Argonautes team up to silence transposable elements in *Arabidopsis*

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The *de novo* silencing of transposable elements in plants and animals is mediated in part by RNA-directed chromatin modification. In flowering plants, AGO4 has been seen as the key argonaute protein in the RNA-directed DNA methylation pathway that links the plant-specific RNA polymerase V with the *de novo* DNA methyltransferase DRM2 (Zhong *et al*, 2014). Two recent papers in *The EMBO Journal* strongly implicate a role for the AGO6 protein in the process of *de novo* silencing.

See also: **C-G Duan *et al*** (March 2015) and **AD McCue *et al*** (January 2015)

The RNA-directed DNA methylation (RdDM) pathway is important for the silencing of active and evolutionarily young transposable elements in *Arabidopsis* (Zhong *et al*, 2012; Marí-Ordóñez *et al*, 2013). Several forward genetic screens for RdDM components have been carried out in *Arabidopsis* using various reporter gene silencing assays and have identified more than 30 genes that play a role in RdDM, including multiple alleles of two related argonaute genes, AGO4 and AGO6 (Zilberman *et al*, 2003; Zheng *et al*, 2007; Eun *et al*, 2011).

In this issue of *The EMBO Journal*, Duan *et al* (2015) address the requirements of AGO4 and AGO6 in the RdDM pathway by conducting whole-genome bisulfite sequencing in *ago4* and *ago6* single mutants, and the *ago4 ago6* double mutant. They find that 53% of the loci identified in the *ago4 ago6* double mutant are hypomethylated to a similar extent in both *ago4* and *ago6* single mutants. A further 42% of loci identified in

the double mutant were also found in *ago4* and *ago6* single mutants, but hypomethylation was more pronounced in the double mutant. From this, Duan *et al* conclude that *ago4* and *ago6* are mutually dependent and therefore function in parallel, rather than in a redundant manner (see Fig 1A).

A plausible explanation for the mutual dependency of AGO4 and AGO6 could be the different expression patterns of AGO4 and AGO6 in *Arabidopsis*. AGO6 is most highly expressed in meristems and in tissue enriched for dividing cells. AGO4, on the other hand, is expressed throughout the plant, including terminally differentiated tissue like leaves, where AGO6 is not expressed (Zheng *et al*, 2007; Havecker

et al, 2010). It is possible that in *ago6* mutants, loss of DNA methylation occurs at an early meristematic stage and thus is maintained in all of the derived cell lineages, including leaves where AGO4 is expressed.

However, over and above this simple model, Duan *et al* provide an additional insight into the non-redundant roles of AGO4 and AGO6, by examining their sub-nuclear localization. They find that AGO4 localization in the nucleoplasm overlaps with RNA polymerase II (Pol II), and not with RNA polymerase V (Pol V) as would be expected from biochemical purification (Zhong *et al*, 2014). In contrast, AGO6 localization in the nucleoplasm overlaps with Pol V and not Pol II. Consistent with these results, the

Figure 1. Argonaute proteins act in parallel to silence transposons.

(A) RNA-directed DNA methylation in *ago4*, *ago6* and *ago4 ago6* mutant backgrounds. At a majority of loci (53%), the *ago4 ago6* double mutant does not exhibit a greater loss of cytosine methylation than either of the single mutants, suggesting AGO4 and AGO6 have parallel and not redundant functions in RNA-directed DNA methylation. Black circles represent methylated cytosines, white circles represent unmethylated cytosines. (B) AGO6 guides DNA methylation at the *Athila6* LTR retrotransposon in a *ddm1* mutant background. *Athila6A* is reactivated in a *ddm1* mutant. After multiple generations, the *Athila6A* element is targeted with DNA methylation in an AGO6- and RDR6-dependent manner. The production of the AGO6-bound small RNAs is probably downstream of easiRNAs which also require RDR6. The molecular link between these pathways remains to be demonstrated. Long terminal repeat (LTR) and intergenic region (IR) promoters are depicted.

authors demonstrate that AGO4 can co-immunoprecipitate Pol II while AGO6 cannot. This suggests that AGO4 and AGO6 may carry out different functions within the nucleus and that both proteins may be required to silence a given transposable element in a single cell. In *D. melanogaster*, two argonaute-like PIWI proteins are required for piRNAs to initiate transposon silencing, leading to the famous ping-pong cycle (Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007). It remains to be determined whether there are any parallels with AGO4 and AGO6.

In another recent publication in *The EMBO Journal*, McCue *et al.* (2015) provide complementary insight into AGO6 function by using a *ddm1* mutant background, where the majority of heterochromatic DNA methylation is lost and re-activation of transposable elements is widespread (Lippman *et al.*, 2004). The authors focus on the *Athila6A* retroelement that they had previously shown to acquire asymmetric DNA methylation in the long terminal repeat (LTR) in *ddm1*, in a manner that depended on AGO6 and the RNA-dependent RNA polymerase RDR6 (Nuthikattu *et al.*, 2013). The rest of the element loses DNA methylation extensively and is strongly transcribed in *ddm1* mutants.

McCue *et al.* performed small RNA sequencing in a *ddm1* background using AGO6-immunoprecipitated RNA and total RNA. They found that AGO6-immunoprecipitated small RNAs overlap the *Athila6A* LTR, including the region of the element that is *de novo* methylated. However, by far the most abundant small RNAs (~90%) targeting *Athila6A* overlap the 3' end of the transposable element and correspond to 21 nucleotide easiRNAs which require AGO1 for biogenesis (see Fig 1B) (McCue *et al.*, 2012; Creasey *et al.*, 2014). Thus, again in this system, two argonaute proteins appear to be important, in this case for the *de novo* silencing of transposons in a *ddm1* background.

In their discussion, McCue *et al.* distinguish the canonical RdDM pathway, which

requires the plant-specific RNA polymerase IV (Pol IV) and RDR2, from the RDR6-RdDM pathway that appears to be important in targeting LTR methylation *de novo* in *ddm1* mutants. McCue *et al.* propose that AGO6 and RDR6 are components of an ancient RdDM pathway that targets actively transcribed elements for DNA methylation. Importantly, McCue *et al.* identify Pol V, but not Pol IV, as interacting genetically with AGO6 to guide LTR methylation, consistent with the immunolocalization results of Duan *et al.* Taken together, it appears that AGO6, along with co-operating partners, is a central player in RdDM in *Arabidopsis*.

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