AGO4 is specifically required for heterochromatic siRNA accumulation at Pol V-dependent loci in *Arabidopsis thaliana*

Feng Wang\textsuperscript{1,2} and Michael J. Axtell\textsuperscript{1,2,*}

\textsuperscript{1} Intercollege Plant Biology Ph.D. Program, Huck Institutes of the Life Sciences, Penn State University, University Park, PA 16802 USA.

\textsuperscript{2} Department of Biology, Penn State University, University Park, PA 16802 USA

* Corresponding author: mja18@psu.edu

Running title: *AGO4*-dependent siRNA accumulation

Significance statement

Genome-wide characterization of *AGO4*-dependent siRNAs revealed that *AGO4* is required for the accumulation of a small subset of heterochromatic siRNAs in *Arabidopsis thaliana*. These *AGO4*-dependent siRNAs are likely secondary het-siRNAs produced by a self-reinforcing loop of RdDM. Slicing-defective *AGO4* is unable to fully complement het-siRNA accumulation from an *ago4* mutant, demonstrating the critical role of *AGO4* catalytic ability in het-siRNA accumulation.

Total word count (References excluded): 4619

Word count breakdown:
Summary

In plants, 24 nucleotide long heterochromatic siRNAs (het-siRNAs) transcriptionally regulate gene expression by RNA-directed DNA methylation (RdDM). The biogenesis of most het-siRNAs depends on the plant-specific RNA polymerase IV (Pol IV), and ARGONAUTE4 (AGO4) is a major het-siRNA effector protein. Through genome-wide analysis of sRNA-seq data sets, we found that AGO4 is required for the accumulation of a small subset of het-siRNAs. The accumulation of AGO4-dependent het-siRNAs also requires several factors known to participate in the effector portion of the RdDM pathway, including RNA POLYMERASE V (POL V), DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) and SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1). Like many AGO proteins, AGO4 is an endonuclease that can 'slice' RNAs. We found that a slicing-defective AGO4 was unable to fully recover AGO4-dependent het-siRNA accumulation from ago4 mutant plants. Collectively, our data suggest that AGO4-dependent siRNAs are secondary siRNAs dependent on the prior activity of the RdDM pathway at certain loci.

Keywords: AGO4 / heterochromatic siRNA accumulation / secondary heterochromatic siRNAs / slicing ability / sRNA-seq
Introduction

24 nucleotide (nt) heterochromatic small interfering RNAs (het-siRNAs) are usually loaded into ARGONAUTE4 (AGO4) to direct repressive chromatic modifications and subsequent transcriptional gene silencing via RNA-directed DNA Methylation (RdDM) (Zilberman et al., 2003; Qi et al., 2006). Het-siRNA-induced transcriptional silencing plays important roles in transposable element silencing, stress responses and genome stability (Law and Jacobsen, 2010; Matzke and Mosher, 2014). The production of het-siRNAs in Arabidopsis thaliana usually requires the plant-specific RNA POLYMERASE IV (Pol IV) (Onodera et al., 2005; Herr et al., 2005; Blevins et al., 2015; Zhai et al., 2015), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) (Xie et al., 2004; Kasschau et al., 2007) and one or more DICER-LIKE (DCL) proteins (most predominantly DCL3; Henderson et al., 2006). A second plant-specific RNA polymerase, Pol V, generates scaffold RNAs targeted by het-siRNAs associated with AGO4 (Wierzbicki et al., 2008; Wierzbicki et al., 2009). This targeting is thought to recruit the de novo DNA methyltransferase DOMAINS REARRANGED 2 (DRM2) to the local chromatin, which acts to catalyze 5-methylation of cytosines (Cao and Jacobsen, 2002; Zhong et al., 2014). The SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) protein interacts with chromatin at Pol V transcribed loci, and recruits Pol IV to promote further siRNA biogenesis specifically from Pol V-dependent regions (Law et al., 2013; H., Zhang et al., 2013). Despite their positions at the effector portion of the RdDM pathway, Pol V and DRM2 are required for the accumulation of a subset of Pol IV-dependent het-siRNAs in Arabidopsis (Pontier et al., 2005; Mosher et al., 2008).
The *Arabidopsis* genome has 10 AGO genes. AGO4, AGO6, AGO8, and AGO9 form a monophyletic clade (Vaucheret, 2008; Mallory and Vaucheret, 2010; Fang and Qi, 2016). AGO8 has been suggested as a pseudogene (Vaucheret, 2008). AGO4 and AGO6 both bind 24 nt het-siRNAs and contribute to the canonical RdDM pathway in a non-redundant fashion (Zheng et al., 2007; Havecker et al., 2010; Duan et al., 2015). AGO6 also binds 21 nt siRNAs and act as a key effector of the non-canonical RDR6-RdDM pathway (McCue et al., 2015; Panda et al., 2016). AGO9, which is primarily expressed in female gametes, interacts with het-siRNAs and silence TEs in female gametes (Olmedo-Monfil et al., 2010). Though AGO4, AGO6 and AGO9 are functionally related, the small RNA profile of an ago4/ago6/ago9 triple mutant has not been reported yet.

