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Arabidopsis ARGONAUTE7 selects miR390 through multiple checkpoints during RISC assembly

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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.)

1st Editorial Decision 03 April 2013

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees agree that the findings are interesting and that the data are clear. However, referee 1 points out several controls and quantifications that should be included, and both referees 2 and 3 indicate that it should be investigated whether other plant miRNA duplexes have 5'A, the central 3 nt and the mismatch, in order to confirm the specificity for Ago7.

Given these positive and constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure.

We also recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

In the manuscript "Arabidopsis ARGONAUTE7 selects miR390 through multiple checkpoints during RISC assembly" Endo et al. report the molecular basis for how the plant Argonaute protein ARGONAUTE7 selects among all miRNAs specifically miR-390 to trigger the production of TAS3 trans-acting siRNAs.
The authors employ an in vitro RISC loading system based on translationally active lysate from evacuolated protoplasts and systematically dissect the structural and nucleotide requirements for miR-390 loading into AGO7.
While the manuscript is a conclusive extension of previous work performed in the Tomari lab and addresses yet another puzzling and important question in how specificity is achieved in RISC loading pathways, some of the experiments require important controls to support the main conclusions. In case the authors can address the concerns (as described in detail below), I recommend publication of the manuscript in EMBO Reports.

Major points:

- In vitro translation was used to generate FLAG-tagged versions of AGO7 and AGO1, which were subsequently used to test in vitro assembly of specific miRNAs. While the in vitro assembly faithfully recapitulates the specificity of AGO7 for miR-390 and AGO1 for typical miRNAs (exemplified by miR-166 and miR-171), the authors never show a Western blot analysis to confirm that the AGO proteins are expressed in their full length.
- It is unclear how the authors quantify the loading and maturation efficiency when comparing different miR-390 variants. Several controls are missing before one can assume a truly quantitative assessment of assembly when comparing different duplexes: First of all, it is not clearly stated in Figure legends or Materials & Methods if FLAG-AGO7 IP was used in the experiments shown in Figure 2, 3, and S2 (Please clarify in Figure legends). If, as I assume, this is the case, then Western blot analysis should be shown as controls to confirm equal amounts of FLAG-AGO7 were immunoprecipitated at the given time points. Second, it is unclear how the authors account for different small RNA labeling efficiencies (e.g. small RNAs starting with C are expected to be labeled less efficiently when using T4-PNK). In none of the experiments do the authors provide input controls. A straightforward way of controlling for labeling efficiencies would be using scintillation counter measurements of all aliquots before IP to normalize for input material. Finally,

data provided in supplemental Figures (Fig. S2 B and D) should be quantified (similar to Fig. 2 C and D) to confirm the statements of AGO7 assembly and maturation efficiencies claimed in the main text.

Minor points:
- Page 8 last paragraph " ... by swapping the central 3-nt region (Fig 3B-D, lane 4)..." should refer to lane 5 not 4.

Referee #2:

The manuscript by Endo et al. provides biochemical evidence that the 5' A and 3-nt central region of miR390/miR390* confer the specificity of AGO7 binding to miR390. The data are clear and well-presented and should be of interest to the RNAi field. The following suggestions should be considered when the authors revise the manuscript.
1) The author should check whether similar 3-nt central region is present in other plant miR/miR*s. This will strengthen the paper's main conclusion that the binding specificity of AGO7 is determined by the 3-nt central region.
2) The fact that Chimera3 but not 2 can be loaded into AGO7 infers that the primary sequence of the 3-nt central region may be important. The authors should generate a series of mutants to test this possibility.
3) miR390 can also be loaded into other AGOs (AGO2 and AGO4) that prefer 5'A, it would be interesting whether the 3-nt central region also promotes the loading of miR390 into these AGOs.

Referee #3:

This manuscript reports on in vitro Argonaute-loading assays for plant AGO7, which is specific for the miR390 miRNA. The authors identify two features of the miR390/miR390* duplex that allow AGO7 to selectively bind just this single miRNA: A 5'-A residue, and a critical G-A central mismatch in the miR390/miR390* duplex. The manuscript is well written, the data are clear, and the conclusions that are drawn are appopriate from the data presented. Overall it is a solid paper of interest to the field. I have a few minor comments for the authors to consider:

1. Are there any other plant miRNA/miRNA* duplexes with both 5'-A and the critical miR390-like central G-A mismatch? If so, why aren't these others loaded onto AGO7 (or are they)?

2. Figure 3 shows that the g7-9 flip actually increases the loading efficiency. Can the authors speculate on why this might be?

