3b). This characteristic was unlikely to be an artefact of the GRO-seq procedure, as no such preference was observed in transcripts that mapped to mRNA regions (Supplementary Fig. 3c,d). To test whether the +10 U signature was specific to nascent RNAs with certain lengths, we examined the nucleotide preferences within different size ranges. We found a +10 U signature in all size ranges tested from 30-nt RNAs to RNAs longer than 70 nt, with the strongest signature in 40–50-nt long reads (Supplementary Fig. 3e–i).

In Arabidopsis, AGO4 shows slicer activity in vitro and interacts directly with Pol V. In addition, AGO4-associated 24-nt siRNAs are highly enriched for 5' adenines. Thus, we hypothesized that the 5' end of Pol V transcripts is often defined by an AGO4 slicing event, and that the U at position 10 in Pol V transcripts corresponds to a 5' A in AGO4 24-nt siRNAs (Fig. 3c). We plotted the sequence composition of previously published AGO4-associated 24-nt siRNAs that mapped to our identified Pol V transcript sites and observed a strong 5' enrichment for A (80.53%) (Fig. 3d). If Pol V transcripts are sliced at 10 nt from the 5' end of AGO4 siRNAs, we should detect sense–antisense siRNA–Pol V transcript pairs separated by 10 nt and a corresponding 10 nt of complementary sequence (Fig. 3c). We plotted the distance between each 5' end of AGO4 siRNAs and the 5' end of its Pol V transcript neighbours on the opposite strand. Consistent with our hypothesis, we found a strong peak of AGO4-associated 24-nt siRNA 5' ends at 10 nt downstream from the Pol V 5' end (Fig. 3e). Overall, 78.07% of AGO4-associated 24-nt siRNAs had a Pol V-dependent transcript partner detected in GRO-seq whose 5' end could be mapped 10 nt away on the complementary strand.

To determine whether the slicing-associated +10 U signature was dependent on 24-nt siRNAs, which are transcribed by Pol IV, we examined the Pol V transcript sequence composition in the Pol IV mutant nrdp1. We found that in nrdp1, the U preference at position 10 was completely abolished (Fig. 3f). Instead, we observed the conventional +1 A/U and a –1 U/A 5' signature (Fig. 3f) similar to other RNA polymerases and to mRNA GRO-seq reads in wild type or in the nrdp1 mutant (Supplementary Fig. 3c,d). These results strongly support the hypothesis that the +10 U signature is due to 24-nt siRNA-dependent slicing of Pol V transcripts.

**Fig. 4 | Slicing of Pol V transcripts requires AGO4/6/9.** a–h. The relative nucleotide bias of each position in the upstream and downstream 20 nt of nascent transcripts captured with GRO-seq in Ws (a), ago4/Col-0 (b), ago4/Ws (c), ago4/6/9 (d), ago4/wtAGO4 (e), ago4/D742A (f), ago4/6/9/wtAGO4 (g) and ago4/6/9/D742A (h) over Pol V-dependent regions. Replicates were merged in a–h. Arrows indicate the relative nucleotide bias 10-nt downstream from the 5' ends of nascent transcripts captured with GRO-seq. i. The percentage of U presented over the genomic average at position 10 from the 5' end of AGO4 siRNAs and the 5' end of AGO4-associated 24-nt siRNA 5' ends at 10 nt downstream from the Pol V 5' end (Fig. 3e). Overall, 78.07% of AGO4-associated 24-nt siRNAs had a Pol V-dependent transcript partner detected in GRO-seq whose 5' end could be mapped 10 nt away on the complementary strand.

