RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family

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Recent studies have identified a conserved WG/GW-containing motif, known as the Argonaute (AGO) hook, which is involved in the recruitment of AGOs to distinct components of the eukaryotic RNA silencing pathways. By using this motif as a model to detect new components in plant RNA silencing pathways, we identified SPT5-like, a plant-specific AGO4-interacting member of the nuclear SPT5 (Suppressor of Ty insertion 5) RNA polymerase (RNAP) elongation factor family that is characterized by the presence of a carboxy-terminal extension with more than 40 WG/GW motifs. Knockout *SPT5-like* mutants show a decrease in the accumulation of several 24-nt RNAs and hypomethylation at different loci revealing an implication in RNA-directed DNA methylation (RdDM). Here, we propose that SPT5-like emerged in plants as a facultative RNAP elongation factor. Its plant-specific origin and role in RdDM might reflect functional interactions with plant-specific RNA Pols required for RdDM.

Keywords: *Arabidopsis*; AGO4; SPT5; RdDM; silencing

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INTRODUCTION

The discovery of small non-coding RNAs with specific regulatory roles has changed our views on the regulation of eukaryotic gene expression. Small RNAs (sRNAs), 20–25 nt in length, are essential effectors of RNA silencing, a process with crucial roles in many cellular processes. Although their mode of action might vary, plant and animal sRNAs usually act as sequence-specific guides to target endogenous messenger RNAs (mRNAs) for cleavage or translational repression, a process known as post-transcriptional gene silencing (PTGS; Jones-Rhoades *et al*, 2006). They can also

guide DNA and histone methylation, allowing heterochromatin assembly at homologous DNA loci, a process referred to as transcriptional gene silencing (TGS) in plants and fission yeast (Zaratiegui *et al*, 2007).

To achieve their regulatory functions, sRNAs recruit Argonaute (AGO)-containing RNA-induced silencing complexes (RISCs) that carry out effector functions, leading to gene silencing at the transcriptional or post-transcriptional level. MicroRNAs/sRNAs associate with AGOs to form RISCs that direct PTGS (Peters & Meister, 2007), heterochromatic sRNAs interact with AGOs to form RNA-induced initiation of transcriptional silencing complexes (RITSs) in *Schizosaccharomyces pombe* (Verdel *et al*, 2004), whereas a hypothetical RITS-like complex probably guides RNA-directed DNA methylation (RdDM) in plants (Qi *et al*, 2006), leading to TGS. RISC- and RITS-associated AGO components have ‘slicing’ activity thought to initiate silencing through cleavage of mRNA/nascent transcripts bearing sRNA-complementary sites (Qi *et al*, 2006; Peters & Meister, 2007).

Several studies have recently provided new insights into the function and composition of RISC/RITS by identifying proteins that are likely to act with, or downstream from, AGOs in RNA-mediated interference (RNAi)-based silencing pathways. These are human GW182 (*Homo sapiens* glycine/tryptophan-rich protein.182; HsGW182) for the miRNA pathway (Behm-Ansmant *et al*, 2006), *S. pombe* TAS3 (SpTAS3) for the yeast TGS pathway (Partridge *et al*, 2007) and *Arabidopsis thaliana* nuclear RNA polymerase V largest subunit (AtNRPE1), the largest subunit of RNA polymerase V (PolV), for the plant RdDM pathway (Li *et al*, 2006). It has recently been shown that the HsGW182, SpTAS3 and AtNRPE1 proteins indeed show conserved AGO-binding capacity through a new WG/GW-containing peptide of similar amino-acid composition, also known as the ‘AGO hook’ (El-Shami *et al*, 2007; Till *et al*, 2007). We have shown that the homologous WG/GW-rich regions of AtNRPE1 and HsGW182 are functionally interchangeable, interacting with AGOs in a tryptophan-dependent manner (El-Shami *et al*, 2007). These observations suggest that the AGO hook is an evolutionarily and functionally conserved

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Previously, we used an empirical approach of classical sequence comparison to identify proteins containing AGO hook motifs in various organisms, including *Arabidopsis* (El-Shami *et al.*, 2007).

