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Cyclophilin 40 Facilitates HSP90-mediated RISC Assembly in Plants

Taichiro Iki, Manabu Yoshikawa, Tetsuo Meshi and Masayuki Ishikawa

Corresponding author: Masayuki Ishikawa, National Institute of Agrobiological Sciences

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(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 12 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise that it has taken longer than usual to have your manuscript reviewed but this is in part due to the current high seasonal submission rate. I have now received the report from the final referee and I enclose their reports below. As you will see from their comments the referees are all positive regarding the description of CYP40 and PP5 in miRISC assembly. However there are a couple of experimental issues that need to be addressed and these surround the origin of the studied proteins and the CYP40 domain analysis in RISC assembly. Should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REVIEWS

Referee #1

In this manuscript, Iki et al. report that TPR domain-containing proteins, especially CYP40 and PP5, associate with AGO1 and modulate binding of small RNA duplexes to AGO1, using a unique in vitro system based on evacuolated tobacco protoplasts. CYP40 has been genetically implicated in RNA silencing, and this study nicely points out a biochemical function of CYP40 in RISC assembly. Overall, the experiments are well performed and the results support the authors' conclusions.

Major points:
1. The authors argue that, among TPR-containing proteins, CYP40 and PP5 have specific positive and negative functions, respectively, in RISC assembly. One major drawback of this study is that AGO1 and molecular chaperons are from tobacco, whereas TPR-containing proteins are from Arabidopsis. Consequently, the observed positive/negative/neutral functions of the TPR-containing proteins in RISC assembly may as well be due to their coincidental interspecific (in)compatibilities, rather than their functional specificities. I recommend the authors to clone TPR-containing proteins from tobacco and confirm their conclusions, at least for some key experiments.

2. The data for CYP40 domain analysis (Fig. 7) is ambiguous and unconvincing, because the difference among WT and the mutants are only marginal. I understand that this experiment is technically challenging, because of the high background (presumably in part due to endogenous CYP40 activity). Is it possible to perform this experiment using lysate from the cells where endogenous CYP40 is knocked down? Alternatively, given that PP5 (and/or CsA) inhibits RISC assembly, the authors could quantitatively evaluate the potency of the CYP40 mutants to compete with and counteract PP5 (and/or CsA). Or else, in the worst-case scenario, omitting this entire Figure and related discussion should not drastically weaken this study.

Minor points:
1. It appears premature to conclude that CYP40 and other TPR-containing proteins do not affect removal of passenger strands, unless the data in Fig. 3C is presented in a more quantitative (and preferably more kinetic) manner. Note that the effects of CYP40 and PP5 in Figs. 3A and B, which the authors rely on to conclude that these proteins do affect duplex loading, are also rather mild.

2. Related to Major point #2: It would be important to confirm that CYP40 can offset the inhibitory effect of CsA (and FK506) in RISC assembly, because specificities of these drugs in this experimental system are unknown.

Referee #2

Review Iki et al.

While intense genetic work has been carried out in Arabidopsis regarding the mode of action of miRNA and their main effector, AGO1, very little is known of the composition and modulation of the AGO1-miRISC, yet this information crucial to fully grasp miRNA biology in plants. This situation is largely due to the current paucity of cell free systems that have expedited this question in fly and human cells. Iki and colleagues have developed such a system in BY2 protoplasts and have already provided important information, in their 2010 paper, on the role of HSP70 and HSP90 in facilitating AGO1-RISC assembly. They now expand their initial discovery by providing a set of elegant and unambiguous data implicating Cyclophylin 40 in this process. This, incidentally, sheds light on the previous identification of SQUINT (a Cyp40 homolog) by the Poethig group, using a forward genetics approach.

I have, in fact, very little comments to make on the manuscript, as the biochemistry is impeccable and the model proposed by the authors well supported by their experiments. One could regret a complete lack of reverse genetics in Arabidopsis, which wouls have perhaps strengthened the conclusions on the PP5 and other TPR proteins found to associate with AGO1 by mass spectrometry. However, the shear amount of data delivered in this single manuscript warrants on its own publication in EMBO.

My recommendation, therefore, is to accept the paper as is, after the following few grammar points have been taken into account:

Line 220 : that...each protein forms a complex..

Line 255 : were not affected

Line 340 : replace overexpression by over-accumulation.

Line 365-372 : the single sentence is very long and needs to be broken down.

Referee #3

The manuscript by Iki et al. describes an in vitro study of RISC assembly using a cell free extract from evacuolated tobacco BY-2 protoplasts (BYL), which was previously used by the same authors to demonstrate the role of HSP90 in AGO1 RISC assembly. By employing ATPγS, an ATP analog that binds to the ATP-binding pocket of HSP90 but is slowly hydrolyzed, they trapped the HSP90-AGO1 complex, a transient complex during RISC assembly and identified FKBP62 and PP5 as proteins that are associated with ATP-bound HSP90-AGO1. FKBP62 and PP5 are tetratricopeptide repeat (TPR) domain containing proteins. Since TPR domains are known to associate with HSP90, they also studied additional proteins with TPR domains, such as CYC40, FKBP65, TPR1, TPR2, and TPR7. Further biochemical studies were conducted mostly on CYC40 and, to some extent, PP5.

