SPT5-like, a new component in plant RdDM

Plant DNA is extensively methylated at cytosine residues; however, unlike in animals, the methylation occurs not only at CG sites but also at CNG and CHH—in which H represents A, C or T—sites. A significant proportion (~30%) of this cytosine methylation in *Arabidopsis* is dependent on the RNA-silencing pathway known as RNA-directed DNA methylation (RdDM; Matzke *et al*, 2009). RdDM has a crucial role in silencing retrotransposons and endogenous repeats, thereby maintaining genome stability. It probably also has a role in regulating the transcriptional activities of plant genes that contain, or are adjacent to, transposons or repetitive elements, many of which might have a role in stress responses. In addition, RdDM is required for the maintenance and systemic transmission of post-transcriptional gene silencing in plants (Brosnan *et al*, 2007; Eamens *et al*, 2008), presumably by causing modification of the transgene DNA that is necessary for producing secondary small-interfering RNAs (siRNAs; Daxinger *et al*, 2009). The existence of RdDM was initially demonstrated using exogenous viral RNAs (Wassenegger *et al*, 1994; Jones *et al*, 1999), and was subsequently shown to be mediated by 24-nucleotide (nt) siRNAs, which form the predominant population of endogenous small RNAs (Kasschau *et al*, 2007; Mosher *et al*, 2008). siRNAs can also be generated by viruses and transgenes in plants to direct RdDM (Mette *et al*, 2000; Wang *et al*, 2001). These siRNAs, which are known as repeat-associated or heterochromatic siRNAs, direct RdDM by providing the sequence specificity necessary for multi-protein complexes to bind to and methylate target DNA sequences. RdDM requires several protein factors for both the upstream biogenesis of the 24-nt siRNAs and the downstream *de novo* cytosine methylation (Matzke *et al*, 2009). Among these proteins are two plant-specific DNA-dependent RNA polymerases, Pol IV and Pol V, which act at the different steps of RdDM (Fig 1). In this issue of *EMBO reports*, Thierry Lagrange and colleagues identify the elongation factor Suppressor of Ty insertion 5-like (SPT5-like) as a new component of the Pol V complex and an important effector of the RdDM pathway in plants (Bies-Etheve *et al*, 2009).

The essential eukaryotic DNA-dependent Pols I, II and III are required for the production of large ribosomal RNAs, messenger RNAs and some small-regulatory RNAs (such as 5S RNAs, transfer RNAs and several small-nuclear RNAs), respectively. In addition, the *Arabidopsis* genome contains two other polymerases: Pol IV (initially known as Pol IVa) and Pol V (initially known as Pol IVb). These two polymerases are not essential for plant viability, as their loss-of-function mutants show normal growth in controlled environments, and instead function specifically in the RdDM pathway. Pol IV produces transcripts that are copied by RNA-dependent RNA polymerase II (RDR2) into double-stranded RNA (dsRNA), which

is processed by DICER-LIKE 3 (DCL3) to produce 24-nt heterochromatic siRNAs (Fig 1). The template for Pol IV-mediated transcription remains unknown, although both methylated DNA and dsRNA have been proposed (Daxinger *et al*, 2009). Pol V is not essential for siRNA biogenesis, but can enhance siRNA accumulation from some genomic loci in *Arabidopsis* (Mosher *et al*, 2008). In the preferred model for RdDM (Fig 1), Pol V synthesizes nascent transcripts from genomic loci that have been modified by the SNF-type chromatin-remodelling protein, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1). siRNAs associated with ARGONAUTE 4 (AGO4) bind to Pol V nascent transcripts to bring the silencing machinery to the vicinity of the chromatin at target loci, thereby facilitating *de novo* DNA methylation and chromatin silencing (Wierzbicki *et al*, 2008). This Pol V-dependent silencing could, in some cases, stimulate the Pol IV-dependent siRNA production by providing a template for Pol IV transcription. Therefore, Pol IV functions upstream in RdDM to produce and amplify the small-RNA trigger for silencing, whereas Pol V acts downstream to transcribe non-coding RNAs that provide scaffolds for attracting silencing complexes and could also be involved in the reinforcement of silencing through a positive-feedback loop. Several independent studies have shown that Pol IV and Pol V share many of their small subunits with Pol II, and the remaining subunits are functionally diversified variants of Pol II counterparts (He *et al*, 2009; Huang *et al*, 2009; Ream *et al*, 2009). The differential functions of these two polymerases are likely to be determined by the extended carboxy-terminal domain of the Pol V largest subunit, NRPE1; this contains neighbouring tryptophan-glycine/glycine-tryptophan residues (WG/GW repeats) that have been shown to interact specifically with AGO4 (El-Shami *et al*, 2007), which is a downstream effector protein in RdDM that also binds to siRNAs. Indeed, as described by Bies-Etheve *et al* (2009), the WG/GW motif was successfully used as a model to identify a new factor that functions in conjunction with Pol V in RdDM.