According to the current model of the RNA-directed DNA methylation (RdDM) pathway, the biogenesis of het-siRNAs depends on Pol IV, RDR2, and primarily DCL3 (Law and Jacobsen, 2010; Matzke and Mosher, 2014), while AGO4 is not directly required for the biogenesis of het-siRNAs. However, the accumulation of certain het-siRNAs was shown to be dependent on AGO4 in previous reports (Qi et al., 2006; Havecker et al., 2010). It has been hypothesized that the accumulation of a subset of het-siRNAs depends on AGO4-mediated target slicing (Qi et al., 2006). All 10 Arabidopsis AGOs have a conserved Asp-Asp-His (DDH) or Asp-Asp-Asp (DDD) motif thought to form a catalytic center for cleavage of target RNA. The target-slicing ability of AGO1 and AGO7 has been confirmed *in vivo* (Vaucheret, 2008; Fang and Qi, 2016). AGO10 can slice miRNA target *in vitro*, but
it is still unclear if the slicer-activity is required for its function in plants (Ji et al., 2011; Zhu et al., 2011). AGO4, which specifically binds 24nt het-siRNAs, can slice synthetic het-siRNA targets \textit{in vitro} (Qi et al., 2006) as well as the passenger-strand of het-siRNA duplexes \textit{in vivo} (Ye et al., 2012). The \textit{in vitro} and/or \textit{in vivo} slicing ability of AGO4 is abolished by mutagenesis of the presumed catalytic triad (Qi et al., 2006; Ye et al., 2012). However, the genome-wide effects of AGO4 slicing on global small RNA accumulation have not been previously reported.

\textbf{Results}

\textbf{Accumulation of a subset of 24 nt het-siRNAs depends on AGO4 in \textit{Arabidopsis}}

To systematically study the profile of AGO4-dependent het-siRNAs and the effect of AGO4 catalytic activity on het-siRNA accumulation, we expressed wild-type AGO4 (\textit{pAGO4:FLAG-AGO4-DDH, wtAGO4} hereafter) or slicing-defective AGO4 (\textit{pAGO4:FLAG-AGO4-DAH, D742A} hereafter) driven by the native AGO4 promoter in both the \textit{ago4-4} single mutant background and the \textit{ago4-4/ago6-2/ago9-1} triple mutant background in \textit{Arabidopsis} (Fig S1a). Three T3 transgenic plants with comparable levels of protein accumulation (Fig S1b) were used to prepare three biological replicate \textit{sRNA-seq} libraries. It is worth noting that \textit{ago4-4} is in the Ws background, while \textit{ago6-2} and \textit{ago9-1} are in the Col-0 background. We therefore prepared three replicate control \textit{sRNA-seq} libraries from both Ws and Col-0. We merged \textit{sRNA-seq} libraries from the same genotype and aligned them to reference genome to study the overall small RNA size distribution in tested samples. Loci dominated by 24 nt small RNAs were the most
abundant in all tested genotypes, and the fractions of small RNA from 24 nt small RNA-dominated loci were similar across different genotypes (Fig S2). siRNA-seq libraries from all backgrounds were then merged, aligned to the reference genome, followed by de novo definition of expressed small RNA clusters. The 24 nt siRNA clusters that were de novo annotated are listed in Data S1.

We first examined siRNA accumulation in the Ws background, to compare ago4-4 to the wild-type. A differential expression analysis was performed by comparing raw read counts from our de novo annotated small RNA loci for all libraries in Ws background. A principal component analysis (PCA) plot was prepared to visualize the overall differences between samples (Fig 1a). The biological replicates were grouped together, indicating good reproducibility (Fig 1a). ago4-4/AGO4 grouped closely with the Ws wild-type, suggesting complementation of small RNA accumulation by expression of AGO4 in the ago4-4 background (Fig 1a). ago4-4 and ago4-4/D742A were distinct from each other and from the wild-type and ago4-4/AGO4 genotypes (Fig 1a).

Most differentially accumulated clusters were dominated by 24 nt siRNAs (Fig 1b). In ago4-4, 2,912 clusters were down-regulated relative to wild-type; we defined these as AGO4-dependent siRNA clusters (Fig 1b). Most of these (2,879) were dominated by 24 nt siRNAs. In contrast, only 121 clusters were down regulated in ago4-4/AGO4, indicating nearly full complementation of small RNA accumulation by AGO4 (Fig 1b). Intriguingly, an intermediate amount of clusters (1,541, Fig 1b) was down-regulated in
ago4-4/D742A, which suggested that slicing-defective AGO4 partially recovers the accumulation of AGO4-dependent small RNAs. Most 24 nt-dominated siRNA clusters are not AGO4-dependent. Only about 18% of the de novo annotated 24 nt siRNA clusters, which contained about 22% of small RNAs in Ws wild-type, were dependent on AGO4 (Fig 1c).

Accumulation of small RNAs in AGO4-dependent clusters requires NRPE1, DRM2 and SHH1

We classified the 16,061 de novo annotated 24 nt siRNA-dominated clusters into different groups based on AGO4-dependency (Data S1). As stated above, 2,879 24 nt-dominated siRNA clusters were AGO4-dependent (FDR=0.01). We found another 1,359 24 nt-dominated clusters that were clearly AGO4-independent (FDR=0.01). Another 354 24 nt-dominated clusters were up-regulated in ago4-4 (FDR=0.01), and the AGO4-dependency of the remaining 11,469 24 nt-dominated siRNA clusters could not be reliably inferred using our strict statistical tests, primarily due to low expression levels. We analyzed sRNA-seq accumulation from the AGO4-dependent and AGO4-independent clusters using data from nrpd1-4, nrpe1-12, drm2-2, and shh1-1 mutants (Law et al., 2013), using accumulation of clusters overlapping high-confidence MIRNA loci (Kozomara and Griffiths-Jones, 2014) as a control (Fig 2a). Note that NRPD1 and NRPE1 encode the catalytic sub-units of Pol IV and Pol V, respectively. In nrpd1, siRNA accumulation was strongly down-regulated in both AGO4-dependent and AGO4-independent clusters (Fig 2a). In contrast, AGO4-dependent clusters were much more strongly affected in the nrpe1,
drm2, and shh1 backgrounds compared to AGO4-independent clusters (Fig 2a). We then normalized small RNA accumulation in AGO4-dependent and AGO4-independent het-siRNA clusters based on wild-type plants. We observed significantly reduced small RNA accumulation (Mann-Whitney test, p<0.01) in AGO4-dependent clusters relative to AGO4-independent clusters in all analyzed RdDM mutants except nrpd1 (Fig 2b). Using small RNA-seq data from nrpe1-1 plants (Lee et al., 2012), we defined 2,827 NRPE1-dependent small RNA clusters, the majority of which overlapped AGO4-dependent siRNA clusters (Fig 2c). This extent of overlap far exceeded the number expected by random chance (Fig 2d). Collectively, these data indicate that the subset of 24 nt dominated siRNA loci that depend on AGO4 for accumulation are those that are also dependent on NRPE1, DRM2, and SHH1.