Fig 2 | DNA methylation defects in *spt5-like* knockout mutants. (A) Western blot and Coomassie blue staining on wild-type (WT) and mutant flower protein extracts (~20 µg) using an SPT5-like specific antibody. (B) DNA methylation at the *AtSN1* locus in *spt5-like* and control genotypes Col-0 and *nrpe1-11* (*polV*). Left panel, *HaeIII*-digested DNA was used as a template for PCR reactions using *AtSN1* primers (412 and 413) and control primers. Cut 1 primers (400 and 402) should not allow DNA amplification if the digestion is complete. Control primers (410 and 411) allow equilibration of DNA quantities used as templates. Right panel, DNA methylation was analysed by bisulphite sequencing. Histograms represent the percentage of CG, CNG and CNN methylation in the indicated genotypes. (C) DNA methylation at the 5S ribosomal RNA locus in *spt5-like* and Col-0. Genomic DNA was digested with *HpaII* or *HaeIII* and blots were hybridized with a 5S ribosomal RNA repeat probe. (D) DNA methylation at the *solo LTR* locus and IG/LINE expression in *spt5-like* and control genotypes Col-0, *nrpd1-4* (*polIV*) *nrpe1-11*. Left panel, genomic DNA was digested by various methylation-sensitive enzymes before being used for PCR amplification using *solo LTR*-specific primers (769 and 770). Input indicates amplification from equal quantities of undigested DNA. Right panel, RT-PCR performed on Col-0, *nrpe1-11* and *spt5-like-1* messenger RNA. gDNA correspond to amplification from genomic Col-0 DNA. +/– RT indicates whether or not transcriptase treatment was carried out. GAPAs (glyceraldehyde 3-dehydrogenase A) have been used to check equilibration. All primers are described in supplementary Table 1 online. Ab, antibody; *AtSN1*, *Arabidopsis thaliana* short interspersed element 1; IG/LINE, intergenic/long interspersed element; LTR, long terminal repeat; RT-PCR, reverse transcription-PCR; SPT5, Suppressor of Ty insertion 5; Und., undigested DNA.

One of these, encoded by gene *At5g04290*, shows considerable similarity to SPT5 proteins, which are conserved throughout eukaryotes and form an SPT4–SPT5 complex that is an essential nuclear RNAPII elongation factor (Hartzog et al, 1998). There are in fact three genes in the *Arabidopsis* genome encoding SPT5 family proteins, two of which are highly similar (*At4g08350* and *At2g34210*), whereas the third, encoded by gene *At5g04290*, has an additional long C-terminal extension, containing 44 WG/GW motifs, encoded by two large exons (Fig 1A and B; supplementary Fig S3A online). Reverse transcription-PCR analysis indicated that all three *SPT5-type* genes are ubiquitously expressed in *Arabidopsis* organs (supplementary Fig S1A online and data not shown).

Fig 1A shows the domain organization of human and *Arabidopsis* SPT5s and the new SPT5-like protein. All have a conserved amino-terminal acidic domain, and central SPT4 and RNAPII-binding domains (Guo et al, 2008; Yamaguchi et al, 1999). There is no significant similarity in the C-terminal region, which is composed exclusively of WG/GW motifs in the SPT5-like protein. In contrast to the considerable divergence of AGO hooks in NRPE1 (El-Shami et al, 2007), WG/GW motifs in SPT5-like are

highly similar, containing two slightly different conserved motifs (supplementary Fig S1B online). We identified putative homologues of SPT5 and SPT5-like in rice by sequence comparison, correcting the predicted structure of the latter after identification of additional 3' exons (supplementary Fig S2 online). Phylogenetic analysis of representative SPT5 sequences from plants and other eukaryotes groups the classical SPT5 proteins from animals and plants, with distinct branches that correspond to the yeast- and the plant-specific SPT5-like proteins (supplementary Fig S1C online).

To investigate the importance of the SPT5-like WG/GW-rich domain in AGO binding, we generated fusion proteins containing either six wild-type (glutathione *S*-transferase–SPT51–WG6; GST–SPT51–WG6) or mutant (GST–SPT51–FG6) versions of the SPT5-like repeats fused with GST and monitored the ability of these constructs to interact either with AGO1 or with AGO4 compared with the GST control protein. Pull-down assays indicated that GST–SPT51–WG6, but not GST–SPT51–FG6, interacts specifically with AGO4 with high affinity (Fig 1C), showing that SPT5-like is indeed an AGO hook-containing protein. Interestingly, under the same experimental conditions, AGO1 failed to bind to the

GST-SPT5I-WG6 fusion protein, indicating that the SPT5-like WG-containing platform shows distinct binding specificity for AGO proteins. As a single AGO hook motif is sufficient for binding AGO (El-Shami *et al*, 2007) and as SPT5-like contains the largest WG/GW platform in *Arabidopsis*, we infer that SPT5-like should have considerable AGO4-binding capacity.

