Materials and Methods

Establishment of NRPE1 and SPT5L Gene constructs, plant material and growth conditions.

The NRPE1(Pol V^{FL}) construct was previously described in El-Shami et al. 2007. NRPE1-WG (PolV+) and NRPE1-AG (POLV-) were obtained by swapping the WG rich region of the CTD with synthetic multimers of wild-type WG or mutant AG motifs from the consensus (El-Shami et al. 2007) in a 2xFlag-containing pCambia 1300 vector. SPT+ and SPT- constructs are based on a genomic fragment including the *SPT5L* promoter and ORF with or without the AGO-hook platform. A full-length *SPT5L* DNA fragment (SPT+) was amplified using primers 721 and 722 cloned into the pGEM-T easy vector (Promega) and then excised with *SalI/SmaI* to be cloned into a binary vector CTL235 (a Flag HA derived pCambia 1300 vector). To generate the SPT- truncated version, the 3' end of the SPT+ construct was swapped by *NheI* and *SmaI* digest and insertion of the 643-1011 PCR fragment, previously digested with the same enzymes. Plants were transformed by the floral dipping method using *Agrobacterium tumefaciens* GV3121 transformed strains (Clough and Bent 1998). All primers used are referenced in Supplemental_Table_S1.

For plant growth, seeds were stratified at 4°C for 2 days before growth on soil at 23°C with 16-h light and 60-70 % relative humidity. *nrpe1-11*, *nrpe1-3*, and *spt5l-1* were respectively described in (Bies-Ethève et al 2009; El-Shami et al 2007; Lahmy et al 2009). The two mutant backgrounds (*nrpe1-11* and *spt5l-1*) were used to generate transgenic plants. The myc-Ago4 line (mAGO4) and *ago4-1* were previously reported (Li et al 2006; Zilberman et al 2003). E1/mAGO4 and e1/mAGO4 lines were obtained by crossing an mAgo4 line that had been backcrossed in a Col0 genetic background with the *nrpe1-11* mutant line. We then retrieved both offsprings, expressing similar levels of cmycAGO4 in either a WT or *nrpe1* mutant backgrounds.

Protein extraction, immunodetection and immunoprecipitation

Total plant protein extracts (up to 100mg) were ground in liquid nitrogen with 200□□l SDS-PAGE loading buffer. Coomassie staining was used to calibrate loadings. Proteins were separated on SDS/PAGE gels and blotted onto PVDF membrane (Immobilon-P, Millipore). Protein blot analysis was performed using the Immobilon Western chemiluminescent HRP substrate (Millipore). The antibodies used in this study were HRP-coupled HA-specific antibody (H6533, Sigma), HRP-coupled FLAG-specific antibody (A8592, Sigma), HRP-coupled myc-specific antibody (A5598, Sigma) at 1/10000 dilution. Antibodies against SPT5L, AGO4, NRPE1, NRPE5 and DRD1 were engineered by Eurogentec and used at specific dilutions (S1 Text). Mouse or rabbit secondary antibodies (Cat #1706516 and 1706515, Biorad) were used at 1/10000 dilution.

For protein immunoprecipitation, 200mg of inflorescences were homogenized in BC100 (50 mM Tris-HCl at pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.1% v/v NP40, 10% glycerol, 10 nM MG132, Complete EDTA-free protease inhibitor cocktail from Roche. Cell debris were removed by centrifugation at 18,000*g* for 30 min at 4°C. The clarified lysate (750□□l) was incubated for 2 h at 4°C at 5 rpm, with anti-Flag M2 agarose beads (A2220, Sigma). After centrifugation at 1,800 g x for 1 min, the resin was washed 3 times with 1ml extraction buffer and resuspended in 50□l of SDS-PAGE loading buffer. Aliquots of these fractions and from inputs were subjected to protein analysis by Western blotting.

DNA methylation analysis and Whole-Genome Bisulfite Sequencing

Genomic DNA was extracted from 100mg of flowers with DNeasy Plant mini Kit (Qiagen). For Chop-PCR experiments, 20ng of digested or undigested DNA were amplified with primers indicated in Supplemental_Table_S1. For bisulfite sequencing analysis at specific

loci, the bisulfite conversion was performed on 500ng of DNA using the Epitect bisulfite kit (Qiagen) according the manufacturer's instructions with slight modifications. Samples were submitted to bisulfite conversion (95° 2min, followed by 8 cycles of 75°C 2 hrs, 95°C 1min). DNA was eluted with 2x20□l of elution buffer. PCR reactions were performed with 1,5 µl of treated DNA with Hot start Takara polymerase (Takara) in 25µl reaction (94°C 5 min, followed by 40 cycles of 94°C (45s), 50°C to 53°C depending on the primers (45s) and 72°C (1min), with a final elongation of 10min at 72°C). PCR products purified with Geneclean kit (MP Biochemicals) were cloned into pGEM-T Easy vector (Promega). 16 to 24 clones per sample were sequenced and analyzed using the web application Cymate (Hetzl et al 2007).