An AGO4 catalytic residue is required for full accumulation of most AGO4-dependent 24 nt siRNAs

We then compared complementation of siRNA accumulation from AGO4-dependent clusters between the wtAGO4 and AGO4-D742A transgenic lines. Small RNA accumulation was recovered in wtAGO4 from nearly all AGO4-dependent clusters, but only from a small subset of loci in the slicing-defective AGO4-D742A plants (Fig 3a). We defined AGO4-D742A complemented loci as those that were significantly down-regulated in the ago4-4 background but not in the ago4-4/AGO4-D742A transgenic plants (Fig 3b). Conversely, AGO4-D742A non-complemented loci were defined as those that were significantly down-regulated in both ago4-4 and ago4-4/AGO4-D742A (Fig 3b). By this
measure, half (49.9%) of the AGO4-dependent siRNA loci required AGO4 catalytic activity for their accumulation. Detailed examination of accumulation levels revealed that recovery was generally not to full wild-type levels at loci designated as complemented by AGO4-D742A (Fig 3c). We conclude that the catalytic ability of AGO4 is important for full accumulation of most AGO4-dependent 24 nt siRNAs, but to varying degrees at different loci.

**Slicing-defective AGO4 partially complements small RNA accumulation in the ago4-4/ago6-2/ago9-1 triple mutant**

AGO4, AGO6, and AGO9 have related but non-redundant functions in gene silencing, and all three can bind 24 nt siRNAs (Havecker et al., 2010). We obtained the triple mutant ago4-4/ago6-2/ago9-1 and analyzed small RNA expression levels from inflorescence tissue. Significant ecotype-specific changes in small RNA accumulation levels were observed between Ws (the parental background of the ago4-4 allele) and Col-0 (the parental background of the ago6-2 and ago9-1 alleles) (Fig S3a). About 15% of the small RNA clusters had significant differential accumulation (FDR = 0.01) when comparing Ws and Col-0 (Fig S3b). Different small RNA accumulation in these DE clusters was presumably caused by the different genetic backgrounds. We therefore excluded these loci from our analyses.

When analyzing the remaining, ecotype-insensitive small RNA clusters, we observed that the Col-0, Ws, and ago4-4/wtAGO4 samples were tightly grouped (Fig 4a). This
demonstrates both the effective removal of clusters that have ecotype-specific differences in accumulation, as well as strong complementation by the wtAGO4 transgene. While ago4-4/6-2/9-1/wtAGO4 strongly diverged from ago4-4/6-2/9-1, ago4-4/6-2/9-1/D742A showed only minimal differences from ago4-4/6-2/9-1 (Fig 4a). This implies that introduction of wild-type AGO4, but not a slicing-defective AGO4, can rescue much of the small RNA accumulation defects of the triple mutant. Full elimination of AGO4-clade AGOs didn't affect accumulation of the majority of 24 nt siRNA clusters: About 22% (3005/13602) of the 24 nt siRNA clusters in Col-0 were AGO4/AGO6/AGO9-dependent, and these clusters contributed only about 15% of the small RNA reads (Fig 4b). Only about 24% (719/3005) of the AGO4/AGO6/AGO9-dependent clusters were not complemented by wtAGO4 (Fig 4c), indicating that AGO6 and/or AGO9 are required for accumulation from relatively few clusters. Similar to the single-mutant analysis (Fig 3), many of the AGO4/AGO6/AGO9-dependent clusters were not complemented by AGO4-D742A (Fig 4c). In addition, even the set of loci that were designated as complemented by AGO4-D742A still generally showed less accumulation than observed with the wtAGO4 transgene (Fig 4d). Overall, these analyses demonstrate that AGO4 is required for the accumulation of a much larger number of siRNAs compared to AGO6 and AGO9 in inflorescences, and that the slicing activity of AGO4 is required for full accumulation of most of these siRNAs.

Discussion

Most 24 nt siRNAs do not require AGO4, AGO6, or AGO9 for accumulation
AGO4 is required for 24 nt small RNA at some loci, but not others (Zilberman et al., 2003; Qi et al., 2006). Our genome-wide analysis confirms this observation, and quantifies the extent of the dichotomy: Most 24 nt siRNA loci are unaffected by loss of AGO4, while only a small subset have siRNA accumulation defects. Even when all three functional members of the AGO4 clade (Vaucheret, 2008) are removed in the ago4-4/6-2/9-1 triple mutant, accumulation of 24 nt siRNAs from most loci is unaffected. This situation seems to contrast to the relationship between the major Arabidopsis miRNA-binding Argonaute AGO1 and miRNAs: In the null mutant ago1-3, accumulation of the majority of miRNAs is decreased (Vaucheret et al., 2004; Arribas-Hernández, Kielpinski, et al., 2016). Why might the majority of 24 nt siRNAs maintain stable accumulation levels in the absence of AGO4, AGO6, and AGO9? One possibility is that they are stabilized by AGO3. Despite not being a member of the AGO4 clade, Arabidopsis AGO3 is primarily associated with 24 nt siRNAs, and can partially complement the DNA methylation defects seen in the ago4 mutant (Z., Zhang et al., 2016). Alternatively, many 24 nt siRNAs might be stabilized by association with non-AGO RNA binding proteins, or perhaps not require protein binding at all.