In addition to NRPE1, the WG/GW motif was also found in the human protein GW182 (Behm-Ansmant *et al*, 2006) and in the *Schizosaccharomyces pombe* TAS3 protein (Partridge *et al*, 2007), both of which probably act with AGOs in RNA-silencing pathways. Therefore, this motif is known as the AGO-hook. Having previously demonstrated that the WG/GW-rich regions of human GW182 and *Arabidopsis* NRPE1 interact with AGOs (El-Shami *et al*, 2007), which suggests a general role of WG/GW-containing proteins in RNA silencing, Bies-Etheve and colleagues designed an ingenious approach to identify novel factors in the gene-silencing pathway. They searched the *Arabidopsis* genome for genes that encode proteins with a WG/GW motif, and identified the *At5g04290* (*SPT5-like*) gene that acts as a new component of the Pol IV/Pol V RNA-silencing machinery.

SPT5-like is homologous to the SPT5 proteins present in all eukaryotes, which form an SPT4–SPT5 complex that is an essential RNA Pol II elongation factor. Three genes in the *Arabidopsis* genome have homology to *SPT5*; however, *SPT5-like* is unique as it encodes a protein with an additional long carboxy-terminal

extension that contains 44 WG/GW motifs. Pull-down assays performed with glutathione-S-transferase (GST) fusions of the SPT5-like protein show that SPT5-like interacts with AGO4 with high affinity. It also co-immunoprecipitates with AGO4 in cell extracts, suggesting an *in vivo* interaction. The pull-down assays failed to detect an interaction between SPT5-like and AGO1, which is an effector protein in small RNA-directed post-transcriptional silencing pathways, suggesting that SPT5-like is an AGO4-interacting member of the nuclear Pol elongation factor family and functions specifically in the RdDM pathway. Indeed, loss-of-function mutants of SPT5-like show a defect in DNA methylation in two known RdDM target loci, although it is not as strong as in the

◀ **Fig 1** | Model of RNA-directed DNA methylation in plants. RNA polymerase (Pol) IV transcribes either methylated DNA or dsRNA to produce single-stranded RNA, which is copied to dsRNA by RDR2. The dsRNA is processed by DCL3, possibly assisted by a DRB, into 24-nt siRNAs that are methylated at the 2'-O-hydroxyl by the small-RNA methylase HEN1 and subsequently loaded to AGO4. Chromatin remodelling by DRD1 is required to initiate transcription by Pol V, which also involves the function of the SPT5-like elongation factor (SPT5I) and possibly AGO4, which interacts physically with Pol V and SPT5I through their carboxy-terminal WG/GW motifs. siRNAs bound to AGO4 interact with nascent Pol V transcripts by base pairing, thereby recruiting chromatin-modifying enzymes, including the *de novo* cytosine methyltransferase DRM2, to adjacent DNA. The modified chromatin could act as a Pol IV template for further siRNA production, generating a positive-feedback loop to reinforce the silencing. Modified from Eamens *et al*, 2008, Wierzbicki *et al*, 2008, Matzke *et al*, 2009 and Bies-Etheve *et al*, 2009. AGO4, ARGONAUTE 4; DCL3, DICER-LIKE 3; DRB, DOUBLE-STRANDED RNA-BINDING PROTEIN; DRD1, DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1; DRM2, DOMAIN REARRANGED METHYLASE 2; dsRNA, double-stranded RNA; HEN1, HUA ENHANCER 1; nt, nucleotide; Pol IV, RNA polymerase IV; RdDM, RNA-directed DNA methylation; RDR2, RNA-dependent RNA polymerase II; siRNA, small-interfering RNA; SPT5I, suppressor of Ty insertion 5-like; WG/GW, neighbouring tryptophan-glycine/glycine-tryptophan residues.

nrpe1 mutant. The levels of methylation in the retrotransposon AtSN1 and in the 5S ribosomal DNA (5S rDNA) cluster are both significantly reduced. Interestingly, methylation of the solo long terminal repeat (LTR) locus was not affected by mutation in SPT5-like, suggesting that there is specificity in the RdDM targets of the SPT5-like-AGO4 pathway.

