Specific but Interdependent Functions for Arabidopsis AGO4 and AGO6 in RNA-directed DNA Methylation


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<td>Submission date:</td>
<td>05 July 2014</td>
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Andrea Leibfried

1st Editorial Decision 28 July 2014

Thank you for submitting your manuscript entitled 'Specific and Interdependent Functions for Arabidopsis AGO4 and AGO6 in RNA-directed DNA Methylation'. I have now received reports from three referees, which are enclosed below.

As you will see, all referees find your study interesting and referee #1 supports publication of a revised version of your manuscript here. However, referee 2 and 3 think that your conclusions are not sufficiently supported, and they further raise technical issues. I will not restate all points here, but essentially referee 2 thinks that an important control is missing and that the data from Stroud et al. (PMID 23313553) should be discussed and included in your bioinformatics analysis. Referee 3 points out that the key conclusions in the current manuscript need to be significantly supported by better data and further experiments, and that an additional analysis should be performed to better support the proposed stepwise mechanism of Ago4 and Ago6 action.

Given the comments provided, I would like to invite you to submit a revised version of the manuscript, addressing all concerns of the referees. The referees give very constructive comments and an analysis along the lines suggested by them would be very important for further proceedings here. Please contact me in case of further questions.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
REFEREE COMMENTS

Referee #1:

This is a very interesting paper examining the extent to which ARGONAUTE4 (AGO4) and its paralog, AGO6, act redundantly in the RNA-directed DNA methylation (RdDM) pathway in plants. RdDM requires two plant-specific, RNA polymerase II-related RNA polymerases called Pol IV and Pol V, which are responsible, respectively, for producing guide siRNAs and scaffold RNAs that base-pair with AGO-bound siRNAs and target DNA methylation.

Previous work has suggested that AGO4 and AGO6 are redundant players in the RdDM pathway, but a comprehensive analysis on a genome-wide scale has not yet been carried out to verify this supposition. The authors have now performed such a study and obtained unexpected results indicating that AGO4 and AGO6, contrary to prevailing thought, operate mostly non-redundantly in regulating DNA methylation and siRNA-production at the majority of RdDM target loci. Data are presented demonstrating that the two AGO proteins function in spatially segregated pathways that appear to be mediated by different AGO-Pol combinations.

I cannot find much to criticize about this paper. The experiments have been well planned and the data, as presented, are convincing and well-interpreted. The paper is well-written and it provides a coherent model supporting sequential AGO6 and AGO4 activity and associations with different Pols in the RdDM pathway. This paper fills an important gap in our knowledge of the roles of these two AGO proteins in RdDM and will make a significant contribution to the field.

Minor comment:
1. It is stated on p. 4 that a direct interaction between AGO4 and DRM2 has not been shown. A publication reporting that DRM2 is in a complex with AGO4 has just appeared: Zhong et al (2014) Cell 157: 1050-1060.

Referee #2:

The study by Duan and colleagues addresses the roles of ago4 and ago6 in RNA-dependent DNA methylation largely by genome wide bisulfite sequencing, bioinformatic analysis, immunohistochemistry and biochemistry.

Genome-wide bisulfite sequencing was performed in Arabidopsis ago4-6 and ago6-2 single mutants, and an ago4-6 ago6-2 double mutant. 3678, 3731 and 4097 hypomethylated loci were identified in the respective mutant backgrounds. Approximately 80% of the loci require Pol-IV for DNA methylation.

53% of the loci identified in the ago4-6 ago6-2 double mutant are hypomethylated to a similar extent in both ago4 and ago6 single mutants. This group is enriched in "Gene" loci. 42% of loci identified in the double mutant were also identified in both ago4 and ago6 single mutants, but hypomethylation was greater in the double mutant. From this, the authors conclude that AGO4 and AGO6 are mostly mutually dependent and not redundant.

Immunostaining demonstrates that AGO4 and AGO6 associate with different RNAPs depending on the nuclear context. AGO4 and Pol V co-localize in perinucleolar regions, while AGO6 and Pol II are absent. In the nucleoplasm, AGO6 associates with Pol V, while AGO4 associates with Pol II.

The data presented in this study provides an interesting insight into ago4 and ago6 function during RdDM. The idea of ago4 and ago6 acting in concert during RdDM is not a new one, but the data presented here is perhaps the strongest to date.