**AGO4, AGO6 and AGO9 are required for the slicing of Pol V transcripts.** Given that AGO4 is the main argonaute involved in RdDM, we tested whether AGO4 is also required for slicing of Pol V transcripts. By performing GRO-seq in the ago4-5 mutant in the Col-0 background (ago4/Col-0) and the ago4-4 mutant in the Wassilewskija (Ws) background (ago4/Ws), we observed that the +10 U slicing signature of Pol V transcripts was reduced by 13.26% in ago4-5 relative to wild-type Col-0 and by 12.37% in ago4-4 relative to wild-type Ws (Figs. 3b and 4a–c). The remaining slicing signature in ago4 mutants is probably due to redundancy of AGO4 with two other close family members, AGO6 and AGO9. Thus, we also performed GRO-seq in the ago4-4/ago6-2/ago9-1 (ago4/6/9) triple-mutant background. The +10 U signature in
ago4/6/9 mutants was completely abolished (Fig. 4d,i), suggesting a complete lack of slicing.

Previous work showed that the Asp-Asp-His (DDH) catalytic motif of AGO4 is required for slicing of RNA transcripts in vitro. Thus, we performed GRO–seq in plants containing either a wild-type AGO4 transgene (wtAGO4) expressed in ago4/Ws or in the ago4/6/9 triple mutant, or a slicing-defective AGO4 (D742A) mutant expressed in ago4/Ws or in the ago4/6/9 triple mutant. We found that wtAGO4 largely complemented the +10 U slicing signature in the ago mutants, whereas the AGO4 D742A catalytic mutant failed to restore the +10 U signature (Fig. 4e–i). To rule out the possibility that the elimination of the +10 U Pol V slicing signature in the ago mutants is caused by the elimination of the +1 A nucleotide preference of 24-nt siRNAs, we analysed previously published small RNA-seq data sets corresponding to the same collection of ago mutant–transgene combinations. We found that all mutants and mutant–transgene combinations retained a strong enrichment of A at position 1 of the 24-nt siRNAs, indicating that both slicing and Pol V transcript levels are affected in ago4/6/9 triple mutants, although we cannot rule out minor levels of slicing that do not involve U–A pairing or by other AGO proteins.

**SPT5L is required for the slicing of Pol V transcripts.** There are several proteins in the RdDM pathway whose precise function is unknown but that act at some point downstream of the biogenesis of siRNAs, including SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5L; also known as KTF1), DOMAINS RARRANGED METHYLTRANSFERASE 3 (DRM3), INVOLVED IN DE NOVO 2 (IDN2), IDN2-LIKE 1 and 2 (IDL1 and IDL2), SNF2-RING-HELCASE-LIKE 1 and 2 (FRG1 and FRG2) and SU(VAR)3-9-RELATED 2 (SUVR2). Mutations in these genes all show a partial reduction of DNA methylation associated with the RdDM pathway, rather than a complete loss of RdDM as seen in strong mutants, such as nrpd1 or nrpe1 (refs 30–34). To examine whether any of these components are involved in the slicing of Pol V transcripts, we performed GRO–seq in mutant backgrounds, including spt5l, drm3, idn2, idn2/idl1/idl2, frg1/frg2 and suvr2. We observed that all mutants retained a strong +10 U slicing signature (Figs. 5a–e and 6a), except for the spt5l mutant, which completely eliminated the slicing signature (Figs. 5f and 6a). A trivial explanation for the lack of +10 U slicing signature in spt5l would be that this mutant eliminated 24-nt siRNAs or eliminated the enrichment of A at the 5′ nucleotide of 24-nt siRNAs. However, we found only a moderate (although significant) reduction of 24-nt siRNA abundance (Fig. 5b) and a strong remaining +1 A nucleotide preference (Fig. 5c,d) in spt5l. These results reveal a novel role for SPT5L in the slicing of Pol V transcripts.

We also analysed the effect of each of the mutants on the overall levels of Pol V GRO–seq signals (Fig. 6c), and, as a control, examined their effects on the background levels of GRO–seq signals at the top 1,000 expressed Pol II genes (Supplementary Fig. 4i). Although the drm3, idn2, idn2/idl1/idl2, frg1/frg2 and suvr2 mutants showed only minor effects on overall Pol V transcript levels, spt5l showed a strong reduction.