Whole-genome bisulfite sequencing libraries were made using TruSeq DNA LT kit (Illumina) and Epitect Bisulfite Kit (Qiagen) as described previously (Du et al ; 2015). All libraries were sequenced by HiSeq 2000 system (Illumina) per manufacture instructions. Single-end 50-bp-long reads were obtained from the sequencer. Bioinformatic processing of the sequencing data was carried out as previously described in Zhong et al 2014. When calculating percent methylation at RdDM targets, RdDM targets were defined as NRPE1 binding sites previously identified in Zhong et al 2012. Briefly, sequencing reads were mapped to the TAIR10 assembly of the Arabidopsis genome using BSseeker pipeline (Chen et al 2010) and methylation ratios of cytosines were calculated as #C/(#C+#T).

Chromatin immunoprecipitation on formaldehyde crosslinked samples

The ChIP procedure was adapted from (Wierzbicki et al 2008). 2g of inflorescences were homogenized in 25mL of Honda buffer and filtered onto two layers of Miracloth (Millipore). 1% formaldehyde was added and the samples were rotated 15 min at 4°. The crosslink was stopped by adding glycine (0,125N final) and rotating for 10 min at 4°C. Following a 15 min centrifugation (2000g, 4°C), the nuclei were washed three times in Honda buffer, lysed in

1mL of Nuclei Lysis buffer and sonicated using a Bioruptor (Diagenode). 20 □g of chromatin was diluted and incubated with 5□l of monoclonal anti-Flag M2 antibody (F1804, Sigma), anti-AGO4, anti-NRPE5 or anti-NRPE1 antibodies (the peptides used to generate the antibodies are listed in Supplemental_Table_S1) on a rotator overnight, at 4°C. 50□l of washed Dynabeads ProteinA/G (LifeTechnologies) were then added and incubated for 2h at 4°C before the washes. After reversing the cross-link by a 10 min incubation at 95°C followed by a 1h treatment with ProteinaseK, the immunoprecipitated DNA was subjected to qPCR analysis using primers listed in Supplemental_Table_S1, using the Takyon no ROX SYBR Master Mix blue dTTP kit (Eurogentec) on a Light Cycler 480 II machine (Roche Diagnostics).

Chromatin immunoprecipitation on UV laser crosslinked samples (LChIP).

After UV laser crosslink of nuclei and lysis in Nuclear Lysis Buffer, immunoprecipitation was performed as for conventional ChIP with slight modifications. Two additional washes with LiCl wash buffer (0,25M LiCl, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8) were performed before the two washes with TE. The immunoprecipitated DNA was recovered from 2 or 3 immunoprecipitations started with 20mg of chromatin either by addition of 100 µl of Chelex 100 followed by a 10 min incubation at 95°C, an extensive treatment with 2µl of Proteinase K at 55°C for 2h and a 10 min incubation at 95°C or by elution at 65°C in elution buffer (1% SDS, 0,1M NaHCO3) followed by a 2h treatment with Proteinase K, phenol-chloroform extraction and ethanol precipitation. The immunoprecipitated DNA was subjected to qPCR as described for conventional ChIP, using primers described in the Supplemental_Table_S1.

RNA Extraction and Analysis

RT-PCR analysis. Total RNAs isolated from inflorescences with the RNeasy Plant kit (Qiagen) were subjected to DNAse treatment (RQ1 Promega) and used to synthesize first-strand cDNAs using GoScript reverse transcriptase (Promega). 400 ng of treated RNAs and random primers were employed to synthetize cDNAs according to manufacturer's instructions. Equal amounts of cDNAs were controlled by actin transcript amplification.

qRT-PCR analysis. RNAs were isolated from inflorescences using 1ml of TRIzol reagent (Invitrogen) for 200mg tissues (Law et al. 2013). Absence of DNA contamination was determined with no reverse transcriptase added to the reaction. 1,2 to 1,5 □g of RNA were used for reverse transcription with gene specific primers and Superscript III (Invitrogen). Real-time qPCR analyses were performed using the Takyon no ROX SYBR Master Mix blue dTTP kit (Eurogentec) on a Light Cycler 480 II machine (Roche Diagnostics). Relative transcript accumulation was calculated using the ΔΔCt methodology, and *ACTIN2* as internal control (Livak and Schmittgen, 2001). Average ΔΔCt represents three experimental replicates with standard errors. Primers used are listed in Supplemental_Table_S1.

Northern blot. RNAs were isolated from inflorescences using 1ml of TRIzol reagent (Invitrogen) for 100mg tissues, according to manufacturer's instructions. A PEG precipitation step was added to enrich in siRNAs. 10-15 µg of total RNAs or 5 µg of siRNAs were run on 15% polyacrylamide-7M urea gels, transferred onto Hybond-NX membranes (Amersham Biosciences) and cross-linked with EDC (Sigma). Membranes were blocked using 10ml of PerfectHyb™ Plus Hybridization Buffer (Sigma) and probed either with 5'end radiolabeled oligonucleotides or with α–^{32}P dCTP probes (Prime-a-Gene. Labeling System, Promega). 10 pmoles of each oligonucleotide probe was end-labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega). Other probes are labelled with 25ng as recommended by manufacturer's instructions. The probes used are reported in Supplemental_Table_S1.

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