Major concerns
1) I am concerned that some of the "Gene" loci that have been identified as hypomethylated in all 3 mutant backgrounds may be natural epialleles and they may not require the mutation in the argonaute genes being studied. I am reminded of a study by David Baulcombe's lab (Havecker et al., 2012) where they found epialleles in an ago5 background but realized they were not actually dependent on loss of ago5, but these loci exhibit variable DNA methylation even within the Columbia accession. Also the recent population epigenomic papers (e.g. Schmitz et al, 2013) show Arabidopsis has significant epigenomic variation.

Are the "Gene" loci that are enriched in "Group III" known targets of RdDM? Do they have 24 nt small RNAs from small RNA-seq data, and what proportion of them are Pol IV and Pol V dependent for DNA methylation?

Related to this, which data were used as a wild type Columbia control? It would be best to make a control Columbia library side by side to the mutants. This is not clear and should be rectified.

2) A recent study (Stroud et al, 2013) has profiled ago4-5 and ago6-2 using genome wide bisulfite sequencing, akin to the current study. I think that the Stroud study should be cited in the current paper, and a critique of their analysis included.

The Stroud paper ranks about 20 RdDM mutants by the loss of drm1/2 dependent DNA methylation. The different mutants are placed in the classes "slightly reduced", "reduced" and "eliminated", with regards to the amount of drm1/2-dependent DNA methylation that has been lost. ago4 is in "eliminated" where as ago6 is in "reduced". How do you reconcile this observation with your conclusion that ago4 and ago6 are mutually dependent?

Is it possible that the ago4-6 used in the current study is a weaker allele than the ago4-5 used in the Stroud study?

The Stroud data is publically available so it should be reanalyzed using the same analysis pipeline to see how the new data sets compare with what is already available, and the current "gold-standard" for the Argonaute mutants.

This is important for the clarity of this study, and also the wider RdDM community.

Minor concerns

Bisulfite data

3) Please include the bisulfite conversion efficiency

4) How were TEs defined in the bioinformatics analyses? Were ATxTE numbers or ATxG numbers used? Please state. This has important consequences for the analyses because the TAIR10 ATxG list does not contain all TEs that have ATxTE numbers (as a lot with ATxTE numbers are transposon fragments). This could severely affect the number of loci that are described as "Genic", "TE" and "Intergenic".

5) How many biological repeats of the bisulfite libraries were performed? Please state.

6) It is not sufficient to say that DNA was sent to BGI and processed. Exactly how was the DNA extracted and how were the bisulfite libraries prepared? Please include a reference to the BGI protocol or outline methods more explicitly.

Immunostaining

7) Have foci been counted in the immunostaining experiments or is the data purely qualitative? No counting data is included. If the conclusions are based on qualitative observations, I would
recommend the n values next to the images are removed as that data has not been properly taken into account during the analysis.

Related to this, some of the images in the manuscript are highly pixelated, especially in Figure 5. Is this a problem with data export and import to the manuscript or is it an issue with the quality of the imaging?

Referee #3:

In this manuscript Duan et al. report that two paralogous Argonaute proteins, AGO4 and AGO6, which mediate gene silencing via RNA-directed DNA methylation (RdDM) in Arabidopsis, exhibit hitherto unrecognized, non-redundant functions in the RdDM pathway. The authors use genome-wide methylome, protein immunolocalization and cytological data to argue that AGO4 and AGO6, respectively, occupy different subnuclear domains.

Admittedly, previous studies have hinted at non-redundant roles for AGO4 and AGO6 in RdDM. The partial redundancy of these highly similar proteins was explained by tissue-specific expression patterns of AGO4 versus AGO6 proteins, and the possibility of locus-specific recruitment (Zheng et al. 2007, Havecker et al. 2010, Eun et al. 2011). Duan et al. have uncovered an additional layer to this non-redundancy, namely that AGO4 and AGO6 subnuclear localizations specify their distinct roles in RdDM. The mechanism proposed here, involving sequential action of AGO4 and AGO6, has intriguing implications for nucleoplasmic-perinucleolar trafficking of AGO-bound siRNAs and the coordinated action of two different AGO proteins in distinct steps required for chromatin-based gene silencing.