AGO4-dependent siRNAs are likely secondary siRNAs

Two models have been proposed to explain why some 24 nt siRNAs are dependent on AGO4. Qi et al. (2006) hypothesized that AGO4-dependent siRNAs might reflect target slicing-dependent secondary siRNA biogenesis similar to that which is sometimes observed from miRNA targets (Fei et al., 2013). In this model, double-stranded RNA
could be synthesized from using AGO4-sliced primary transcripts, which are then further processed into 24 nt secondary siRNAs by DCL3. Because Pol V makes chromatin-associated, long non coding RNAs that are targeted by AGO4 (Wierzbicki, 2012), the sliced-secondary siRNA model predicts that AGO4-dependent siRNAs would also be NRPE1-dependent. Our analysis shows that this prediction is supported by the data: most NRPE1-dependent siRNA clusters are also AGO4-dependent, and vice-versa. However, we also found that AGO4-dependent siRNAs also tend to be DRM2-dependent. This isn't an obvious prediction of the sliced-secondary siRNA model because DRM2, a de novo DNA methyltransferase, is thought to be recruited to chromatin in the vicinity of an AGO4-Pol V interaction. An alternative model proposed that an initial wave of de novo AGO4/Pol V-dependent DNA methylation at a locus could subsequently recruit Pol IV and thus produce secondary siRNAs in a self-reinforcing loop (Pontier et al., 2005). Our observation that ago4, nrpe1, drm2, and shh1 were all required for accumulation of the same subsets of 24 nt siRNA loci is fully consistent with the self-reinforcing loop model for secondary het-siRNAs. Intriguingly, much stronger reduction of het-siRNA accumulation was observed in nrpd1-4 than in shh1-1, suggesting that SHH1 may be specifically required for guiding Pol IV to the regions targeted by AGO4-dependent, self-reinforcing silencing.

**On the role of AGO4-catalyzed slicing**

A full description of the functions of AGO4-catalyzed endonuclease activity (e.g. slicing) remains elusive. In other systems, two general functions of AGO-catalyzed slicing have been described: Slicing of passenger strands during AGO-loading of a small RNA duplex
(Matranga et al., 2005), and slicing of target RNAs (Qi et al., 2005). For Arabidopsis AGO1, both in vitro and in vivo experiments demonstrate that AGO1-catalyzed slicing is not required for miRNA loading, but is required for many aspects of target regulation (Iki et al., 2010; Carbonell et al., 2012; Arribas-Hernández, Kiepinski, et al., 2016; Arribas-Hernández, Marchais, et al., 2016). In contrast, in vitro and in vivo data have demonstrated that AGO4-catalyzed slicing is required for passenger strand removal during siRNA loading and subsequent nuclear localization of the AGO4-siRNA complex (Ye et al., 2012). Although AGO4 can slice a free target RNA in vitro (Qi et al., 2006), to our knowledge there is no direct evidence of AGO4-catalyzed slicing of Pol V target RNAs in vivo. Our analysis showed that the catalytic capability of AGO4 is critical for the full accumulation of nearly all AGO4-dependent siRNAs. Many siRNAs were not rescued at all by slicing-defective AGO4, and even those that showed some degree of complementation almost never recovered to the extent allowed by complementation with the wild-type AGO4. The dependency of AGO4-dependent siRNAs upon AGO4-catalyzed slicing could be fully explained by defects in siRNA loading (Ye et al., 2012). In either the sliced-secondary siRNA or self-reinforcement secondary siRNA models, lack of proper loading and subsequent nuclear localization of the 'primary' siRNAs would prevent accumulation of the AGO4-dependent sub-population.

Ye et al. reported that passenger strand removal mediated by AGO4 slicing is required for nuclear location of AGO4 (Ye et al., 2012). Why could any complementation occur at all in the slicing defective mutant AGO4-D742A in our study? One hypothesis is that the
passenger strand removal for proper AGO4-loading may not be completely dependent on slicing. AGO1-mediated slicing is not required for the unwinding of miRNA/miRNA* duplexes during AGO1-loading (Iki et al., 2010; Carbonell et al., 2012; Arribas-Hernández, Kielpinski, et al., 2016; Arribas-Hernández, Marchais, et al., 2016). Slicing-independent miRNA loading may be efficient because of the mismatches and bulges in common in miRNA/miRNA* duplexes (Iki et al., 2010). In the case of AGO4-loading, where siRNA duplexes are perfectly complementary, a slicing-independent mechanism might still contribute to passenger strand removal, but with a much lower efficiency.

Whether or not AGO4-catalyzed slicing occurs at the targeting stage (e.g. in the nucleus upon targeted Pol V transcripts) remains unclear. If so, it would seem to present difficulties for the current model of RdDM, which supposes that a stable tethering of AGO4-siRNA complexes to nascent RNAs is required to recruit DRM2 to the vicinity. Conversely, if slicing is not used at the targeting stage, the challenge becomes understanding how it is prevented in vivo, given that in vitro AGO4-siRNA complexes are perfectly competent to direct target cleavage (Qi et al., 2006). Resolution of these questions is an important goal for the future that will further illuminate the mechanisms of RdDM.