The existence of the WG/GW motifs in Pol V, but not in Pol IV, implies that SPT5-like is more likely to function as an elongation factor for Pol V transcription than for Pol IV transcription. This was confirmed by the effect of the *SPT5-like* mutation on 24-nt siRNA accumulation. The *spt5-like* mutation had no effect on the production of 24-nt small RNAs from the two Pol IV-dependent loci analysed (TR2558 and siRNA02). By contrast, the accumulation of 24-nt siRNAs in all four Pol IV/Pol V-dependent loci (AtSN1, SimpleHAT2, 45S and 5S rDNA) was decreased in the *spt5-like* mutants to a level similar to that detected in an *nrpe1* mutant. On the basis of these observations, the authors suggest that SPT5-like is a variant elongation factor required for Pol V activity. They propose that SPT5-like might have evolved as a facultative elongation factor with a dual role in RdDM modulating the processivity of Pol V and ensuring the availability of AGO4 during the course of Pol V transcription.

The same *SPT5-like* gene was recently isolated from cauliflower (*Brassica oleracea*) by Huang *et al* (2009) as part of the Pol V complex using immunoprecipitation, which was a different approach. Besides identifying homologues of the 12 Pol II subunits or their variants, these authors identified several non-polymerase proteins from NRPE1 immunoprecipitates, including one (At5g04290, which is SPT5-like) that has been annotated as a KOW domain transcription factor-family protein (KTF1). KTF1 has KOW motifs, which are present in many ribosomal proteins and transcription factors, and contains a reiterated WG/GW motif that is a potential AGO-binding platform. Consistent with the observations of Bies-Etheve *et al*, Huang *et al* demonstrated that NRPE1-dependent siRNAs and cytosine methylation at an

A. thaliana short interspersed element 1 (AtSN1) locus were reduced in a *ktf1* mutant, although not as strongly as in an *nrpe1* mutant. At the 5S rDNA locus and the two LTR loci analysed, the level of DNA methylation was reduced in *nrpe1* and *ktf1* mutants, although the level of siRNA production from these loci was either unaffected (for LTR in *nrpe1*) or reduced only slightly (for 5S rDNA). This uncoupling of siRNA production from DNA methylation in both *nrpe1* and *ktf1* mutants supports a direct functional interaction between the two, and is consistent with Pol V acting mainly as a downstream effector in RdDM. The SPT5-like protein was not isolated by Ream and colleagues, who successfully identified most of the Pol II subunits or their variants in the Pol IV and Pol V complexes using epitope-tagged NRPD1 and NRPE1 subunits (Ream *et al.*, 2009). The failure to isolate SPT5-like in this experiment could be due to a loose association of the protein with the Pol V complex, preventing its efficient pull-down by FLAG purification.

The absence of Pol IV and Pol V in animals suggests that these higher organisms use a different mechanism from that used by plants to mediate chromatin silencing. The restriction of DNA methylation to CG sites catalysed by a maintenance DNA methyltransferase, and the absence or low levels of 24-nt small RNAs, suggest a lack of RdDM-induced chromatin changes in animals. However, the finding that Pol V forms a Pol II-like complex that includes Pol II-like elongation factors raises the possibility that Pol II might function in RNA silencing-directed chromatin regulation in animals. Indeed, there is evidence that Pol II can function in RNA-directed chromatin modification in *S. pombe*, in which Pol II transcripts act as both precursors for siRNAs and scaffolds for interactions with siRNAs (Bühler *et al.*, 2006; Irvine *et al.*, 2006). In *Arabidopsis*, a large proportion of cytosine methylation is independent of the Pol IV-mediated and Pol V-mediated RdDM pathway (Matzke *et al.*, 2009). It is conceivable that DNA methylation and chromatin silencing at these loci could involve mechanisms similar to those of animals and depend on Pol II transcription. The structural similarity—and a potential functional overlap—of Pol IV and Pol V with Pol II make likely the identification of additional Pol II components or their variants in the plant chromatin-silencing machineries.

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