That said, the manuscript requires significant revision to improve clarity and refine the data interpretation. Firstly, the authors leave the reader guessing as to how cytological analyses were used to reach key conclusions about AGO4 and AGO6 co-localization with two distinct RNA polymerase enzymes (Major concern #1). Secondly, diagnostic loci used to show deficiency for different molecular steps in the RdDM pathway are not chosen consistently across all assay types (Major concern #2). Finally, the manuscript over-interprets cytological results involving the detection of TATA-binding protein (TBP) and its nuclear localization vis-à-vis the AGO4 and AGO6 effector proteins (Major concern #3).

Major concerns:

1. (Figures 4, 5, 6) It is hard to discern how the authors came to their conclusions about co-localization of AGO4 and AGO6 with either Pol II or Pol V, respectively, based on the images provided. How do the authors define perinucleolar versus nucleoplasmic? Outline and arrow overlays indicating subnuclear compartments in the images, as well as indications as to what are considered overlapping signals would be helpful. Is the nucleolus evident in every image that these conclusions derive from? The authors should also define what the numbers next to the cytological images mean (number of total nuclei analyzed ... or number showing a particular phenotype?). In summary, these data must be interpreted more transparently if they are to be used in support of the authors’ concluding model.

2. There is a disconnect between the RdDM target loci selected for molecular analysis (e.g., siRNA production, transcriptional derepression, and Pol V transcription) and those loci categorized into Groups I-IV based on dependency of DNA methylation on AGO4 and/or AGO6 (methylome analysis, Figures 1 & 2). For example, in Figure 7 the authors show example loci where AGO6, but not AGO4 reduce Pol V transcript levels. How these loci relate to the Groups I-IV is not stated, but based on the methylation patterns in Figure S4, it appears that they may represent more than one of the groups. Similarly, loci chosen for siRNA quantification (Fig. 8C) and transcriptional derepression (Fig. 3D) are an apparently arbitrary selection. The authors should chose representative loci from each of the Groups I-IV and assay siRNA production, transcript derepression, and Pol V transcription, all from the same locus. Assays exist to examine all of these molecular readouts at AtSN1 and IGN5 loci, for example, making this task feasible. Doing these assays in a coordinated approach...
fashion would help the authors narrow down which step(s) in the pathway AGO4 and AGO6 function in and demonstrate the mechanistic implications (if any) of the Group I-IV categorizations for their proposed model.

3. The logic behind the experiment to determine the overlap of AGO4 and AGO6 with TBP is unclear. Most mRNA genes transcribed by Pol II lack TATA boxes in their promoters. For Arabidopsis thaliana the fraction of protein-coding genes with discernible TATA boxes was estimated at 29% (see Molina and Grotewold 2005). Furthermore, the authors' conclusion that colocalization of AGO4 and Pol II is not associated with mRNA transcription appears at odds with their own finding that the majority (~70%) of CHH methylation affected by AGO4 and AGO6 occurs over genic regions. Finally, without knowledge of how Pol II (or Pol V) overlaps with TBP in these cytological assays, it is hard to see how this experiment can be informative.

Minor issues:

1. In the intro, the authors state "Although a direct interaction between AGO4 and DNA methyltransferase DRM2 has not been shown..." This statement is rather misleading, because an in vivo interaction between AGO4 and DRM2 proteins, perhaps as part of a larger complex, has been reported in Zhong et al. 2014 Cell 157(5):1050-60. The authors should incorporate the findings of this study into their intro, relevant sections of the discussion, as well as the model figure.

2. In the background information where known functions of Arabidopsis Argonaute proteins are described, it would also be worth noting that 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 (see Wei et al. 2012 Cell 149(1):101-12).

3. On Page 6, "Similarly, AGO4 dysfunction causes DNA hypomethylation at loci where DNA methylation is AGO4-dependent..." Should the second AGO4 be AGO6?

4. Methods section Co-immunoprecipitation, p. 15 "NRPE1 protein was detected using anti NRPB1-antibody..." Should NRPE1 be NRPB1?