Experimental procedures

Plant materials and growth condition

All Arabidopsis thaliana plants were grown at 21°C with 16 h light/8 h dark. ago4-4 (FLAG_216G02) was from INRA T-DNA transformants in the Wassilevskija (Ws)
ecotype. **ago6-2** (SALK_031553) and **ago9-1** (SALK_127358) were from Salk T-DNA transformants in the Columbia-0 (Col-0) ecotype. The **ago4-4/ago6-2/ago9-1** triple mutant was generated by crossing **ago4-4** to **ago6-2** first, and crossing the **ago4-4/6-2** double mutant to **ago9-1**. Homozygous mutants were selected by genotyping using primers that specifically amplify T-DNA inserted alleles. All the genotyping primers are listed in Table S1.

**Cloning of wild-type and slicing-defective AGO4**

cDNA encoding **AGO4** (*AT2G27040*) was amplified from *Arabidopsis thaliana* cDNA in Col-0 ecotype. A **FLAG** tag was inserted at the 5' of **AGO4** cDNA right after start codon by PCR. The **FLAG**-tagged **AGO4** sequence was sub-cloned into the pGII0179 vector. A ~2 kb DNA sequence located upstream of the start codon of **AGO4** in Col-0, and a ~500 bp DNA sequence downstream of stop codon of **AGO4** in Col-0, were further sub-cloned into **AGO4** expression vector as native promoter and terminator (**pAGO4:FLAG-AGO4**).

Mutagenesis of the catalytic motif of **AGO4** was performed by overlapping extension PCR. Primers with desired changes, which encode alanine instead of aspartic acid at the 742th amino acid position of **AGO4**, were used to introduce slicing defective mutation. The wild-type **AGO4** sequence in **AGO4** expression vector was then swapped by mutagenized **AGO4** to generate slicing-defective **AGO4** expression vector (**pAGO4:FLAG-AGO4-D742A**). The hygromycin-B phosphotransferase gene was inserted into both wild-type and slicing-defective **AGO4** expression vectors for hygromycin resistance selection in transgenic plants. All primers used for subcloning are listed in Table S1.
Plant transformation and transgenic plant selection

Wild-type or slicing-defective AGO4 expression vector was introduced into ago4-4 or the ago4-4/ago6-2/ago9-1 background by floral dip with Agrobacterium tumefaciens strain GV3101 bearing the pSOUP plasmid and designated expression vectors. Transgenic plants were selected on 1/2 strength Murashige-Skoog plates supplemented with 15mg/L Hygromycin-B. Independent transgenic lines with single insertion were selected in the T2 generation. Homozygous lines with comparable wild-type or slicing-defective AGO4 protein accumulation in the T3 generation were further selected to prepare sRNA-seq libraries.

sRNA-seq library preparation

Libraries were constructed by using 1µg total RNA extracted from Arabidopsis immature inflorescence tissue as described in Wang et al. (2016). Three biological replicates from each genotype were prepared. Raw data have been deposited at NCBI GEO under accession number GSE79119 (Col-0 samples) and GSE87333 (all other samples). Details for sRNA-seq libraries are listed in Table S2.

Differential expression analysis

sRNA-seq data sets, including libraries from wild-type AGO4 and slicing-defective AGO4 transgenic lines in ago4-4 and ago4-4/ago6-2/ago9-1 background, mutant controls of ago4-4 and ago4-4/ago6-2/9-1, wild-type controls of Col-0 and Ws, were merged and run
with ShortStack 3.3 (Johnson et al., 2016) with options --adapter TGGAATTC --mincov 50. All sRNA-seq libraries were aligned to the Arabidopsis TAIR10 reference genome.

A matrix of raw read counts from de novo annotated small RNA clusters in all three biological replicates of different genotypes were used for differential expression analysis with the R package DESeq2 (Love et al., 2014). Clusters with at least a 2-fold change relative wild-type at a 1% false discovery rate were defined as differentially expressed.

To identify differentially expressed clusters in nrpe1 compared to Col-0, sRNA-seq data sets from a previous study (Lee et al., 2012) with three biological replicates of nrpe1-1 and three biological replicates of Col-0 were analyzed with the same pipeline as described above, except that small RNA clusters were previously annotated by analyzing the AGO4-related data sets. sRNA-seq libraries used in this analysis are listed in Table S2.

**Heatmap of small RNA accumulation in AGO4-dependent clusters**

To generate the heatmap for small RNA accumulation visualization, we first transformed read per million (RPM) data in AGO4-dependent clusters with the equation $E = \log_2(R_i/R_m)$, where $E$ is the input for heatmap, $R_i$ is the RPM of a cluster in a sRNA-seq library, $R_m$ is the mean RPM of a cluster across different sRNA-seq libraries been analyzed for the heatmap. The matrix of transformed RPM was then used for heatmap preparation with the R package heatmap (Kolde, 2015).
Euler diagrams

All Euler diagrams in this study were prepared with eulerAPE 3.0 (Micallef and Rodgers, 2014).

Small RNA accumulation in *nrpd1-4, nrpe1-12, drm2-2, shh1-1, and ago4-4*

sRNA-seq libraries from a study (Law et al., 2013) containing samples from *nrpd1-4, nrpe1-12, drm2-2, shh1-1* and Col-0 were aligned to the *Arabidopsis* TAIR10 genome using ShortStack 3.3 (Johnson et al., 2016) with a locifile specifying small RNA clusters which were defined in the AGO4 sRNA-seq data sets. The 3’ adapters were removed with the option --adapter TGGAATTC. Before log2 transformation, a value of 0.5 was added to all raw counts. Log2 transformed RPMs of 24 nt siRNA clusters from *nrpd1-4, nrpe1-12, drm2-2* and *shh1-1* as well as Col-0 were plotted to illustrate small RNA accumulation in 24 nt siRNA clusters. Log2 transformed RPMs of high-confidence miRNA genes were also plotted. The linear regression and 95% predicted intervals were calculated based on the distribution of high-confidence miRNA genes. Small RNA accumulation at 24 nt siRNA loci in indicated RdDM mutants was then normalized to corresponding wild-type plants, with equation \( N = \log_2 (\text{RPM}_{\text{mutant}} / \text{RPM}_{\text{WT}}) \). Statistical differences between AGO4-dependent and AGO4-independent clusters were tested using the Mann-Whitney U test.

