Specific but interdependent functions for *Arabidopsis* AGO4 and AGO6 in RNA-directed DNA methylation

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Abstract

Argonaute (AGO) family proteins are conserved key components of small RNA-induced silencing pathways. In the RNA-directed DNA methylation (RdDM) pathway in *Arabidopsis*, AGO6 is generally considered to be redundant with AGO4. In this report, our comprehensive, genomewide analyses of AGO4- and AGO6-dependent DNA methylation revealed that redundancy is unexpectedly negligible in the genetic interactions between AGO4 and AGO6. Immunofluorescence revealed that AGO4 and AGO6 differ in their subnuclear co-localization with RNA polymerases required for RdDM. Pol II and AGO6 are absent from perinucleolar foci, where Pol V and AGO4 are co-localized. In the nucleoplasm, AGO4 displays a strong co-localization with Pol II, whereas AGO6 co-localizes with Pol V. These patterns suggest that RdDM is mediated by distinct, spatially regulated combinations of AGO proteins and RNA polymerases. Consistently, Pol II physically interacts with AGO4 but not AGO6, and the levels of Pol V-dependent scaffold RNAs and Pol V chromatin occupancy are strongly correlated with AGO6 but not AGO4. Our results suggest that AGO4 and AGO6 mainly act sequentially in mediating small RNA-directed DNA methylation.

Keywords argonautes; DNA methylation; epigenetics; RdDM; RNA polymerases

Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Plant Biology; RNA Biology

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Introduction

Argonaute (AGO) proteins are core components of small RNA-induced silencing pathways, which negatively regulate gene expression in a sequence-specific manner. In the post-transcriptional gene silencing (PTGS) pathway, AGO proteins bind to small RNAs such as miRNAs, tasiRNAs, or piRNAs and mediate gene silencing through degradation and/or translational suppression of the target mRNAs (Mallory & Vaucheret, 2010; Castel & Martienssen, 2013). In the transcriptional gene silencing (TGS) pathway, AGO proteins bind to siRNAs or piRNAs and subsequently facilitate the formation of repressive chromatin at loci that show complementarities to the small RNAs (Mallory & Vaucheret, 2010; Castel & Martienssen, 2013).

AGO proteins are highly conserved and present in various life forms ranging from Archaea to humans (Fagard *et al.*, 2000; Song *et al.*, 2004; Qi *et al.*, 2006; Tolia & Joshua-Tor, 2007). Each AGO protein is characterized by three conserved domains: PAZ, MID, and PIWI. The PAZ domain recognizes and binds the 3' end of small RNAs (Yan *et al.*, 2003; Lingel *et al.*, 2004; Ma *et al.*, 2004). The MID domain contains a 5'-phosphate-binding pocket that binds to a guide small RNA at the 5' phosphate (Ma *et al.*, 2005; Mi *et al.*, 2008; Montgomery *et al.*, 2008; Frank *et al.*, 2010). The PIWI domain is structurally similar to RNase H and, in some AGO proteins, exhibits endonuclease activity (Song *et al.*, 2004; Rivas *et al.*, 2005; Wang *et al.*, 2008). While the capacity to bind small RNAs is a common feature for all AGO proteins, different eukaryotic AGO proteins often have unique functions, which can arise from multiple factors such as distinct biochemical activities or differential association with specific types of small RNAs (Farazi *et al.*, 2008).

Arabidopsis encodes 10 AGO proteins that are only partially understood. Among the characterized *Arabidopsis* AGO proteins,

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AGO1 is the major AGO protein that mediates miRNA- and tasiRNA-induced PTGS (Bohmer et al, 1998; Mallory & Vaucheret, 2010); AGO2 is required for the DNA methylation induced by 21-nt siRNAs at a subset of non-canonical RNA-directed DNA methylation (RdDM) target loci (Pontier et al, 2012); AGO2 also binds viral siRNAs and is involved in antiviral defense response (Harvey et al, 2011; Jaubert et al, 2011). In addition, AGO2 has been implicated in binding small RNAs that direct DNA double-stranded break repair through a process that also involves Pol IV and V (Wei et al 2012). AGO7 binds miR390 and controls production of TAS3 tasiRNAs (Montgomery et al, 2008); AGO10 specifically sequesters miR166/miR165 to regulate shoot apical meristem development (Zhu et al, 2011); and AGO4, AGO6, and AGO9 are involved in the RdDM pathway that requires 24-nt siRNAs.

RdDM confers transcriptional repression via the formation of heterochromatin, characterized by DNA methylation and repressive histone modifications (Zilberman et al, 2003; Zheng et al, 2007; Olmedo-Monfil et al, 2010). In the canonical RdDM pathway, the complementary pairing of 24-nt siRNAs with nascent scaffold RNAs guides the DNA methylation complex to its target loci (Law & Jacobsen, 2010; Zhang & Zhu, 2011; Pikaard et al, 2012; Matzke & Moshier, 2014). Production of nearly all the 24-nt siRNAs requires Pol IV, a plant-specific DNA-dependent RNA polymerase (Zhang et al, 2007). According to the current paradigm, Pol IV transcription produces single-stranded non-coding RNAs, which serve as substrates for RDR2 (RNA-dependent RNA polymerase 2) production of double-stranded RNAs that are subsequently cleaved by DCL3 (dicer-like 3) and loaded onto AGO4, AGO6, or AGO9 (Matzke et al, 2009; Law & Jacobsen, 2010; Zhang & Zhu, 2011; Pikaard et al, 2012). In parallel to siRNA production, Pol V generates long, non-coding RNAs that, before being released from the chromatin, function as scaffold RNAs for the recruitment of AGO-siRNA complexes (Wierzbiński et al, 2008). In addition to Pol IV and Pol V, Pol II can also generate 24-nt siRNAs and scaffold RNAs at some intergenic, low-copy-number, repeat loci (Zheng et al, 2009). AGO4 can be cross-linked to scaffold RNAs, supporting the model of siRNA-scaffold RNA pairing (Wierzbiński et al, 2009). Both Pol V and Pol II possess an argonaute-binding motif and have been shown to interact with AGO4 (El-Shami et al, 2007; Zheng et al, 2009). In addition, AGO4 co-localizes with Pol V in perinuclear foci (Pontes et al, 2006) and with Pol II in the nucleoplasm (Gao et al, 2010). Cytological analyses revealed co-localization of AGO4 and DRM2, together with other RdDM components in distinct subnuclear foci (Li et al, 2006, 2008; Pontes et al, 2006; Gao et al, 2010). A recent study revealed that AGO4 can be co-purified with DRM2 in co-immunoprecipitation assays (Zhong et al, 2014). Additionally, AGO4 and DRM2 both co-immunoprecipitate with RDM1 (RNA-directed DNA methylation 1) (Gao et al, 2010). Therefore, AGO4 has been assigned a critical role in targeting *de novo* DNA methylation in the RdDM pathway.