5. In Figure 2A, a brief description of what each group represents in the legend would be helpful.

6. In Figure S1 B, what is shown in the Venn diagrams?

Point-to-point response to reviewers:

Question 1 of Reviewer #1:

"It is stated on p. 4 that a direct interaction between AGO4 and DRM2 has not been shown. A publication reporting that DRM2 is in a complex with AGO4 has just appeared: Zhong et al (2014) Cell 157: 1050-1060."

As suggested by reviewer, we have updated this information in the revised manuscript (see page 4 in revised manuscript).

Question 1 of Reviewer #2:

"I am concerned that some of the "Gene" loci that have been identified as hypomethylated in all 3 mutant backgrounds may be natural epialleles and they may not require the mutation in the argonaute genes being studied. I am reminded of a study by David Baulcombe’s lab (Havecker et al., 2012) where they found epialleles in an ago5 background but realized they were not actually dependent on loss of ago5, but these loci exhibit variable DNA methylation even within the Columbia accession. Also the recent population epigenomic papers (e.g. Schmitz et al, 2013) show Arabidopsis has significant epigenomic variation.

Are the "Gene" loci that are enriched in "Group III" known targets of RdDM? Do they have 24 nt small RNAs from small RNA-seq data, and what proportion of them are Pol IV and Pol V dependent for DNA methylation?"
Related to this, which data were used as a wild type Columbia control? It would be best to make a control Columbia library side by side to the mutants. This is not clear and should be rectified.”

To make sure the “Gene” loci in Group III are real RdDM target, we compared these loci to the pol IV and pol V target loci. We found 945 (94.3%) of total 1002 loci overlaps with pol IV or pol V and 849 (84.7%) overlaps with both pol IV and pol V (see Fig. S2C and page 7 paragraph 3). These results suggest the majority of Gene loci in Group III are RdDM targets but not epigenomic variation.

To find out whether the “Gene” loci that are enriched in Group III have 24nt siRNAs, we analyzed two published Col-0 small RNA library (Zhang et al., 2014; Johnson et al., 2014) as wild type control. In parallel, we also examined “Gene” loci that are targeted by Pol IV and Pol V. We found that the 24-nt small RNAs in one Col-0 small RNA library (Zhang et al., 2014) mapped to 12%, 18.4% and 19.3% “Gene” loci of ago4-6 double mutant Group III, nrd1-3 and nrdp1-11, respectively. In another Col-0 small RNA library (Johnson et al., 2014), the percentage of 24 nt small RNA in the “Gene” loci of Group III, pol IV and pol V are 9%, 15% and 16%, respectively. This result indicates the small RNAs enrichment in ago4-6 double mutant Group III gene loci is comparable to that of Pol IV and Pol V mutants, further supporting these Gene loci are regulated by RdDM pathway.

In this study, we used Col-0 methylome data published by our lab (GSE44209, this information is available in Materials and Methods). Experimental conditions are the same as described for GSE44209. We agree with reviewer that sequencing the Col-0 control side by side to the mutants will be better for this study. But unfortunately whole genome bisulfite sequencing is costly and thus we were not able to do side by side wild type control each time. So we validated the methylome results by individual bisulfite sequencing of a group of randomly chosen hypomethylation loci (Fig. S1B). In addition, we also used another published wild type control (Stroud et al., 2013 Cell) to re-analyze methylome of the ago4-6 ago6-2 double mutant. We got 3351 hypo-DMR loci. Among them, Group I has 12 loci, Group II has 177 loci (61 are AGO4 specific and 116 are AGO6 specific), Group III has 2032 loci and Group IV has 1130 loci. This pattern is highly similar to what we obtained based on the Col-0 control we used in this study, indicating that our data analysis is solid.

Reference:

Question 2 of Reviewer #2:
"A recent study (Stroud et al, 2013) has profiled ago4-5 and ago6-2 using genome wide bisulfite sequencing, akin to the current study. I think that the Stroud study should be cited in the current paper, and a critique of their analysis included.
The Stroud paper ranks about 20 RdDM mutants by the loss of drm1/2 dependent DNA methylation. The different mutants are placed in the classes "slightly reduced", "reduced" and "eliminated", with regards to the amount of drm1/2-dependent DNA methylation that has been lost. ago4 is in "eliminated" where as ago6 is in "reduced". How do you reconcile this observation with your conclusion that ago4 and ago6 are mutually dependent?
Is it possible that the ago4-6 used in the current study is a weaker allele than the ago4-5 used in the Stroud study?
The Stroud data is publically available so it should be reanalyzed using the same analysis pipeline to see how the new data sets compare with what is already available, and the current "gold-standard" for the Argonaute mutants.
This is important for the clarity of this study, and also the wider RdDM community."