This reduction was even greater than that seen in the Pol IV mutant nrpd1, which is a strong RdDM mutant that shows a much greater reduction in DNA methylation than spt5l. This result suggests that SPT5L has a role in Pol V transcript stability and/or production. SPT5L is a homologue of the Pol II elongation factor SPT5 (ref. 35). It has been shown to interact with the Pol V complex, but its precise role in the RdDM pathway has been unclear. Our finding that both slicing and Pol V transcript levels are affected in spt5l mutants suggests that SPT5L has a dual role in the processing and utilization of Pol V transcripts.

**Discussion**

In this work, we show that Pol V transcripts are frequently sliced in a siRNA-dependent and SPT5L-dependent manner. Because the slicing signature is present in Pol V transcripts that are in the process of transcribing, it is clear that this slicing occurs co-transcriptionally. Mutations in the gene encoding AGO4 that affect the catalytic residues required for slicing show a partial loss of RdDM similar to spt5l mutants, suggesting that the slicing step is required for efficient RdDM. However, it is also clear that slicing is not required for all RdDM, as spt5l mutants seem to abolish slicing and yet show only a partial loss of CHH methylation at RdDM sites. AGO4 can also physically interact with DRM2, which provides an alternative mechanism for slicing of Pol V transcripts.
mechanism by which AGO4–siRNA complexes can promote RdDM. This suggests a dual mechanism by which AGO4 can promote DRM2 activity, through both Pol V transcript slicing and interaction with DRM2 (Fig. 6f).

SPT5L contains a region rich in WG repeats (called the AGO hook) that is capable of binding to AGO4 (ref. 23). AGO4 also interacts with a similar WG repeat region within the largest subunit of Pol V23. It has been recently shown that deletion of the WG repeats of SPT5L, or deletion of the WG repeats of Pol V, still allow AGO4 recruitment and RdDM. However, simultaneous deletion of both WG repeat regions abolishes RdDM, indicating that the WG-rich domains of SPT5L and Pol V are redundantly required for AGO4 recruitment43. This genetic redundancy also indicates that the role of SPT5L in AGO4 recruitment is unlikely to account for its requirement for Pol V-dependent regions in Col-0, spt5l and nrpd1. *P < 0.05 (Welch two sample t-test). n = 2,365. c, d, The relative nucleotide bias of each position for all 24-nt siRNAs in Col-0 (c) and spt5l (d) generated over Pol V-dependent regions. e, Abundance of nascent transcripts over Pol V-dependent regions in Col-0, nrpd1, nrpe1, nrpd1/6/9, spt5l, drm3, frg1/2, idn2/id1/id2, idn2 and suvr2. *P < 0.05 (Welch two sample t-test). n = 2,365. f, Proposed model for slicing of Pol V transcripts. For the box plots in b and e: the middle line represents the median; boxes represent the 25th (bottom) and 75th (top) percentiles; and bars represent the minimum and maximum points within 1.5× interquartile range.

In *Drosophila*, similar slicing patterns were observed in the AGO3–repeat-associated siRNA ‘ping-pong’ pathway in which AGO3 directs cleavage of its cognate mRNA target across from nucleotides 10 and 11, measured from the 5′ end of the small RNA guide strand, followed by the generation of secondary small RNAs from mRNA targets43,44. Thus, one hypothesis is that sliced Pol V nascent transcripts, clearly supports an RNA-targeting model, whereby the siRNAs target the nascent Pol V RNA rather than binding directly to the DNA. This is also supported by the conclusive data in fission yeast suggesting siRNA–RNA interactions45–47. Once the AGO4–siRNAs have bound to nascent Pol V RNAs and slicing has occurred, one possible function is that the resulting sliced RNAs or siRNA–sliced RNA duplexes have a signalling role, perhaps through specific RNA-binding proteins, in the targeting of the DRM2 methyltransferase to methylate chromatin (Fig. 6f). This model is attractive because slicing represents the integration of the activities of the upstream Pol IV-driven siRNA biogenesis pathway and the downstream Pol V-driven non-coding RNA biogenesis pathway, which could provide additional accuracy and specificity for DNA methylation targeting. Another possibility is that slicing promotes the recycling of AGO–siRNA complexes and/or Pol V transcripts to promote iterative cycles of targeting of DNA methylation through AGO4–DRM2 interactions42. Future studies aimed at understanding the biochemical details of the interaction of AGO4-bound siRNAs and Pol V targets are likely to shed additional light on the mechanisms of DNA methylation control.