Author contributions

MJA conceived of the project. FW generated transgenic plants, constructed small RNA-seq libraries and performed data analysis. MJA and FW wrote the manuscript.
Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors thank Penn State University Genomic Core Facility for small RNA-seq services, and all members of the Axtell Lab for constructive comments.

Supporting information

Figure S1. Expression of wild-type AGO4 and slicing-defective AGO4 proteins in transgenic plants

Figure S2. Overall size profiles of small RNAs in tested genotypes

Figure S3. Divergence of small RNA accumulation between Col-0 and Ws

Table S1. Primers used in this study

Table S2. Data sources and accession numbers of *Arabidopsis thaliana* sRNA-seq libraries

Data S1. *de novo* annotated 24 nt siRNA clusters in this study

Funding

US National Science Foundation [1121438 to M.J.A.]; purchase of the Illumina HiSeq2500 used for small RNA-seq was funded by a major research instrumentation award from the US National Science Foundation [1229046 to M.J.A.].
References


Iki, T., Yoshikawa, M., Nishikiori, M., Jaudal, M.C., Matsumoto-Yokoyama, E.,


McCue, A.D., Panda, K., Nuthikattu, S., Choudury, S.G., Thomas, E.N. and Slotkin,


Figure legends

Figure 1. Identification of AGO4-dependent small RNA clusters in Arabidopsis thaliana

(a) Principal component analysis demonstrating overall relationships between sRNA-seq libraries in Ws background.

(b) Number of differentially expressed (DE) clusters in the indicated genotypes and small RNA clusters compared with Ws wild-type. DE clusters were defined as clusters with at least 2-fold change compared to wild-type at a false discovery rate of 1%.

(c) Percentage of AGO4-dependent clusters and AGO4-dependent small RNAs in 24 nt siRNA loci. AGO4-dependent clusters were defined as clusters with at least 2-fold less accumulation in ago4-4 compared with Ws.

Figure 2. AGO4-dependent and AGO4-independent 24 nt siRNA clusters in other RdDM mutants

(a) Small RNA accumulation from AGO4-dependent and AGO4-independent 24 nt siRNA clusters in indicated RdDM mutants. Log2 transformed reads per million (RPM) in indicated genotypes were plotted. A linear regression (solid line) and associated 95% prediction interval (dashed lines) was plotted based upon accumulation from clusters overlapping high-confidence MIRNA loci.
(b) Normalized small RNA accumulation in AGO4-dependent and AGO4-independent clusters in indicated RdDM mutants. Boxplots show medians (horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches), other data out to 1.5 times the interquartile range (whiskers) and outliers (dots). Asterisks indicate significant differences (Mann-Whitney U test, p<0.01) between AGO4-dependent and AGO4-independent clusters in the indicated mutant.

(c) Venn diagram showing the overlap of AGO4-dependent and NRPE1-dependent 24 nt siRNA clusters.

(d) Percentage of overlap between AGO4-dependent and NRPE1-dependent clusters. Overlaps expected by random chance were estimated by by randomly choosing 2827 and 2879 clusters from all 24 nt siRNA clusters. The mean and standard deviation (n=10) of randomly overlapping percentages are shown.

Figure 3. Slicing-defective AGO4-D742A partially complements small RNA accumulation from AGO4-dependent siRNA loci

(a) Heatmap showing normalized (log2-transformed and mean-centered) small RNA accumulation from AGO4-dependent clusters in indicated genotypes and replicates.

(b) Euler diagram showing overlaps between down-regulated small RNA clusters (FDR=0.01) in the indicated genotypes compared to Ws wild-type. C: Complemented by the AGO4-D742A transgene; NC: Not complemented by the AGO4-D742A transgene.

(c) Normalized small RNA accumulation levels from AGO4-dependent loci that were complemented or not complemented by the AGO4-D742A transgene. The ratio of small
RNA accumulation in indicated genotypes over that in the Ws wild-type was computed and then log2-transformed. Boxplots show medians (horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches), other data out to 1.5 times the interquartile range (whiskers) and outliers (dots).

**Figure 4. Slicing-defective AGO4-D742A partially complements small RNA accumulation in the ago4-4/6-2/9-1 background**

(a) Principal component analysis demonstrating overall relationships between all sRNA-seq libraries from ecotype-insensitive small RNA clusters. The first two principal components were shown for tested samples.

(b) Percentage of AGO4/AGO6/AGO9-dependent clusters and AGO4/AGO6/AGO9-dependent small RNAs in 24 nt siRNA loci. AGO4/AGO6/AGO9-dependent clusters were defined as clusters with at least 2-fold less accumulation in ago4-4/ago6-2/ago9-1 compared with Col-0.

(c) Euler diagram showing the number of significantly down-regulated small RNA clusters (FDR=0.01) in indicated genotypes compared with Ws wild-type. C: AGO4-D742A complemented clusters in ago4-4/6-2/9-1 background; NC: AGO4-D742A non-complemented clusters in ago4-4/6-2/9-1 background.

(d) Normalized small RNA accumulation in AGO4-D742A complemented and non-complemented clusters. The ratio of small RNA accumulation in indicated genotypes over that in the Ws wild-type was computed and then log2-transformed. Boxplots show medians.
(horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches),
other data out to 1.5 times the interquartile range (whiskers) and outliers (dots).
Figure 1. Identification of AGO4-dependent small RNA clusters in Arabidopsis thaliana

(a) Principal component analysis demonstrating overall relationships between sRNA-seq libraries in Ws background.