When comparing ago4-5 and ago6-2 effects on RdDM pathway, 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 100bp genomic segments. As suggested by the reviewer, we re-analyzed their ago4-5 and ago6-2 methylome (Stroud et al., 2013) by using the same analysis pipeline reported in this study. The re-analysis results showed that DNA
methylation at 1636 genomic loci, are dependent on both AGO4 and AGO6. The majority (62%) of these loci displayed similar cytosine (including CG, CHG, and CHH) methylation levels between \textit{ago4-5} and \textit{ago6-2}. Loci where AGO4 and AGO6 are mutually dependent in regulating DNA methylation are characterized by 1) \textit{ago4} and \textit{ago6} mutants show similar reduction in DNA methylation levels, and 2) \textit{ago4 ago6} double mutant does not show additive effects comparing to the single mutants. Therefore, the re-analysis results of Stroud et al. (2013) are consistent with our observation of the interdependence between AGO4 and AGO6, although an \textit{ago4-5 ago6-2} double mutant is unavailable for a complete re-analysis.

Analysis of several commonly studied RdDM loci revealed similar DNA methylation levels between \textit{ago4-5} and \textit{ago4-6} (see Fig. S9), indicating that, at least at the examined loci, \textit{ago4-6} is not a weaker allele compared to \textit{ago4-5}. We described the reanalysis results and our comments in the revised “Discussion” part (see page 12 and 13).

**Question 3 of Reviewer #2:**

“Please include the bisulfite conversion efficiency”

The bisulfite conversion rate of Col-0 and the five mutants used in this study are

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In the revised manuscript we have added the information into the materials and methods part (page 14 paragraph 3).

**Question 4 of Reviewer #2:**

“How were TEs defined in the bioinformatics analyses? Were ATxTE numbers or ATxG numbers used? Please state. This has important consequences for the analyses because the TAIR10 ATxG list does not contain all TEs that have ATxTE numbers (as a lot with ATxTE numbers are transposon fragments). This could severely affect the number of loci that are described as “Genic”, “TE” and “Intergenic”. “

We used both ATxTE and ATxG numbers for TE annotation. 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.

We stated this in the material and methods of the revised manuscript (page 15 paragraph 2).

**Question 5 of Reviewer #2:**

“How many biological repeats of the bisulfite libraries were performed? Please state.”

In this study we did not have methylome data for biological repeats. We note that other published data also do not have methylome data for biological repeats (e.g., Stroud et al., 2013 Cell; Julie et al., 2013 Nature). To validate whole-genome bisulfite sequencing results, we performed bisulfite sequencing at individual loci before doing genetic analysis. Results from individual bisulfite sequencing are consistent with whole-genome bisulfite sequencing (Fig. S1B). In addition, we reanalyzed \textit{ago4-6 ago6-2} double mutant by using another wild type control that was published by another lab (Stroud et al., 2013), and the results are similar to what we had presented in the first submission.

**Question 6 of Reviewer #2:**

“It is not sufficient to say that DNA was sent to BGI and processed. Exactly how was the DNA extracted and how were the bisulfite libraries prepared? Please include a reference to the BGI protocol or outline methods more explicitly.”
The information has been added into the Material and Methods (see page 15). Genomic DNA was extracted from 14-day-old seedlings using the Plant DNeasy Maxi Kit from Qiagen 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 100-300bp by Sonication.
2. DNA-end repair, 3'-da overhang and ligation of methylated sequencing adaptors.
4. Desalting, size selection, PCR amplification and size selection again.
5. Qualified library for sequencing.

Question 7 of Reviewer #2:
“Have foci been counted in the immunostaining experiments or is the data purely qualitative? No counting data is included. If the conclusions are based on qualitative observations, I would recommend the n values next to the images are removed as that data has not been properly taken into account during the analysis. Related to this, some of the images in the manuscript are highly pixelated, especially in Figure 5. Is this a problem with data export and import to the manuscript or is it an issue with the quality of the imaging?”