To assess further the interaction between SPT5-like and AGO4 *in vivo*, whole-cell extracts generated from the previously reported Myc-AGO4 line (Li *et al*, 2006) were immunoprecipitated with Myc antibodies and analysed using a specific SPT5-like antibody (Ab72; see Fig 2A; supplementary Fig S3 online). Fig 1D shows that SPT5-like coimmunoprecipitates with Myc-AGO4, therefore supporting the interaction between these proteins. Taken together, our results indicate that SPT5-like is an authentic AGO hook-containing protein that interacts with AGO4 in *Arabidopsis*.

spt5-like mutants show specific DNA methylation defects

To investigate the functional role of SPT5-like, two independent homozygous mutant lines, *spt5l-1* and *spt5l-2*, were characterized. None of the mutant alleles showed a readily discernible phenotype. The T-DNA insertion position for each mutant is indicated in supplementary Fig S3A online. No full-length *SPT5-LIKE* transcript was detected by reverse transcription-PCR in either of the mutant lines (supplementary Fig S3B online). Furthermore, no SPT5-like protein was detected using either Ab71 or Ab72 SPT5-like antibodies, indicating that *spt5l-1* and *spt5l-2* are probably null mutants (Fig 2A; supplementary Fig S3A,C online). Both SPT5-like antibodies revealed a prominent 150–180 kDa protein doublet with the apparent mass of the largest protein being slightly greater than the predicted molecular size of SPT5-like (~150 kDa; Fig 2A; supplementary Fig S3C online). Whatever the explanation for the apparent increase in the molecular weight of the detected protein, the fact that these two bands are absent in the knockout insertion lines shows their specificity and confirms the accumulation of SPT5-like protein in wild-type extracts.

As AGO4 is known to be involved in RdDM and SPT5-like is a nuclear protein (data not shown), we tested the impact of *spt5-like* mutation on DNA methylation at repeated endogenous loci. The retrotransposon *Arabidopsis thaliana* short interspersed element 1 (*AtSN1*) locus is protected against cleavage by the methylation-sensitive enzyme *HaeIII* in wild-type plants. A similar reduction in protection, indicating decreased methylation, is seen in both *spt5-like* mutants compared with the wild type (Fig 2B, left panel). The reduction is, however, not as strong as that seen in the *nrpe1-11* mutant (Fig 2B, left panel). Decreased methylation at the *AtSN1* locus was confirmed by bisulphite sequencing, which indicated a reduction by about 43% of CNG (C, cytosine; N, adenine, thymine or cytosine; G, guanine) and 32% of both CG and CNN methylation in the *spt5l-1* mutant compared with the wild type (Fig 2B, right panel; supplementary Fig S4 online). DNA methylation defects in both *spt5-like* mutants were also observed for the 5S rDNA cluster for which digestions with *HpaII* and *HaeIII* allow the monitoring of both CG and CNN methylation, respectively (Fig 2C). Interestingly, DNA methylation assays of the solo *LTR* (long terminal repeat) locus, a well-known target of the RdDM pathway (Huettel *et al*, 2006), indicate that the two *spt5-like* mutants, in contrast to the *nrpd1-4* and *nrpe1-11* mutants, do not lose DNA methylation (Fig 2D, left panel).

Fig 3 | Short interfering RNA accumulation defect in *spt5-like* mutants. Small RNA blot assays for U6 and various endogenous siRNAs in *spt5-like*, *nrpd1-4* and *nrpe1-11* mutants. Blots were stripped and reprobed multiple times with probes indicated on the right. siRNA accumulation dependency on polIV or polV is indicated on the left. U6 was used as an equilibration control. *AtSN1*, *Arabidopsis thaliana* short interspersed element 1; *nrpd1*, nuclear RNA polymerase IV largest subunit; *nrpe1*, nuclear RNA polymerase V largest subunit; siRNA, short interfering RNA; SPT5, Suppressor of Ty insertion 5.