Arabidopsis AGO4, AGO6, and AGO9 are closely related family members (Morel et al, 2002). AGO9 specifically silences transposable elements (TEs) in the female gametophyte in a non-cell-autonomous manner (Olmedo-Monfil et al, 2010). Although the DNA methylation phenotype is unclear in the *ago9* mutant, AGO9 has been suggested to function through Pol IV-dependent 24-nt siRNAs because it preferentially binds 24-nt siRNAs and because a double mutant with dysfunctional Pol IV and Pol V resembles the

ago9 mutant in that they both are defective in specifying the cell fate in the ovule (Olmedo-Monfil et al, 2010). Like AGO4, AGO6 has been clearly shown to regulate DNA methylation through the RdDM pathway (Zilberman et al, 2003; Zheng et al, 2007; Eun et al, 2011). Mutants defective in either AGO4 or AGO6 were both isolated in the same genetic screen for mutants with defective RdDM (Zheng et al, 2007; He et al, 2009). This indicates that AGO4 and AGO6 can have non-redundant roles in regulating the same RdDM target, although AGO6 is generally considered to be redundant with AGO4 in regulating RdDM (Ghildiyal & Zamore, 2009; Eun et al, 2011). It remains unclear, on a whole-genome scale, to what extent AGO4 and AGO6 function non-redundantly in the RdDM pathway. In fact, although AGO4 and AGO6 are known to differ in preference for siRNAs from different heterochromatin-associated loci (Havecker et al, 2010), the genomewide functional integration and/or diversification of these two closely related paralogs in regulating DNA methylation has yet to be explored.

Results

Genomewide profiling of AGO4- and AGO6-dependent DNA methylation

To profile the genomewide methylation regulated by AGO4 and/or AGO6, we performed whole-genome bisulfite sequencing using *ago4-6* and *ago6-2* mutant plants in the Col-0 background. Similar to the AGO4 mutation, the AGO6 mutation resulted in DNA hypomethylation in mature leaves (Supplementary Fig S1A), even though it was primarily expressed in root and shoot meristems (Havecker et al, 2010; Eun et al, 2011). Dysfunction of AGO4 or AGO6 causes DNA hypomethylation at 3,678 or 3,731 loci, respectively (Fig 1). Both methylome results were validated by individual bisulfite sequencing of a group of randomly chosen hypomethylation loci (Supplementary Fig S1B). In both *ago4-6* and *ago6-2* mutants, DNA hypomethylation was most obvious in the CHH context (Fig 1A and B), which is consistent with CHH methylation being the hallmark of RdDM activities and with these genes functioning in RdDM.

Approximately 80 and 82% of loci with hypomethylated DNA identified in *ago4-6* and *ago6-2* mutant plants, respectively, overlap with loci where DNA methylation is Pol IV dependent (Supplementary Fig S2A). Similar patterns were observed when AGO4 and AGO6 target loci were compared with Pol V target loci (Supplementary Fig S2A). These results further support the functions of AGO4 and AGO6 in the RdDM pathway. *ROS1* gene expression is reduced in many *Arabidopsis* mutants defective in RdDM components (Huetel et al, 2006; Li et al, 2012; Martinez-Macias et al, 2012). We found that dysfunction of either AGO4 or AGO6 decreases *ROS1* expression (Supplementary Fig S2B), further suggesting that both AGO proteins are required for RdDM. AGO6 dysfunction causes global DNA hypomethylation at loci where DNA methylation is AGO4 dependent (Fig 1A and C). Similarly, AGO4 dysfunction causes DNA hypomethylation at loci where DNA methylation is AGO6 dependent (Fig 1B and C). Even with a stringent filtering criterion (loci that are called "overlap loci" must show $\geq$ twofold reduction in methylation levels in both mutants compared to Col-0), both AGO4 and AGO6 are clearly required for DNA methylation at 2,537 loci (Fig 1C), which accounts for 68 and 69% of DNA

Figure 1. Comparative profiling of AGO4- and AGO6-dependent methylomes.

- A Heatmap depiction of the 3,731 DNA hypomethylation loci in *ago4-6*. Each hypomethylated region corresponds to a colored horizontal bar, and the bars are clustered numerically into a column (y-axis). Cytosines were examined as CG, CHG, and CHH. The color-scaled methylation levels indicate the ratios of each type of methylated cytosines over total cytosines of the same type within the examined hypomethylated regions.
- B Heatmap depiction of the 3,678 DNA hypomethylation loci in *ago6-2*.
- C Overlapping patterns of hypomethylated loci in *ago4-6* and *ago6-2*. Upper panel, Venn diagram showing the numbers of the following three types of hypomethylation loci: (1) loci where *ago4-6* shows lower methylation levels than *ago6-2* (green alone); (2) loci where *ago4-6* and *ago6-2* show equal methylation levels (green and red overlap); and (3) loci where *ago6-2* shows lower methylation levels than *ago4-6* (red alone). Lower panel, box plots showing methylation levels at the three groups of loci.

hypomethylation loci identified in *ago4-6* and *ago6-2*, respectively. Therefore, AGO4 and AGO6 mostly act non-redundantly in regulating DNA methylation in the RdDM pathway.