1st Revision - authors' response 07 May 2013

Point-by-point response to the Referee's comments

Referee #1:

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Major points:

In vitro translation was used to generate FLAG-tagged versions of AGO7 and AGO1, which were subsequently used to test in vitro assembly of specific miRNAs. While the in vitro assembly faithfully recapitulates the specificity of AGO7 for miR-390 and AGO1 for typical miRNAs (exemplified by miR-166 and miR-171), the authors never show a Western blot analysis to confirm that the AGO proteins are expressed in their full length.
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We appreciate Referee's comment. As the Referee pointed out, FLAG-AGO7-IP was used in Figure 2, 3, and S2. We have now clarified this in Figure Legends, and representative Western blot analyses of immunoprecipitated AGO proteins are now shown in Figure 1C, D and 2B. For all the experiments, we quantified radiolabeled miRNA duplexes by using phosphorimager, and normalized the concentrations by appropriate dilution before they are used for in vitro RISC assembly assays. This is now clearly stated in Materials and Methods, and representative input controls are now shown in Figure 1C and 2B.

Finally, data provided in supplemental Figures (Fig. S2B and D) should be quantified (similar to Fig. 2C and D) to confirm the statements of AGO7 assembly and maturation efficiencies claimed in the main text.

The data originally in Fig. S2B and D are now quantified and the graphs are presented.

Minor points:
Page 8 last paragraph " ... by swapping the central 3-nt region (Fig 3B-D, lane 4)..." should refer to lane 5 not 4.

We thank the Referee for pointing this out. This has now been amended.

Referee #2:

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1) The author should check whether similar 3-nt central region is present in other plant miR/miR*s. This will strengthen the paper's main conclusion that the binding specificity of AGO7 is determined by the 3-nt central region.

Among 299 *Arabidopsis* miRNA precursors registered in miRBase, only miR390/miR390* duplex bears both 5′ A and the G-A mismatch at position 11. This is now clearly stated in the text.

2) The fact that Chimera3 but not 2 can be loaded into AGO7 infers that the primary sequence of the 3-nt central region may be important. The authors should generate a series of mutants to test this possibility.

We greatly appreciate the Referee's comment. Our preliminary results suggest that a single base-pair mutation adjacent to the central G-A mismatch in miR390/miR390* duplex impairs AGO7-RISC assembly, supporting the idea that the primary sequence of the 3-nt central region is indeed important. Our results from the 3-nt "flip" mutant series are also consistent with this idea. Notably, however, it is well known that the types of nearest neighbor base pairs drastically affect the three-dimensional conformation of a given single mismatch (Davis *et al*. NAR. 2011; e.g., UAC AGG shows an A-G mismatch in the 5′(A)H/3′(G)S pairing, antiparallel, trans orientation with XI hydrogen bonding, while CAC CGG shows an A-G mismatch in the 5′(A)W/3′(G)W pairing, antiparallel, cis orientation with VIII hydrogen bonding). Thus, we reason that it will be extremely challenging to determine whether the primary sequence per se or the three-dimensional conformation of the 3-nt central region is critical for AGO7-RISC assembly, and only future structural studies should be able to address this issue.

3) miR390 can also be loaded into other AGOs (AGO2 and AGO4) that prefer 5'A, it would be interesting whether the 3-nt central region also promotes the loading of miR390 into these AGOs.

We appreciate the Referee's suggestion. We have now experimentally tested if the 3-nt central region of miR390/miR390* duplex affects loading into AGO2. As shown in Supplementary Fig S1, AGO2 efficiently incorporated the miR390 mutant with the flipped central 3 nt, as well as the wild-type miR390. Thus, unlike AGO7, AGO2 does not inspect the central region for RISC assembly. This is consistent with the previous finding that AGO2 incorporates a wide variety of 5′ A small RNAs including viral siRNAs (Mi et al. Cell, 2008, Mongomery et al. Cell, 2008, Takeda et al. Plant Cell Physiology, 2008).

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2. Figure 3 shows that the g7-9 flip actually increases the loading efficiency. Can the authors speculate on why this might be?

Unfortunately, we don't have a good answer for why the g7-9 flip increases the loading efficiency. At least, no equivalent sequence as the g7-9 flip can be found in the known *Arabidopsis* miRNAs, and miR390 seems to be the best natural substrate for AGO7.

2nd Editorial Decision 14 May 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal. However, as you will see below, referee 3 recommends that you include the full gels for the Western Blot data in the paper. We offer the publication of source data for all figures, and I think this would be a good opportunity here. Can you please send us the full blots for the relevant figures and we will upload them as source data and link them to their main figures. Thank you very much.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Finally, we provide a short summary of published papers on our website to emphasize the major findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFEREE REPORTS:

Referee #1

The authors have addressed all the points raised in the initial review. I recommend publication of the manuscript as it is.
I do however recommend the authors to show for representative examples the full western blot data (in addition to the provided cropped data in the main figures) to avoid potential criticism that major truncations of in vitro translated Argonaute proteins could potentially affect in vitro loading assays.

Referee #3

In this revision, the authors have adequately addressed my small list of concerns.