Methods

**Plant materials and growth.** Col-0 was used as the wild-type genetic background for this study unless specified. The mutant alleles of *nrpd1*–4 (*SALK_083051*), *nrpd1*–12 (*SALK_033852*), *sppt5l*–1 (*SALK_001254*), *drm3*–1 (*SALK_136439*), *frg1*–2 (SALK_012288) and *idn2*–1 (SALK_136439), *idn2*–1 (SALK_075378) and *idn2*–1 (SALK_012288) triple mutants were described before48. *Ds*, *ago4*/*Ds*, *ago4*/*Ds*, *ago4*/*wtAGO4*, *ago4*/*D742A*, *ago4*/*D742A* were described previously49. All plants were grown...
on soil under long-day conditions (16 h of light, 8 h of dark). Inflorescence tissues with both floral buds and open flowers were collected and used for the GRO–seq procedure. T-DNAs were confirmed by PCR-based genotyping.

**Nuclei isolation.** Approximately 10 g of inflorescence and meristem tissue was collected from plants and immediately placed in ice-cold grinding buffer (300 mM sucrose, 20 mM Tris, pH 8.0, 5 mM MgCl₂, 5 mM KCl, 0.2% Triton X-100, 5 mM β-mercaptoethanol and 35% glycerol). Nuclei were isolated as described previously. Briefly, samples were ground with an Omni International General Laboratory Homogenizer at 4°C until well homogenized, filtered through a 250-μm nylon mesh, a 100-μm nylon mesh, a miracloth and finally a 40-μm cell strainer before being split into 50-ml conical tubes. Samples were spun for 10 min at 2,520 × g, the supernatant was discarded and the pellets were pooled and resuspended in 25 ml of grinding buffer using a Dounce homogenizer.

The wash step was repeated at least once more, and nuclei were resuspended in 1 ml of freezing buffer (50 mM Tris, pH 8.0, 5 mM MgCl₂, 20% glycerol and 5 mM β-mercaptoethanol).

**GRO–seq.** Approximately 5×10⁷ nuclei in 200 μl of freezing buffer were run-on in 3x NRR reaction buffer with 330 μg/mL T7 RNA polymerase (Roche, cat. no. 170-6891-01) and 100 μg/mL wtAGOT, ago4/D742A, ago4/6/wtAGOT and ago4/6/9/D742A, approximately 3×10⁷ to 5×10¹¹ nuclei were used. To minimize run-on length, the limiting cytidine triphosphate (CTP) concentration was reduced to a final concentration of 20 mM. Reactions were stopped after 5 min to minimize run-on length (~5–15 nt) while still incorporating 5-bromouridine 5'-triphosphate (BrUTP) at 750 μM to yield 750 μM Trizol IS (Fisher Scientific), and RNA was purified according to the manufacturer's manual. Without fragmentation or Terminator treatment, nascent RNA was enriched twice for BrUTP by BrUTP by using a customized Perl script for downstream analysis. Raw AGO4 RIP-seq data were obtained from a previously published data set (GSM707686)32. Reads were then trimmed for Illumina adaptors using Cutadapt (v1.9.1) and mapped to the TAIR10 reference genome using Bowtie (v1.1.0)30, allowing only one unique hit (±1) and zero mismatch.