AGO4 and AGO6 predominantly function non-redundantly in the RdDM pathway

To dissect the genetic interactions between AGO4 and AGO6, we generated the *ago4-6 ago6-2* double mutant plants and compared its methylome with those of the single mutants. A total of 4,097 loci were identified as hypomethylated in the *ago4-6 ago6-2* double mutant compared to wild-type Col-0 (Fig 2A; Supplementary Table S1). These hypomethylated loci were categorized into four groups based on DNA methylation patterns: Group I are loci where DNA hypomethylation is observed in the double mutant but not in the single mutants, indicating that DNA methylation at these loci is redundantly regulated by AGO4 and AGO6; Group II are loci where DNA hypomethylation occurs in one of the two single mutants but not in the other, and DNA methylation is not further decreased in the double mutant, indicating that DNA methylation at these loci requires AGO4 or AGO6 specifically; Group III are loci where DNA methylation is similarly reduced in the two single mutants and the double mutant shows no additive effects on DNA hypomethylation, indicating that AGO4 and AGO6 are each required to mediate RdDM at these loci; and Group IV are loci where DNA hypomethylation is observed in the single mutants while the double mutant shows more severe DNA hypomethylation, indicating more complex genetic interactions between the two AGO proteins.

Among the 4,097 hypomethylated loci in the *ago4-6 ago6-2* double mutant, only 15 (0.4%) were categorized into Group I (Fig 2A and B; Supplementary Fig S3), indicating that AGO4 and AGO6 function mostly non-redundantly in the RdDM pathway.

Group II consists of 191 loci. The numbers of AGO4- and AGO6-specific loci are 66 (1.6%) and 125 (3.1%), respectively (Fig 2A and B; Supplementary Fig S3). Group III consists of 2,174 regions that account for the majority (53.1%) of the 4,097 hypomethylated regions (Fig 2A and B; Supplementary Fig S3). AGO4 and AGO6 are each required to confer DNA methylation at Group III loci; thus, AGO4 and AGO6 mainly play distinct but cooperative roles, for instance, with a sequential relationship, in the RdDM pathway. Group IV consists of the remaining 1,717 (41.9%) loci, where DNA hypomethylation was observed in *ago4-6*, *ago6-2*, and *ago4-6 ago6-2*, while the double mutant exhibited a further reduction in DNA methylation levels compared to the single mutants (Fig 2A and B; Supplementary Fig S3). Together, these results indicate that AGO4 and AGO6 are mostly mutually dependent, instead of being redundant, in their regulation of DNA methylation.

To examine whether the mutual dependence between AGO4 and AGO6 might be preferentially associated with certain types of genomic loci, we categorized the DNA hypomethylation loci into transposons (TEs), genic regions (Genes), or intergenic regions (IGRs). Among the 4,097 hypomethylation loci identified in the *ago4-6 ago6-2* double mutant, 33% (1,347) are gene loci, 44% are TE loci, and 23% are IGR loci (Fig 2C). Notably, 76% (1,002) of the hypomethylated gene loci exhibited the pattern that is characteristic of Group III loci (Fig 2C; Supplementary Table S1). Among these 1,002 loci, 94% loci are DNA methylation targets of Pol IV and/or Pol V (Supplementary Fig S2C). Thus, DNA hypomethylation at these loci indicates defects in AGO4/6-mediated RdDM rather than natural epialleles.

The canonical RdDM pathway involves 24-nt siRNAs that are almost exclusively Pol IV dependent. The Pol IV mutant *nrrpd1-3* and the Pol V mutant *nrrpe1-11* share 83% of their hypomethylated loci (Fig 3A), whereas 82% of hypomethylated loci in the *ago4-6 ago6-2* double mutant overlap with those identified in *nrrpd1-3* or

Figure 2. Dissection of genetic interactions between AGO4 and AGO6 based on comparative methylome analyses.

- A** Pie chart showing proportions of different groups of hypomethylation loci in the *ago4-6 ago6-2* double mutant. Group I are loci where DNA hypomethylation is observed in the double mutant but not in the single mutants, indicating that DNA methylation at these loci is redundantly regulated by AGO4 and AGO6; Group II are loci where DNA hypomethylation occurs in one of the two single mutants but not in the other, and DNA methylation is not further decreased in the double mutant, indicating that DNA methylation at these loci requires AGO4 or AGO6 specifically; Group III are loci where DNA methylation is similarly reduced in the two single mutants, and the double mutant shows no additive effects on DNA hypomethylation, indicating that AGO4 and AGO6 are each required to mediate RdDM at these loci; and Group IV are loci where DNA hypomethylation is observed in the single mutants while the double mutant shows more severe DNA hypomethylation, indicating more complex genetic interactions between the two AGO proteins.
- B** Box plots showing methylation patterns of different groups of loci as categorized in (A).
- C** Gene regions targeted by AGO4 and AGO6 are enriched in Group III loci. Y-axis shows the numbers of DNA hypomethylation loci in *ago4-6 ago6-2*. TEs: transposons; Gene: genic regions; IGR: intergenic regions that do not overlap with TEs.

nrpe1-11 (Fig 3A). These patterns suggest that AGO4- and AGO6-mediated DNA methylation is predominantly dependent on Pol IV and Pol V function in the canonical RdDM pathway. On the other hand, 62% of hypomethylated loci in *nrpd1-3* or *nrpe1-11* overlap with hypomethylation regions in the *ago4-6 ago6-2* double mutant (Fig 3A). Although this pattern supports the importance of AGO4 and AGO6 in RdDM, it also indicates that DNA methylation at some RdDM target loci does not require AGO4 and AGO6. At loci where DNA methylation is regulated by AGO4 and/or AGO6, simultaneous dysfunction of AGO4 and AGO6 drastically reduces but does not abolish DNA methylation in the CHH context (Fig 3B; Supplementary Fig S4). At many of these loci, *nrpd1-3* and *nrpe1-11* mutants display a further reduction in DNA methylation levels compared to the *ago4-6 ago6-2* double mutant (Fig 3B; Supplementary Fig S4). These results suggest that, besides AGO4 and AGO6, other AGO proteins such as AGO9 may contribute to DNA methylation at these loci.