The numbers next to the images indicate the total number of nuclei analyzed. The proportion of nuclei showing the presented phenotype to total nuclei was shown in parentheses. This is now clarified in figure legends after revision. The low resolution of Figure 5B was because the image was captured in a low resolution microscope. We replaced the image with a high resolution one. Two representative nuclei are shown in the figures.

Question 1 of Reviewer #3:
“(Figures 4, 5, 6) It is hard to discern how the authors came to their conclusions about co-localization of AGO4 and AGO6 with either Pol II or Pol V, respectively, based on the images provided. How do the authors define perinucleolar versus nucleoplasmic? Outline and arrow overlays indicating subnuclear compartments in the images, as well as indications as to what are considered overlapping signals would be helpful. Is the nucleolus evident in every image that these conclusions derive from? The authors should also define what the numbers next to the cytological images mean (number of total nuclei analyzed … or number showing a particular phenotype?). In summary, these data must be interpreted more transparently if they are to be used in support of the authors’ concluding model.”

Nuclei were stained with DAPI and nucleoli can be identified by an absence (or low intensity) of DAPI staining. A fluorescence signal focus was defined as “perinucleolar” if it overlaps or is clearly adjacent to a nucleolus. Other foci were defined as “nucleoplasmic” due to their presence in the nucleoplasm. Each pair of studied proteins was localized by the two pseudocolors red and green. The yellow signals due to the overlap of red and green channels in merged images indicate protein co-localization.

As suggested, we have revised the figures by using dotted outlines that indicate nucleoli as well as arrows that indicate examples of co-localization foci. Only images in which nucleoli were evident were used for analysis. The numbers next to the images indicate the total number of nuclei analyzed. The proportion of nuclei showing the presented phenotype to total nuclei was shown in parentheses. This is now clarified in figure legends after revision.

Question 2 of Reviewer #3:
“There is a disconnect between the RdDM target loci selected for molecular analysis (e.g., siRNA production, transcriptional derepression, and Pol V transcription) and those loci categorized into Groups I-IV based on dependency of DNA methylation on AGO4 and/or AGO6 (methyome analysis, Figures 1 & 2). For example, in Figure 7 the authors show example loci where AGO6, but not AGO4 reduce Pol V transcript levels. How these loci relate to the Groups I-IV is not stated, but based on the methylation patterns in Figure S4, it appears that they may represent more than one of the groups. Similarly, loci chosen for siRNA quantification (Fig. 8C) and transcriptional derepression (Fig. 3D) are an apparently arbitrary selection. The authors should chose representative loci from each of the Groups I-IV and assay siRNA production, transcript derepression, and Pol V transcription, all from the same locus. Assays exist to examine all of these
molecular readouts at AtSN1 and IGN5 loci, for example, making this task feasible. Doing these assays in a coordinated fashion would help the authors narrow down which step(s) in the pathway AGO4 and AGO6 function in and demonstrate the mechanistic implications (if any) of the Group I-IV categorizations for their proposed model.”

We agree with the reviewer that, if information of non-coding RNAs and transcript derepression can be obtained all from the same locus for each group of AGO4/6 target loci, these molecular analyses may provide more mechanistic insights into how AGO4 and AGO6 function in RdDM. However, our attempt in doing this had failed due to technical difficulties mostly in detecting new scaffold RNAs and/or 24nt siRNAs. Alternatively, we examined a group of RdDM loci where 24nt siRNAs or scaffold RNAs have previously been detected. While ago4-6 and ago6-2 mutation have similar effects on releasing transcriptional silencing and reducing 24nt siRNA levels, there are patterns that seem to distinguish AGO6 from AGO4, i.e., AGO6, but not AGO4, is strongly correlated with Pol V-dependent scaffold RNAs levels (Fig. 6A) and with Pol V occupancy in chromatin (Fig. 6B).