(b) Number of differentially expressed (DE) clusters in the indicated genotypes and small RNA clusters compared with Ws wild type. DE clusters were defined as clusters with at least 2-fold change compared to wild type at a false discovery rate of 1%.

(c) Percentage of AGO4-dependent clusters and AGO4-dependent small RNAs in 24 nt siRNA loci. AGO4-dependent clusters were defined as clusters with at least 2-fold less accumulation in ago4-4 compared with Ws.
Figure 2. AGO4-dependent and AGO4-independent 24 nt siRNA clusters in other RdDM mutants

(a) Small RNA accumulation from AGO4-dependent and AGO4-independent 24 nt siRNA clusters in indicated RdDM mutants. A hexbin plot of Log2 transformed reads per million (RPM) in indicated genotypes were plotted. A linear regression (solid line) and associated 95% prediction interval (dashed lines) was plotted based upon accumulation from clusters overlapping high-confidence MIRNA loci.

(b) Normalized small RNA accumulation in AGO4-dependent and AGO4-independent clusters in indicated RdDM mutants. Boxplots show medians (horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches), other data out to 1.5 times the interquartile range (whiskers) and outliers (dots). Asterisks indicate significant differences (Mann-Whitney U test, p<0.01) between AGO4-dependent and AGO4-independent clusters in the indicated mutant.

(c) Venn diagram showing the overlap of AGO4-dependent and NRPE1-dependent 24 nt siRNA clusters.

(d) Percentage of overlap between AGO4-dependent and NRPE1-dependent clusters. Overlaps expected by random chance were estimated by by randomly choosing 2827 and 2879 clusters from all 24 nt siRNA clusters. The mean and standard deviation (n=10) of randomly overlapping percentages are shown.
Figure 3. Slicing-defective\textit{AGO4-D742A} partially complements small RNA accumulation from\textit{AGO4}-dependent siRNA loci

(a) Heatmap showing normalized (log2-transformed and mean-centered) small RNA accumulation from\textit{AGO4}-dependent clusters in indicated genotypes and replicates.

(b) Euler diagram showing overlaps between down-regulated small RNA clusters (FDR=0.01) in the indicated genotypes compared to Ws wild-type. C: Complemented by the\textit{AGO4-D742A} transgene; NC: Not complemented by the\textit{AGO4-D742A} transgene.

(c) Normalized small RNA accumulation levels from\textit{AGO4}-dependent loci that were complemented or not complemented by the\textit{AGO4-D742A} transgene. The ratio of small RNA accumulation in indicated genotypes over that in the Ws wild-type was computed and then log2-transformed. Boxplots show medians (horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches), other data out to 1.5 times the interquartile range (whiskers) and outliers (dots).
Figure 4. Slicing-defective AGO4-D742A partially complements small RNA accumulation in the ago4-4/6-2/9-1 background

(a) Principal component analysis demonstrating overall relationships between all sRNA-seq libraries from ecotype-insensitive small RNA clusters. The first two principal components were shown for tested samples.

(b) Percentage of AGO4/AGO6/AGO9-dependent clusters and AGO4/AGO6/AGO9-dependent small RNAs in 24 nt siRNA loci. AGO4/AGO6/AGO9-dependent clusters were defined as clusters with at least 2-fold less accumulation in ago4-4/ago6-2/ago9-1 compared with Col-0.

(c) Euler diagram showing the number of significantly down-regulated small RNA clusters (FDR=0.01) in indicated genotypes compared with Ws wild-type. C: AGO4-D742A complemented clusters in ago4-4/6-2/9-1 background; NC: AGO4-D742A non-complemented clusters in ago4-4/6-2/9-1 background.

(d) Normalized small RNA accumulation in AGO4-D742A complemented and non-complemented clusters. The ratio of small RNA accumulation in indicated genotypes over that in the Ws wild-type was computed and then log2-transformed. Boxplots show medians (horizontal lines), the 1st-3rd quartile range (boxes), 95% confidence of medians (notches), other data out to 1.5 times the interquartile range (whiskers) and outliers (dots).
Supporting information

Supporting figures

(a)

(b)

Figure S1. Expression of wild-type AGO4 and slicing-defective AGO4 proteins in transgenic plants

(a) Schematic of transgenes. Indicated codons correspond to the catalytic residues required for slicing. Codon color-coded by red represented the mutagenesis of codon 742.

(b) Anti-FLAG immunoblot of FLAG-tagged AGO4 in T3 lines of the indicated transgenic plants. Transgenic lines that were chosen for sRNA-seq library preparation, based on approximately equal accumulation of AGO4 protein, are indicated by arrows.
Figure S2. Overall size profiles of small RNAs in tested genotypes

Fractions of small RNA clusters with different predominant sizes in indicated genotypes are shown. R1, R2 and R3 represent three biological replicates.
Figure S3. Divergence of small RNA accumulation between Col-0 and Ws

(a) Principal component analysis demonstrating overall relationships between sRNA-seq libraries. The first two principal components are shown for tested samples.