In addition to regulating 24-nt siRNA-dependent canonical RdDM, AGO4 and AGO6 also mediate a non-canonical RdDM pathway that depends on 21–22-nt tasiRNAs (Wu *et al*, 2012), which do not require Pol IV for their production. DNA methylation at the *TAS3a* locus is tasiRNA dependent and is substantially decreased by mutation of either AGO4 or AGO6 (Wu *et al*, 2012). We consistently observed that AGO4 and AGO6 mutations both reduce DNA methylation at the *TAS3a* locus (Fig 3C). In addition to *TAS3a*, *TAS1a* and *TAS1c* are two other loci that are subjected to tasiRNA-dependent DNA methylation (Wu *et al*, 2012). We observed that AGO6 mutation strongly depletes DNA methylation at *TAS1a* and *TAS1c*,

whereas AGO4 mutation causes only a slight decrease in DNA methylation at these two loci (Fig 3C). Thus, AGO6 has a more prominent role in mediating tasiRNA-dependent non-canonical RdDM than AGO4.

The non-redundant relationship between AGO4 and AGO6 was also observed at the level of transcriptional silencing. Dysfunction of either AGO4 or AGO6 relieves transcriptional silencing at several transposon loci tested, including *ATSN1*, *AtGP1*, *IGN5*, and *LINE1-4* (Fig 3D). Derepression occurs to different degrees at some loci in *ago4-6* and *ago6-2* single mutants (Fig 3D), but is not additive in the *ago4-6 ago6-2* double mutant (Fig 3D). Therefore, AGO4 and AGO6 function non-redundantly to promote transcriptional silencing of a subset of loci.

Subnuclear spatial segregation of AGO4 and AGO6 in mediating RdDM

Co-localization of distinct RdDM components can be observed within nucleoplasmic and peri-nucleolar foci (Li *et al*, 2006, 2008; Pontes *et al*, 2006; Gao *et al*, 2010). We therefore asked whether AGO4 and AGO6 display similar subnuclear localization patterns in *Arabidopsis* seedlings. Immunostaining of AGO4 and AGO6 in the same nuclei revealed spatial segregation, as indicated by the absence of co-localized signals ($n = 105$) (Fig 4A). Production of non-coding RNAs for RdDM involves the RNA polymerases Pol IV, Pol V, and Pol II. Biogenesis of siRNAs depends predominantly on Pol IV, which does not affect scaffold RNA levels (Wierzbicki *et al*, 2008). In all cells examined ($n \geq 112$), neither AGO4 nor AGO6

Figure 3. AGO4 and AGO6 mediation of canonical and non-canonical RdDM pathways.

A Venn diagram showing the overlapping patterns among DNA hypomethylation loci identified in *ago4-6 ago6-2*, *nrpd1-3*, and *nrpe1-11*.
B DNA methylation levels in different mutants. The 2,174 loci where AGO4 and AGO6 are mutually dependent are numerically clustered (*y*-axis). Cytosines were examined as CG, CHG, and CHH.
C DNA methylation levels at *TAS1a*, *TAS1c*, and *TAS3a* loci. Snapshots from whole-genome bisulfite sequencing results are shown.
D RT-qPCR measurements of transposon RNA levels. Double: *ago4-6 ago6-2* double mutant. *Actin 2* was used as an internal control. RNA levels in the mutants are relative to those in the wild-type (Col-0). Means $\pm$ SD are shown, *n* = 3.

exhibited co-localization with NRPD1, the largest subunit of Pol IV (Fig 4B). These results are consistent with the inference that AGO proteins function downstream of Pol IV-dependent siRNA production. Pol V not only transcribes scaffold RNAs that recruit AGO-siRNA complexes, but also reinforces production of some siRNAs (Mosher *et al*, 2008). AGO6 displays partial co-localization with NRPE1, the largest subunit of Pol V, in the nucleoplasm but not in the peri-nucleolar foci (Fig 5A). Consistent with previous reports (Li *et al*, 2006; Pontes *et al*, 2006), we observed that AGO4 signals overlapped with NRPE1 signals almost exclusively in the peri-nucleolar foci (Fig 5B), where other key RdDM components such as RDR2 and DCL3 have also been observed (Li *et al*, 2006, 2008; Pontes *et al*, 2006; Gao *et al*, 2010).

In addition to Pol IV and Pol V, Pol II can produce scaffold RNAs at some intergenic low-copy-number repeat loci (Zheng *et al*, 2009) and can also initiate siRNA production at inverted repeats (Pikaard *et al*, 2008; Dunoyer *et al*, 2010). Pol II localizes in the nucleoplasm but not at perinucleolar foci (Supplementary Fig S5A; Gao *et al*, 2010). Pol II was previously shown to co-localize with AGO4 in the nucleoplasm (Gao *et al*, 2010). We also consistently observed a

strong co-localization between AGO4 and Pol II in the nucleoplasm (Supplementary Fig S5A). In contrast, Pol II does not co-localize with AGO6 (Fig 5B), which displays extensive co-localization with Pol V in the nucleoplasm but not at perinucleolar foci (Fig 5B). To test whether AGO4-Pol II co-localization is associated with Pol II's function in mRNA transcription, we examined subnuclear localization patterns of TBP, a transcription factor that binds to the TATA box in gene promoters and is thereby indicative of mRNA transcription in TBP-dependent Pol II promoters (Vannini & Cramer, 2012). AGO4 signals did not overlap with signals of TBP (Supplementary Fig S5B), but TBP-Pol II overlapped signals were observed (Supplementary Fig S5C), suggesting that, at least at those TBP-dependent promoters, the co-localization between AGO4 and Pol II is not associated with mRNA transcription. Further, the largest subunit of Pol II, NRPB1, co-immunoprecipitates with AGO4 but not AGO6, which supports a physical interaction between Pol II and AGO4 and is consistent with the immunostaining results (Supplementary Fig S5C). Together, these data reveal a subnuclear spatial segregation of AGO4 and AGO6 in mediating RdDM. Because AGO4 and AGO6 are simultaneously required for DNA methylation at most of the RdDM

Figure 4. AGO4 and AGO6 do not co-localize with each other or with Pol IV.