As suggested by the reviewer, during the revision, we fully examined AtSN1 and IGN5 (see Fig. 3D, 6, 7C and S6). The results show that, at AtSN1 locus, scaffold RNA levels are moderately decreased in both ago4-6 and ago6-2 (Fig. 6A), while Pol V occupancy in chromatin is decreased only in ago6-2 (Fig. 6B). The new results also show that, at IGN5 locus, scaffold RNA levels and Pol V occupancy are reduced in ago6-2 but not ago4-6 (Fig. 6A). Thus the new results are consistent with our other observations that AGO6, but not AGO4, is strongly correlated with Pol V-dependent scaffold RNAs levels and with Pol V occupancy in chromatin. We stated this data in “Result” section in the revision (see page 10). Although these results currently cannot provide mechanistic implications of the Group I-IV categorizations because the examined loci do not correspond to a specific DMR group, the strong correlation between AGO6 and Pol V is supported by cytological analysis (Fig. 5) and thus provides a clue for further understanding the roles of AGO4 and AGO6 in RdDM.

question 3 of reviewer #3:

“The logic behind the experiment to determine the overlap of AGO4 and AGO6 with TBP is unclear. Most mRNA genes transcribed by Pol II lack TATA boxes in their promoters. For Arabidopsis thaliana the fraction of protein-coding genes with discernible TATA boxes was estimated at 29% (see Molina and Grotewold 2005). Furthermore, the authors’ conclusion that co-localization of AGO4 and Pol II is not associated with mRNA transcription appears at odds with their own finding that that the majority (~70%) of CHH methylation affected by AGO4 and AGO6 occurs over genic regions. Finally, without knowledge of how Pol II (or Pol V) overlaps with TBP in these cytological assays, it is hard to see how this experiment can be informative.”

TBP is generally considered as an indicator of Pol II-dependent mRNA transcription (Chen et al., 2004; Ding et al., 2011; Kamenova et al., 2014), although clearly not all mRNA transcription involves TBP. Nonetheless, we have revised the statement “TBP, a transcription factor that specifically binds to the TATA box in gene promoters and is thereby indicative of mRNA transcription (Vannini & Cramer, 2012)” to “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)” (see page 10).

As to the question “the authors’ conclusion that co-localization of AGO4 and Pol II is not associated with mRNA transcription appears at odds with their own finding that that the majority (~70%) of CHH methylation affected by AGO4 and AGO6 occurs over genic regions”, we have carefully checked the manuscript and did not see a conclusion of “the majority (~70%) of CHH methylation affected by AGO4 and AGO6 occurs over genic regions”. Instead, on Page 7 Paragraph 3, we had stated “Among the 4097 hypomethylation loci identified in the ago4-6 ago6-2 double mutant, 33% (1347) are Gene loci, 44% are TE loci, and 23% are IGR loci. Notably, 76% of the hypomethylated Gene loci exhibited the pattern that is characteristic of Group III loci”. Is it possible that the reviewer misunderstood this statement? Nonetheless, at the Gene loci targeted by RdDM, Pol II transcription of the genes and Pol II transcription of the RdDM target regions of the gene loci are separate events.

As suggested by the reviewer, we performed immunostaining assay to check the co-localization pattern between TBP and pol II in the nucleus. We did observe co-localization foci of TBP and Pol II in the nucleoplasm (revised Fig S5C and page 10 paragraph 1). This observation, in contrast to the
absence of co-localization between AGO4 and TBP, supports the notion that Pol II’s co-localization with AGO4 and with TBP are two separate events.

Reference
Ding Y, Avramova Z, Fromm M (2011) Two distinct roles of ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) at promoters and within transcribed regions of ATX1-regulated genes. The Plant cell 23: 350-363

Question 4 of Reviewer #3:
"In the intro, the authors state "Although a direct interaction between AGO4 and DNA methyltransferase DRM2 has not been shown..." This statement is rather misleading, because an in vivo interaction between AGO4 and DRM2 proteins, perhaps as part of a larger complex, has been reported in Zhong et al. 2014 Cell 157(5):1050-60. The authors should incorporate the findings of this study into their intro, relevant sections of the discussion, as well as the model figure."

We rewrote this part in the revised manuscript and cited the paper (see page 4).

Question 5 of Reviewer #3:
"In the background information where known functions of Arabidopsis Argonaute proteins are described, it would also be worth noting that 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 (see Wei et al. 2012 Cell 149(1):101-12)."