(b) MA plot highlighting in red small RNA clusters with at least 2-fold differences (FDR=0.01) between Col-0 and Ws ecotypes.
### Supporting tables

#### Table S1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’ to 3’ sequence</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For genotyping</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.101</td>
<td>CGGAAGTAGTACGCAAACGTGATACTTC</td>
<td><em>AGO4</em> wild-type allele genotyping forward primer</td>
</tr>
<tr>
<td>26.102</td>
<td>CCAATGGGAATGAAAGTCCAA</td>
<td><em>AGO4</em> wild-type allele genotyping reverse primer</td>
</tr>
<tr>
<td>20.80</td>
<td>TCTTAAGTACGCAAATGGTGG</td>
<td><em>AGO6</em> wild-type allele genotyping forward primer</td>
</tr>
<tr>
<td>20.81</td>
<td>ACTCTAAGTACGCAAATGGTGG</td>
<td><em>AGO6</em> wild-type allele genotyping reverse primer</td>
</tr>
<tr>
<td>20.49</td>
<td>CGGTGGAACCCTTGCTGCAA</td>
<td>ago6-2 mutant allele genotyping forward primer</td>
</tr>
<tr>
<td>22.50</td>
<td>TTTTTCCTTTTTGTGGAT</td>
<td><em>AGO9</em> wild-type allele genotyping forward primer</td>
</tr>
<tr>
<td>22.51</td>
<td>AACCTGCCTTCCTTTGCTGCAA</td>
<td><em>AGO9</em> wild-type allele genotyping reverse primer</td>
</tr>
<tr>
<td>20.50</td>
<td>TTTTTCCTTTTGCTGCAA</td>
<td>ago9-1 mutant allele genotyping forward primer</td>
</tr>
<tr>
<td>20.48</td>
<td>TGTTTCACGTTAGCGCCATC</td>
<td>ago9-1 mutant allele genotyping reverse primer</td>
</tr>
<tr>
<td><strong>For subcloning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAGO4F</td>
<td>ACCGGGCGCGTTACAAAGCATTCCGAACATC</td>
<td><em>AGO4</em> promoter forward primer</td>
</tr>
<tr>
<td>pAGO4R</td>
<td>CATACTAGTCTCTCCCTCAAAAGAAGCAAACAC</td>
<td><em>AGO4</em> promoter reverse primer</td>
</tr>
<tr>
<td>AGO4F</td>
<td>GAGACTAGTATGACACTACAAGGATGACGATGA</td>
<td><em>AGO4</em> cDNA forward primer</td>
</tr>
<tr>
<td>AGO4R</td>
<td>TTAAGCAGCGCGCGCTTAGAAATAGAAGAACATC</td>
<td><em>AGO4</em> cDNA reverse primer</td>
</tr>
<tr>
<td>AGO4-ter-F</td>
<td>TTAAGCAGCGCGCGCTTAGAAATAGAAGAACATC</td>
<td><em>AGO4</em> terminator forward primer</td>
</tr>
<tr>
<td>AGO4-ter-R</td>
<td>ACTCTAAGTACGCAAATGGTGG</td>
<td><em>AGO4</em> terminator reverse primer</td>
</tr>
<tr>
<td>D742AF</td>
<td>CATAATTTTCCAGGCTGGCTATGGAAGTGAATC</td>
<td>D742A Mutagenesis forward primer</td>
</tr>
<tr>
<td>D742AR</td>
<td>ATTCAGCCTACAGGCCGCTGAAATATTAGA</td>
<td>D742A Mutagenesis reverse primer</td>
</tr>
</tbody>
</table>
Table S2. Data sources and accession numbers of *Arabidopsis thaliana* sRNA-seq libraries

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Genotype</th>
<th>Ecotype</th>
<th>3’-Adapter (first 8 nt)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSM2086247</td>
<td>Col-0</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2086248</td>
<td>Col-0</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2086249</td>
<td>Col-0</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327936</td>
<td>Ws</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327937</td>
<td>Ws</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327938</td>
<td>Ws</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327939</td>
<td>ago4-4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327940</td>
<td>ago4-4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327941</td>
<td>ago4-4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327945</td>
<td>ago4-4/wtAGO4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327946</td>
<td>ago4-4/wtAGO4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327947</td>
<td>ago4-4/wtAGO4</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327948</td>
<td>ago4-4/D742A</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327949</td>
<td>ago4-4/D742A</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327950</td>
<td>ago4-4/D742A</td>
<td>Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327952</td>
<td>ago4-4/6-2/9-1</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327960</td>
<td>ago4-4/6-2/9-1</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327951</td>
<td>ago4-4/6-2/9-1/wtAGO4</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327952</td>
<td>ago4-4/6-2/9-1/wtAGO4</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327953</td>
<td>ago4-4/6-2/9-1/wtAGO4</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327954</td>
<td>ago4-4/6-2/9-1/D742A</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327955</td>
<td>ago4-4/6-2/9-1/D742A</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM2327956</td>
<td>ago4-4/6-2/9-1/D742A</td>
<td>Col-0/Ws</td>
<td>TGGAATTC</td>
<td>This study</td>
</tr>
<tr>
<td>GSM103235</td>
<td>Col-0</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>Law et al., 2013</td>
</tr>
<tr>
<td>GSM103237</td>
<td>nrpd1-4</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>Law et al., 2013</td>
</tr>
<tr>
<td>GSM103238</td>
<td>nrpe1-4</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>Law et al., 2013</td>
</tr>
<tr>
<td>GSM103240</td>
<td>drm2-2</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>Law et al., 2013</td>
</tr>
<tr>
<td>GSM103239</td>
<td>shk1-1</td>
<td>Col-0</td>
<td>TGGAATTC</td>
<td>Law et al., 2013</td>
</tr>
<tr>
<td>GSM893112</td>
<td>Col-0</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>GSM893113</td>
<td>Col-0</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>GSM893114</td>
<td>Col-0</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>GSM893115</td>
<td>nrpe1-1</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>GSM893116</td>
<td>nrpe1-1</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>GSM893117</td>
<td>nrpe1-1</td>
<td>Col-0</td>
<td>CACTCGGG</td>
<td>Lee et al., 2012</td>
</tr>
</tbody>
</table>
Supporting references