- A AGO4 and AGO6 were visualized by immunofluorescence using their specific antibodies. Yellow signals would be expected in the merged images if the two proteins co-localize, as a result of the overlap of red and green channels. DNA (blue) was stained with DAPI.
- B The largest subunit of Pol IV, NRPD1 (red), was visualized by its specific antibody in cells expressing FLAG-tagged AGO4 or AGO6 (green).

Data information: The perinucleolar part was marked with dashed white lines, and nucleolar dot was marked with white arrow. The number next to the images showing the total number of nuclei with the presence of both red and green fluorescence signals. The percentage shows the proportion of the nuclei with similar co-localization patterns. Qualitative images are shown here.

target loci, these results further suggest the existence of spatially separated yet cooperative RdDM steps in the nucleus.

Functional divergence between AGO4 and AGO6 in regulating siRNAs and scaffold RNAs

Pol V physically interacts with AGO proteins and synthesizes scaffold RNAs that recruit complementary siRNAs. Thus, it is critical for the recruitment of AGO-siRNA complexes. To examine the potential effects of AGO proteins on Pol V-mediated RdDM steps, we quantified scaffold RNA levels in mutants defective in AGO4 or AGO6. We evaluated RdDM loci known to produce Pol V-dependent non-coding RNAs and found that the *ago6-2* mutant displayed reduced levels of Pol V transcripts relative to wild-type plants (Fig 6A; Supplementary Fig S6). In addition, we performed chromatin immunoprecipitation for Pol V and observed reduced occupancy at these loci in *ago6-2* mutant plants compared to wild-type (Fig 6B). In contrast, AGO4 dysfunction does not reduce Pol V transcript levels at the examined loci except the *AtSN1* locus, where Pol V transcripts were moderately decreased in both *ago4-6* and *ago6-2* (Fig 6A). Meanwhile, AGO4 dysfunction does not affect Pol V occupancy in chromatin as examined at the same loci including *AtSN1* (Fig 6B). These results further support the functional divergence between AGO4 and AGO6 and the hypothesis that the two AGO proteins function non-redundantly in the RdDM pathway.

Although most siRNAs are Pol IV dependent, the production of some siRNAs is reinforced by Pol V (Zhang *et al*, 2007; Mosher *et al*, 2008). We wanted to determine whether AGO4 or AGO6 may

Figure 5. AGO4 and AGO6 exhibit different co-localization patterns with Pol V.

- A The largest subunit of Pol V, NRPE1 (red), was visualized by immunofluorescence using its specific antibody in cells expressing FLAG-tagged AGO4 (green). AGO4-NRPE1 co-localized to nucleolar dot (yellow dot, marked with white arrow, upper panel) but not in nucleoplasmic foci (lower panel).
- B NRPE1 (red) was visualized by its specific antibody in cells expressing FLAG-tagged AGO6 (green). The yellow signals due to the overlap of red and green channels in merged images indicate protein co-localization. DNA (blue) was stained with DAPI. AGO6-NRPE1 co-localized to nucleoplasmic foci (yellow dots, marked with white arrow).

Data information: The perinucleolar part was marked with dashed white lines. The number next to the images showing the total number of nuclei with the presence of both red and green fluorescence signals. The percentage shows the proportion of the nuclei with similar co-localization patterns. Representative images are shown here.

preferentially bind to siRNAs that are Pol V dependent. AGO4- and AGO6-bound siRNAs have been identified previously (Havecker *et al*, 2010). We retrieved these siRNAs and examined their levels in our *nrpd1-3* and *nrpe1-11* whole-genome, small RNA sequencing datasets. As expected, Pol IV dysfunction decreases the levels of almost all AGO4- and AGO6-bound siRNAs (Fig 7A), consistent with the inference that both AGO4 and AGO6 function downstream of Pol IV. In contrast, only about 44% of AGO4- or AGO6-bound siRNAs showed reduced levels in *nrpe1-11* (Fig 7B), indicating that neither AGO4 nor AGO6 preferentially associates with Pol V-dependent siRNAs.

In addition to guiding DNA methylation by pairing siRNAs with scaffold RNAs, AGO4 can also contribute to siRNA production through its endonuclease activity within the PIWI domain (Qi *et al*, 2006). We consistently observed decreased siRNA levels in *ago4-6* at several RdDM loci (Fig 7C), where individual 24-nt siRNAs can be quantitatively detected by RT-PCR (Zhang *et al*, 2014). The *ago6-2* mutant also showed reduced 24-nt siRNA levels at the examined loci (Fig 7C), indicating that AGO6 contributes to siRNA production as well. The *ago4-6 ago6-2* double mutant displayed an additive effect on siRNA accumulation at the *At1TE40810* locus (Fig 7C); such an additive effect was not observed at other examined

Figure 6. Differential regulation of scaffold RNAs by AGO4 and AGO6.

A AGO6 dysfunction reduces levels of Pol V-dependent transcripts. RNA levels were measured by RT-qPCR. Means $\pm$ SD are shown, $n = 3$.

B AGO6 dysfunction decreases Pol V occupancy at chromatin of loci where Pol V transcript levels are affected by AGO6. Anti-NRPE1 antibody was used for ChIP assays. Means $\pm$ SD are shown, $n = 3$.