As expected, the adjacent *IG/LINE* (intergenic/long interspersed element) transcript is activated in the *nrpe1-11* mutant, owing to hypomethylation of the solo *LTR* locus, but not in the *spt5l-1* mutant (Fig 2D, right panel). Taken together, our results suggest a target-specific requirement for the AGO-interacting protein SPT5-like in RdDM.

Altered levels of 24-nt sRNA in spt5-like mutants

Cytosine methylation is guided to RdDM targets by 24-nt sRNAs produced by components of the RNAi machinery (Xie *et al*, 2004), and the plant-specific PolIV and PolV (Herr *et al*, 2005; Kanno *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005; Zhang *et al*, 2007). According to these observations and to extend our analysis, we assessed the impact of *spt5-like* null mutations on the accumulation of a set of 24-nt sRNAs that are produced either from PolIV-dependent (*TR2558* and *siRNA02*) or PolIV/PolV-dependent (*AtSN1*, *SimpleHAT2*, *45S* rDNA and *5S* rDNA) loci (Pontier *et al*, 2005; Mosher *et al*, 2008). As expected, all 24-nt sRNAs were absent or greatly reduced in samples from the *nrpd1-4* mutant lacking PolIV, whereas only a subset of the sRNAs, corresponding to PolIV/PolV-dependent loci, was also affected in the *nrpe1-11* mutant (Fig 3). Our analysis showed that although the *siRNA02* and *TR2558* 24-nt sRNAs remain unaffected in both *spt5-like* mutants, the level of 24-nt sRNAs corresponding to *AtSN1* was considerably reduced in these

Fig 4 | Hypothetical model for the action of SPT5-like in RNA-directed DNA methylation. Two possible mechanisms for the mode of action of SPT5-like in RdDM are presented. Both involve the recruitment of AGO4 to PolV and SPT5-like (SPT5L) but differ in the subsequent role of the latter. (A) Recycling of AGO4 through SPT5-like. (B) Recruitment of SPT5L through the AGO hook domain. AGO, Argonaute; DCL3, dicer-like 3; DRM2, domain rearranged methylase 2; dsRNA, double-stranded RNA; me_C, methylated cytosine; RdDM, RNA-directed DNA methylation; RDR2, RNA dependent RNA polymerase 2; siRNA, short interfering RNA; SPT5, Suppressor of Ty insertion 5; Spt5L, SPT5 like.

mutants (Fig 3), an observation that is consistent with the methylation defect observed previously at this locus. Similarly, the accumulation of *SimpleHAT2*, *45S* and *5S* 24-nt sRNAs was also decreased in the *spt5-like* mutants to a level similar to that detected in *nrpe1-11* (Fig 3). Taken together, these results indicate that the methylation defects noted in *spt5-like* mutants are linked to a defect in the production/accumulation of 24-nt sRNA and that the PolIV/PolV-dependent loci are preferentially sensitive to the *spt5-like* mutations.

Previous work has shown that the plant-specific PolV was probably derived by the modification of a duplicated PolIV gene (Luo & Hall, 2007). The WG motifs of NRPDE1 are encoded by two large exons inserted between the Pol and the DCL (defective chloroplast and leaves) domains of the ancestral gene (Pontier et al, 2005). It is intriguing to note that the AGO platform in *Arabidopsis* SPT5-like is also carried by two large exons at the 3' end of the gene. This is therefore the second small gene family in which two forms exist: one resembling other non-plant proteins and the other that contains an AGO-binding platform. All members of the SPT5 family have been implicated in the control of RNAP processivity through direct binding to the core RNAP enzyme (Sims et al, 2004). As the conserved domain structure of SPT5-like suggests an interaction with RNAP, and as it is involved in RdDM, we propose that SPT5-like is a variant elongation factor needed for the activity of PolV (Fig 4). This idea is supported by the 24-nt sRNA analysis, which revealed that most of the loci that specifically require PolV for the accumulation of sRNA are affected in *spt5-like* mutants.