**Whole-genome bisulfite sequencing analysis.** Processed whole-genome bisulfite sequencing data of Col-0 and nrpd1 were obtained from previously published data sets (GSE39901 and GSE3828634). CG, CHG and CHH methylation over different regions was extracted using a customized Perl script.

**Bioinformatic analysis.** GRO–seq analysis. Queq files from the sequencer were demultiplexed and converted to fastq format with a customized script. Paired-end reads were first trimmed for Illumina adaptors and primers using Cutadapt (v1.9.1). After trimming, reads <100 bp long were removed with a customized Perl script. Paired-end reads were then separately aligned to the Arabidopsis reference genome (TAIR10) using Bowtie (v1.1.0)30 by allowing only one unique hit (~1 and up to three mismatches in v 3). Paired-end reads that were aligned to positions within 2,000 bp of each other were considered as correct read pairs, and reads aligned to Watson or Crick strands were separated by a customized Perl script.

**Identification of Pol V-dependent transcripts from GRO–seq data.** To call Pol V-dependent transcripts, the P package DESeq2 (ref. 34) was used. Only reads with at least 4-fold enrichment in Col-0 compared to the nrpd1 and nrpd1/e1 mutant and a FDR of <0.05 were retained. Bins within 200 bp of each other were then merged into Pol V-dependent transcript clusters. To characterize Pol IV dependency on those Pol V-dependent transcript clusters, we checked NRPE1 binding in the nrpd1 mutant. If a Pol V-dependent transcript cluster was not bound by NRPE1 in the nrpd1 mutant while also having a RPKM (reads per kilobase of million) of GRO–seq in nrpd1 of >2, then this site was classified as a Pol IV/Pol V co-dependent site. Conversely, if a Pol V-dependent transcript cluster was also bound by NRPE1 in the nrpd1 mutant while also having a RPKM of GRO–seq in nrpd1 of <1, then this site was classified as a Pol IV-independent Pol V site.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Code availability.** The customized code used in this manuscript can be distributed upon request. Requests should be addressed to S.E.J.

**Data availability.** High-throughput sequencing data that support the findings in this study can be accessed through the Gene Expression Omnibus (GEO) database with accession numbers GSE108078 and GSE100010.

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Author contributions

W.L., J.H., S.H.D. and S.F. performed the GRO−seq experiments. M.G. performed the ChIP-seq experiments. W.L., J.G.-R., Z.Z. and S.F. performed the small RNA-seq experiments. W.L. and M.G. performed the bioinformatics analysis. W.L. and S.F. wrote the manuscript. J.Z., H.Y.K., Z.W. and J.C. assisted in writing the manuscript and discussion.

Competing interests

The authors declare no competing financial interests.

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Experimental design

1. Sample size
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2. Data exclusions
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   Describe whether the experimental findings were reliably reproduced.
   GROseq, ChIPseq and small RNAseq in plants in this paper, pooled floral tissues were used. For Col-0 GROseq, n=6 on independent pooled floral tissues as well as independent run-on experiments were performed. GROseq on other mutants were performed n=2. except for spt5l GROseq were performed n=3. ChIPseq were performed n=1. Small RNAseq were performed n=2.

4. Randomization
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   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
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   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

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See the web collection on statistics for biologists for further resources and guidance.
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7. Software

Describe the software used to analyze the data in this study.

Cutadapt (v 1.9.1) was used to trim adaptors.
Bowtie (v1.1.0) was used to align GRO-seq, ChIP-seq, samll RNA-seq data.
MACS2 (v 2.1.1.) was used for ChIP-seq peak calling.
NGSplot (v 2.41.4) was used for visualizing ChiP-seq, GRO-seq data.
R package DESeq2 was used for calling Pol V-dependent transcripts regions.

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Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Anti-NRPE1 antibody generated by Covance that recognizes the peptide NNCDDKKNSETESDAAAWG-C as described before in Ream, T.S., et al, Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II. Molecular Cell, 33, 192-203 (2009)

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