Figure 7. The effects of AGO4 and AGO6 on siRNAs.

A Heatmap depiction of the dependence of AGO4- and AGO6-bound siRNAs on Pol IV. Identities of siRNAs were retrieved from Havecker *et al* (2010), and siRNAs were grouped into 1,210 AGO4-bound clusters and 1,486 AGO6-bound clusters (see Materials and Methods for details), each of which corresponds to a colored horizontal bar; the bars are stacked numerically into a column (y-axis). Information on siRNA levels in Pol IV mutant and wild-type plants was retrieved from Zhang *et al* (2013).

B Heatmap depiction of the dependence of AGO4- and AGO6-bound siRNAs on Pol V.

C Quantification of individual 24-nt siRNAs by TaqMan small RNA assays. Double: *ago4-6 ago6-2* double mutant. *snoR101* was used as an internal control. RNA levels in the mutants were relative to those of the wild-type (Col-0). Error bars indicate SD, $n \geq 3$.

RdDM loci (Fig 7C). Together, these observations are consistent with non-redundant roles of AGO4 and AGO6 in regulating siRNA production at the majority of RdDM target loci.

Discussion

AGO4 and AGO6 have been considered redundant in regulating RNA-directed DNA methylation. In this study, we performed genomewide quantitative analyses of DNA methylation in *Arabidopsis* mutants defective in AGO4, AGO6, or both. Our results revealed that redundancy is unexpectedly negligible (0.4%) between AGO4 and AGO6. At most of their target loci, AGO4 and AGO6 are each required to confer DNA methylation, indicating that the two AGO proteins have distinct yet cooperative functions. Consistent with these proteins functioning non-redundantly, AGO6 and AGO4 display differential effects on Pol V-dependent scaffold RNA levels and Pol V occupancy. In addition, subnuclear localization patterns of AGO4 and AGO6 provide support for spatially segregated activities in the RdDM pathway. AGO4 and AGO6 differ in their subnuclear co-localization with Pol II and Pol V. In perinucleolar foci where Pol V and AGO4 show co-localization, Pol II and AGO6 are absent. In the nucleoplasm where AGO4 co-localizes with Pol II, AGO6 preferentially co-localizes with Pol V. These patterns suggest spatially segregated RdDM activities, which appear to be mediated through different AGO–Pol combinations (Supplementary Fig S7).

AGO4 and AGO6 have different expression patterns. A GUS reporter gene under the control of the AGO4 promoter ($P_{AGO4}:GUS$) showed ubiquitous expression in the embryo and in mature leaves (Havecker et al, 2010). In the embryo, $P_{AGO6}:GUS$ expression was concentrated in the shoot and root apical meristems and the vascular tissues, whereas $P_{AGO6}:GUS$ expression was not observed in mature leaves (Havecker et al, 2010; Eun et al, 2011). Nevertheless, AGO6 dysfunction resulted in DNA hypomethylation in leaves at transgenic reporter genes (Eun et al, 2011) and at endogenous RdDM target loci (Supplementary Fig S1). AGO4 protein levels are reduced in mutants defective in Pol IV, RDR2, or DCL3, which are RdDM regulators upstream of AGO proteins (Li et al, 2006; Wierzbicki et al, 2009). AGO6 dysfunction does not decrease AGO4 protein level (Supplementary Fig S8; Havecker et al, 2010). Thus, DNA hypomethylation caused by AGO6 dysfunction is not likely mediated through the down-regulation of AGO4 in leaves. Because meristems are the hubs of AGO6 gene expression, it is possible that DNA hypomethylation in the mature leaves of *ago6* mutant is a consequence of hypomethylation in the shoot apical meristem, from which aerial portions develop. Analysis of several commonly studied RdDM loci revealed similar DNA methylation levels between *ago4-5* and *ago4-6* (Supplementary Fig S9), indicating that, at least at the examined loci, *ago4-6* is not a weaker allele compared to *ago4-5*. When comparing *ago4-5* and *ago6-2* effects on RdDM, Stroud et al (2013) examined DRM1/2-dependent loci where DNA methylation showed dependence on both AGO4 and AGO6; DNA methylation patterns were examined in the CHH context within 100-bp genomic segments. We re-analyzed *ago4-5* and *ago6-2* methylomes (Stroud et al, 2013) by using the same analysis pipeline described in this study. The re-analysis showed that DNA methylation at 1,636 genomic loci, of varying sizes up to 2,065 bp, is dependent on both AGO4 and AGO6. The majority (62%) of these loci

displayed similar cytosine (including CG, CHG, and CHH) methylation levels between *ago4-5* and *ago6-2*. Loci where AGO4 and AGO6 are mutually dependent in regulating DNA methylation are characterized by the following: (1) *ago4* and *ago6* mutants show similar reduction in DNA methylation levels, and (2) *ago4 ago6* double mutant does not show additive effects compared to the single mutants. Therefore, the results of re-analysis of Stroud et al (2013) are consistent with our observation of the interdependence between AGO4 and AGO6, although an *ago4-5 ago6-2* double mutant is unavailable for further analysis.

The function of AGO proteins in RdDM may be affected by Pol V in different ways, because Pol V controls RdDM in two ways, that is, by contributing to the transcription of scaffold RNAs and by reinforcing the production of some siRNAs. Our bioinformatics analysis revealed that AGO6-bound siRNAs are not preferentially Pol V dependent. AGO4 interacts with Pol V (Wierzbicki et al, 2009; Havecker et al, 2010) and co-localizes with Pol V at perinucleolar foci (Fig 5B; Li et al, 2006) where the siRNA biogenesis proteins RDR2 and DCL3 were also observed (Pontes et al, 2006). However, like AGO6, AGO4 does not preferentially bind Pol V-dependent siRNAs, as only 43% of AGO4-bound siRNAs were down-regulated by Pol V dysfunction. Therefore, the dependence of siRNAs on Pol V does not distinguish the roles of AGO4 and AGO6 in the RdDM pathway. Except at the AtSN1 locus, we also observed that dysfunction of AGO6, but not AGO4, partially decreases Pol V-dependent transcripts that can serve as scaffold RNAs to recruit AGO–siRNA complexes. It thus appears that AGO6 is more tightly connected with Pol V function than AGO4. Consistent with this notion, dysfunction of Pol V decreases the protein level of AGO6 but not of AGO4 (Supplementary Fig S8; Havecker et al, 2010), while AGO6 displayed a stronger physical association with Pol V than AGO4 (Havecker et al, 2010).