As suggested by the reviewer, we revised the statement “AGO2 also binds viral siRNAs and is involved in antiviral defense response(Harvey et al, 2011; Jaubert et al, 2011)” to “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).” (see page 3)

Question 6 of Reviewer #3:
"On Page 6, “Similarly, AGO4 dysfunction causes DNA hypomethylation at loci where DNA methylation is AGO4-dependent...” Should the second AGO4 be AGO6?"

Yes, the second AGO4 should be AGO6. We corrected it in the revised manuscript.

Question 7 of Reviewer #3:
"Methods section Co-immunoprecipitation, p. 15 “NRPE1 protein was detected using anti NRPB1-antibody...” Should NRPE1 be NRPB1?"

Yes, it should be NRPB1. We corrected it in the revised manuscript.

Question 8 of Reviewer #3:
“In Figure 2A, a brief description of what each group represents in the legend would be helpful.”

We have added a description to the figure legends.
“Group I, loci where DNA hypomethylation is observed in the double mutant but not in the single mutants; Group II, 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; Group III, loci where DNA methylation is similarly reduced in the two single mutants and the double mutant shows no additive effects on DNA hypomethylation; Group IV, loci where DNA hypomethylation is observed in the single mutants while the double mutant shows more severe DNA hypomethylation.”

Question 9 of Reviewer #3:
“In Figure S1 B, what is shown in the Venn diagrams?”
Figure S1B shows the hypo-DMR overlap numbers between ago4, ago6 single mutants and nrpd1-3, nrpe1-11 mutants. We stated it in the revised Fig. S2A.

Thank you for submitting the revised version of your manuscript for consideration by the EMBO Journal. It has now been seen by two of the original referees whose comments are enclosed. As you will see, both referees now support publication, pending minor textural changes. Referee #2 thinks that the discussion should either be less speculative or better distinguish between speculation and conclusions based on actual data. Given this input, I would like to invite you to provide a final version of your manuscript.

I am thus formally returning the manuscript to you for a final round of minor revision, to allow you to easily modify/replace the files. Once we have received them, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

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REFEREE COMMENTS
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Referee #2:

With regards to the bisulfite sequencing data, the authors have addressed all of the issues that we raised. The authors have included much clearer information regarding library construction, sequencing and the bisulfite sequencing analysis. As suggested, the authors have also included analysis of bisulfite data from ago4 and ago6 mutants published by Stroud et al. This re-analysis of published data using the author's own pipeline significantly strengthens the conclusion made by the authors that ago4 and ago6 are mutually dependent. The authors have also clearly demonstrated that the gene loci are not natural epialleles and are RdDM targets.

The authors have clarified the numbers accompanying the Immunofluoresence data and replaced the pixelated image.

The authors state in the discussion:

"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."

This seems like a suitable hypothesis for why ago6 mutants exhibit widespread loss of RdDM in leaf tissue. Rather than pursue this idea, the authors prefer to speculate that:

"Our results suggest that 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."

From the bisulfite data, the authors have clearly demonstrated that ago4 and ago6 do not act redundantly in RdDM. However, the evidence presented in this paper to suggest that they are both
required within a single cell, acting on the same DNA strand, is weak. This concluding statement is purely speculative and cannot really be backed up by the empirical results in this paper. If the authors are keen to present this speculative model, we would suggest the removal of the phrase "our results suggest".

Referee #3:

In this revised manuscript, Duan et al. report that the paralogous silencing factors AGO4 and AGO6, occupy different subnuclear domains and thereby collaborate to mediate RNA-directed DNA methylation in Arabidopsis. The authors have improved the clarity of their original manuscript and performed several additional experiments requested by referees. In response to my primary critiques, Duan et al. have now (1) explained the methodology used for their cytological analyses, (2) completed additional assays at diagnostic loci and (3) provided evidence for TBP and Pol II co-localization as a control for the absence of TBP and AGO4 co-localization. The sequential action of AGO4 and AGO6 proposed in this manuscript, now reinforced by additional evidence, has intriguing mechanistic implications for nuclear-perinucleolar trafficking of siRNAs and the coordinated action of AGO proteins in distinct steps of chromatin-based gene silencing. The author's work will be of interest to scientists in the fields of small RNA and epigenetic gene regulation.

2nd Revision - authors' response 25 November 2014

Thank you for your November 24, 2014 decision letter. We have made some slight modifications to the figures and uploaded them.

Thank you for your consideration of our work.