Our data also indicate that the AGO hook platform of SPT5-like shows specificity towards AGO proteins having a strong preference for AGO4 in our binding assays. As NRPE1 and SPT5-like contain the largest WG/GW platforms in *Arabidopsis*, we propose that a massive recruitment of AGO4 is likely to be essential in the RdDM pathway. In this context, it is possible that SPT5-like has evolved as a facultative elongation factor with a dual role in RdDM, modulating the processivity of PolV and ensuring the availability of

AGO4 during the course of PolV transcription (Fig 4A). This claim is in agreement with the prevailing model, which suggests that base pairing between AGO4-bound siRNA and PolV transcripts is required for RdDM (Wierzbicki et al, 2008; Lahmy et al, 2009). This suggests that the complexity of the PolV-transcribed sequences will be significantly greater than that of the siRNA population that can be loaded simultaneously on the PolV CTD (carboxy-terminal domain; El-Shami et al, 2007; Mosher et al, 2008), requiring a dynamic supply of AGO4. Alternatively, SPT5-like could be acting merely as an SPT5-type transcription elongation factor, of which the post-initiation recruitment on the PolV/AGO4 complex takes place through its WG/GW domain (Fig 4B). Further experiments will be necessary to decipher the exact role of SPT5-like and its Pol partner in RdDM.

METHODS

Plant growth and genetic analysis. For plant growth, seeds were stratified at 4 °C for 2 days before growth at 23 °C with 16-h light on the soil. The *spt5L-1* mutant corresponds to line SALK_001254C (Alonso et al, 2003). The *spt5L-2* mutant corresponds to line SAIL_SAIL_362_G07. *nrpd1-4* and *nrpe1-11* were described by Herr et al (2005) and El-Shami et al (2007).

DNA isolation and analysis. Genomic DNA was extracted from leaves using a Wizard Genomic kit (Promega, Charbonnières-Bains, France) before being used for PCR or Southern blot hybridizations. PCR was performed using 150 ng of digested or undigested genomic DNA. Primers are described in supplementary Table 1 online. All PCRs were performed with an annealing temperature of 55 °C and 30 amplifying cycles, except in some cases described in supplementary Table 1 online. For Southern blots, 5 µg of DNA was used and hybridized with a 5S probe (Pontier et al, 2005).

RNA isolation and analysis. Total RNA was isolated using the Trizol reagent (Invitrogen, Cergy-Pontoise, France). After DNase treatment, cDNA was synthesized with an Affinity Multitemperature cDNA synthesis kit (Stratagene, Amsterdam, The Netherlands) using

an oligodT primer with 1 µg of RNA according to the manufacturer's instructions. For sRNA analysis, total RNA extraction and northern blots were performed as described previously (Pontier et al, 2005), except that NX-Hybond membranes (Amersham Biosciences, Orsay, France) and EDC (Sigma, Saint-Quentin Fallavier, France)-mediated crosslinking were used. The probes used are described in supplementary Table 1 online.

Immunoblot. Total flower protein extracts were obtained following the method described by Pontier et al (2005). Protein samples from wild-type and transgenic lines were quantified and subjected to immunoblot analysis using a 1/1000 dilution of Spt5-like antibodies raised against peptides described in supplementary Table 1 online (Eurogentec, Angers, France).

Sodium bisulphite sequencing. Sodium bisulphite sequencing was performed as described previously (Fraga et al, 2008). Primer sequences are described in the supplementary information online. In total, 17 and 19 clones were sequenced for Col-0 and *spt5l-1*, respectively.

GST-CTD-binding experiments. The multimers were constructed following the strategy described by El-Shami et al (2007). Primers corresponding to each monomer (877/878 for SPT5L-WG1 and 919/920 for SPT5L-FG1) were annealed and subcloned into a *BglII*–*Bam*HI-digested pBluescript vector. After three rounds of cloning, the multimers were introduced into a *Bam*HI–*Hind*III-digested pET41a(+) vector and expressed in BL21. All the GST fusion proteins were purified on glutathione Sepharose 4B beads (Amersham Biosciences) and used in pull-down assays as described previously (El-Shami et al, 2007).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

Note added in proof. In a recent study, Huang et al (2009) identified peptides of SPT5-like in a PolV-purified fraction. This finding is in agreement with the model of action reported here, that SPT5-like acts in RdDM as a PolV-associated SPT5-type elongation factor in plants.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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