AGO4 and AGO6 may act sequentially to mediate siRNA-guided DNA methylation at the majority of RdDM target loci. Perhaps, when loaded with siRNAs, one of the two AGOs guides the formation of a heterochromatic histone mark, and then, the other AGO can guide DNA methylation. It is possible that AGO4–siRNA guides histone modification through association with Pol II-generated scaffold RNAs. The resulting histone mark may allow Pol V to be recruited to generate new scaffold RNAs to pair with AGO6-bound siRNAs. Through the combined actions of AGO4–siRNA and AGO6–siRNA, DRM2 may then be recruited to trigger DNA methylation. These actions would all take place in the nucleoplasm. At the perinucleolar foci where AGO4 is co-localized with Pol V, AGO4 may also directly guide DNA methylation. Future experiments to test this and other models should help us understand how siRNAs promote DNA methylation.

Materials and Methods

Plant materials and growth conditions

Arabidopsis was grown at 23°C and with 16-h light/8-h dark. T-DNA insertion lines salk_071772 (*ago4-6*) and salk_031553 (*ago6-2*) were ordered from the SALK Institute Genomic Analysis Laboratory. The native promoter-driven FLAG-tagged AGO4 and AGO6 transgenic plants were obtained from the laboratory of Dr. David Baulcombe.

Whole-genome bisulfite sequencing and analysis

DNA was extracted from 12-day-old seedlings and sent to BGI (Shenzhen, China) for bisulfite treatment, library preparation, and sequencing. Bisulfite sequencing libraries were prepared using standard Illumina protocols. The brief pipeline of BGI is as follows: (1) fragment genome DNA to 100–300 bp by sonication; (2) DNA-end repair, 3'-dA overhang and ligation of methylated sequencing adaptors; (3) bisulfite treatment by ZYMO EZ DNA Methylation-Gold kit; (4) desalting, size selection, PCR amplification and size selection again; and (5) qualified library for sequencing. The bisulfite conversion rate of Col-0 and the five mutants used in this study are col-0 99.37%, *ago4-6* 99.63%, *ago-2* 99.61%, *ago4-6 ago6-2* 99.59%, *nrpd1-3* 99.51% and *nrpe1-11* 99.32%.

For data analysis, adapter and low-quality sequences ($q < 20$) were trimmed, and clean reads were mapped to the *Arabidopsis* genome (TAIR10) using BRAT-BW (Harris *et al*, 2012) and allowing two mismatches. DNA hypomethylated regions were identified according to Ausin *et al* (2012) with minor modifications. In brief, only cytosines with 4 $\times$ coverage in all libraries were considered. A sliding-window approach with a 200-bp window sliding at 50-bp intervals was used to identify DMRs. Fisher's exact test was performed for methylated versus unmethylated cytosines for each context, within each window, with FDRs estimated using a Benjamini–Hochberg adjustment of Fisher's *P*-values calculated in the *R* environment. Windows with an FDR ≤ 0.05 were considered for further analysis, and windows within 100 bp of each other were condensed to larger regions. Regions were then adjusted to extend to differentially methylated cytosines (DMC) at each border. A cytosine was considered differentially methylated if it showed at least a twofold reduction in methylation percentage in the mutant. The regions were then filtered to include only those with at least 10 DMCs and with at least an average of a twofold reduction in methylation percentage per cytosine.

To dissect genetic interactions between AGO4 and AGO6, the 4,097 hypomethylated loci in *ago4-6 ago6-2* were categorized into four groups based on the DNA methylation (mC) levels in the wild-type, *ago4-6*, *ago6-2*, and *ago4-6 ago6-2* following these steps: (1) loci in which reduction of mC in both *ago4-6* and *ago6-2* is $< 25\%$ were categorized as Group I loci; (2) the remaining loci were then filtered for AGO4-specific loci (mC reduction in *ago4-6* $\geq 25\%$ while mC reduction in *ago6-2* $< 25\%$) and AGO6-specific loci (mC reduction in *ago6-2* $\geq 25\%$ while mC reduction in *ago4-6* $< 25\%$), which are collectively categorized into Group II; (3) then, Group III loci were defined by " mC reduction in *ago4-6 ago6-2* $\leq 125\%$ mC reduction in either *ago4-6* or *ago6-2*" and "the ratio of mC reduction in *ago4-6* to mC reduction in *ago6-2* is between 75 and 125%"; and (4) the loci remaining from step 3 were then assigned to Group IV. To compare *ago* mutants with *nrpd1a-3* and *nrpe1-11*, we re-analyzed methylome data of *nrpd1a-3* and *nrpe1-11* (Zhang *et al*, 2013). A DNA hypomethylated region was categorized as "TE" if it overlapped (≥ 1 bp) a TE. A DNA hypomethylation region was categorized as "gene" if it overlapped a gene and did not overlap any TE. An intergenic region was categorized as "IGR" if it did not fall into the groups of "TE" or "gene". For TE annotation, we used both ATxTE and ATxG numbers. If a region overlapped with ATxTE, it was classified as TE region. If a region did not overlap with ATxTE but overlapped with ATxG which was annotated as transposable

element gene, it also was classified as TE region. Regions having no overlap with ATxTE or transposable element gene (ATxG) and overlapping with protein coding genes were classified as gene region.

Individual bisulfite sequencing

Individual bisulfite sequencing was performed as described previously (Zheng *et al*, 2007). In brief, 2-week-old seedlings were collected for genomic DNA extraction using DNeasy Plant Mini Kit (Qiagen). Purified DNA was subjected to bisulfite conversion reaction using BisulFlash DNA Modification Kit (EPIGENTEK) according to manufacturer's protocol. Bisulfite-converted DNA was used as template to amplify target loci, and PCR products were cloned into pGEM Teasy vector (Promega) for DNA sequencing. For each locus, 15–20 clones were selected for sequencing. The primers were listed in Supplementary Table S2.

Immunostaining analysis

To prepare nuclei, 4 g of 2-week-old seedlings grown on $\frac{1}{2}$ MS plates was chopped, and nuclei were isolated as previously described (Pontes *et al*, 2006) with minor modification. The slides were first fixed in 4% formaldehyde/PBST buffer for 30 min at room temperature and washed three times with PBST. Slides were then blocked in 1% BSA/PBST at 37°C for 30 min. Slides were then exposed to primary antibody in blocking solution overnight at 4°C. After they were washed three times with PBST, slides were incubated with Alexa Fluor fluorescent-labeled secondary antibody. The nuclei were counterstained by ProLong Gold Antifade Reagent with DAPI (P36931, Invitrogen). For double immunostaining, slides were repeatedly blotted with primary and secondary antibodies. Images of nuclear protein localization were captured with a Nikon A1R MP confocal microscope. Images were analyzed using NIS-Elements Ar Microscope Imaging Software and processed by Adobe Photoshop (Adobe Systems). The primary antibodies used in this study included monoclonal antibodies of anti-AGO4 (1:200, Agrisera), anti-AGO6 (1:200, Agrisera), anti-H3K9me2 (1:400, Ab1220, Abcam), anti-H3K27me3 (1:400, Abcam), anti-NRPD1 (1:200, rabbit antibody, a gift from Craig S. Pikaard), and NRPE1 (1:200, rabbit antibody, a gift from Craig S. Pikaard). The following secondary antibodies were diluted at a ratio of 1:400: Alexa fluor 488 goat anti-rabbit IgG (A11008, Invitrogen), Alexa fluor 488 goat anti-mouse IgG (A11001, Invitrogen), Alexa fluor 568 goat anti-mouse IgG (H+L) (A11004, Invitrogen), and Alexa fluor 568 donkey anti-rabbit (A10042, Invitrogen).

Co-immunoprecipitation

Total proteins were extracted from 1 g of inflorescence tissues using IP buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM PMSF, and 1 $\times$ Complete Protease Inhibitor Cocktail Tablets [Roche]). Total proteins were first precleared with Protein G Dynabeads (Invitrogen) at 4°C for 1 h. Anti-FLAG monoclonal antibody (F1804, Sigma) was equilibrated and prebound to Dynabeads at room temperature according to the manufacturer's manual. The anti-FLAG-Dynabead complexes were then added to the precleared total protein supernatant, and the preparation was incubated with rotation at 4°C for 2 h. After the

precipitant was washed with low/high salt (150/500 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.2% SDS, 0.5% Triton X-100, and 2 mM EDTA) and TE buffers, SDS sample buffer was added to the precipitant and boiled for 8% SDS-PAGE electrophoresis. NRPB1 protein was detected using anti-NRPB1 antibody (ab24758, Abcam).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as described previously (Saleh *et al*, 2008). In brief, 4 g of 2-week-old seedling was harvested and cross-linked using 1% formaldehyde. Nuclei were isolated, and lysis nuclei were sonicated using Bioruptor Plus Sonicator (Diagenode). The sonicated chromatin was precleared using Protein IgG Dynabeads (Invitrogen), and antibody was added to the precleared supernatant. After overnight incubation with rotation at 4°C, the chromatin-antibody-Dynabeads were sequentially washed with low salt, high salt, LiCl, and TE buffer. The chromatin-antibody was eluted from Dynabeads using elution buffer (1% SDS and 0.1 M NaHCO₃), and DNA was recovered using standard procedures. Real-time PCR was performed using recovered DNA in the CFX96 Touch™ Real-Time PCR Detection System (185-5196, Bio-Rad). Primer sequences are listed in Supplementary Table S2.

Quantification of Pol V-dependent scaffold RNAs

Total RNA was extracted from 12-day-old seedlings with TRIzol (Invitrogen). DNase-treated RNAs were reverse-transcribed with random hexamers using SuperScript III (Invitrogen). Bio-Rad SYBGreen was used for quantitative RT-PCR. Primer sequences are listed in Supplementary Table S2.

Quantification of individual siRNAs

Small RNAs were extracted by using RNazol RT (Molecular Research Center). The abundance of 24-nt siRNAs was quantitatively detected by using Taqman Small RNA Assays (Applied Bioscience) as described previously (Zhang *et al*, 2014).

Comparison of DNA methylation levels by Chop-PCR

Genomic DNA was extracted from 12-day-old seedlings with the Dneasy Kit (Qiagen). A 500-ng quantity of genomic DNA was incubated overnight with the methylation-sensitive restriction enzyme HaeIII. The digested DNA was used to amplify the RdDM targets by semi-quantitative RT-PCR. Non-digested genomic DNA was simultaneously amplified as controls.

Data availability

The MethylC-Seq data of *ago4-6*, *ago6-2*, and double mutants have been deposited in NCBI's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE56388. The MethylC-Seq and small RNA data of WT, *nrdp1*, and *nrdp1* are downloaded from Zhang *et al*, 2013 (GEO Series accession number GSE44209). The AGO4- and AGO6-associated small RNA data are

downloaded from Havecker *et al*, 2010 (GEO Series accession number GSE16545).

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

CGD, HZ, and JKZ designed research; CGD, XZ, WQ, YJH, BW, ZL, YZ, XW, PW, JZ, GL, NL, and CW performed research; KT performed bioinformatics analysis of DNA methylation and small RNA libraries; CGD, HZ, and JKZ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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