They showed that CYC40 increased the amount of single-stranded siRNA associated with AGO1, i.e. siRISC, in BYL. Furthermore, they showed that the amounts of both duplexed siRNA-bound AGO1 and single-stranded siRNA-bound AGO1 were increased, indicating that CYC40 does not affect the removal of the small RNA passenger strand. They showed that CYC40 associates with HSP90-AGO1 that contains an siRNA duplex. CYC40 promotes the association of HSP90-AGO1 with duplexed siRNA, and duplexed siRNAs also promotes the association of CYC40 with HSP90-AGO1. This pinpoints the step of CYC40 action as the loading of duplexed small RNAs onto AGO1. PP5 has the opposite effect in that it inhibits the loading of duplexed small RNAs onto AGO1.

Overall, the biochemical analyses were beautifully executed and most conclusions are justified. Given that little is known about RISC assembly in plants, the work will be of great interest in the field. A major weakness is the lack of in vivo biological relevance or lack of discussion on the in vivo biological relevance. For CYC40, a previous study using mutants in this gene has documented the role of this gene in miRNA biogenesis and function. It was shown that cyc40 mutants have reduced accumulation of miRNAs, which is consistent with the proposed role of CYC40 in RISC assembly. It is unclear why the authors do not discuss their biochemical findings in the context of the previous genetic studies. For PP5, there have been no prior genetic studies, and this reviewer wonders whether the authors have examined mutants in this gene in terms of miRNA or siRNA accumulation. The prediction based on their biochemical findings would be that pp5 mutants accumulate more miRNAs or siRNAs.

Two minor issues:

One is that there is no description of the anti-CYC40, anti-HSP90, and anti-AGO1 antibodies that were used extensively in the study. It is not known how the specificities of the antibodies were determined.

The other is that the conclusion that CYC40 does not associate with mature RISC containing a single-stranded siRNA guide strand is not justified by the experiments in Figure 4A. In this experiment, the presence of ATPγS would inhibit the production of mature RISC. The lack of association of single-stranded guide RNA with CYC40 could simply be due to low levels of mature RISC.

1st Revision - authors' response 28 September 2011

Response to the referees' comments

Referee #1

Major points:
1. The authors argue that, among TPR-containing proteins, CYP40 and PP5 have specific positive and negative functions, respectively, in RISC assembly. One major drawback of this study is that AGO1 and molecular chaperons are from tobacco, whereas TPR-containing proteins are from Arabidopsis. Consequently, the observed positive/negative/neutral functions of the TPR-containing proteins in RISC assembly may as well be due to their coincidental interspecific (in)compatibilities, rather than their functional specificities. I recommend the authors to clone TPR-containing proteins from tobacco and confirm their conclusions, at least for some key experiments.
Response: We cloned *CYP40*, *PP5*, and *FKBP62/65* cDNAs from *Nicotiana tabacum* (Nt) BY-2 cells; synthesized NtCYP40, NtPP5, and NtFKBP62/65 proteins in evacuolated BY-2 protoplast extracts (BYL); and added them to the *NtAGO1* mRNA-translated BYL to see the effects on NtAGO1-RISC assembly (Supplementary Figure S2C). Like the *Arabidopsis thaliana* orthologs, the addition of NtCYP40, NtPP5, and NtFKBP62/65 showed positive, negative, and neutral effects on RISC assembly, respectively (Supplementary Figure S2D and S2E). We described this result in the revised manuscript (main text, page 11, lines 171-182).

2. The data for CYP40 domain analysis (Fig. 7) is ambiguous and unconvincing, because the difference among WT and the mutants are only marginal. I understand that this experiment is technically challenging, because of the high background (presumably in part due to endogenous CYP40 activity). Is it possible to perform this experiment using lysate from the cells where endogenous CYP40 is knocked down? Alternatively, given that PP5 (and/or CsA) inhibits RISC assembly, the authors could quantitatively evaluate the potency of the CYP40 mutants to compete with and counteract PP5 (and/or CsA). Or else, in the worst-case scenario, omitting this entire Figure and related discussion should not drastically weaken this study.
Response: We thought that the smaller extent of enhancement of RISC formation (1.2-1.3-fold) by the addition of myc-tagged CYP40 (wild-type) (previous Figure 7C and D; current Figure S5B and C) compared to that of non-tagged CYP40 in Figure 3A and B (ca. 1.5-fold) might be due to the myc-tag. So, we performed the Figure 7C and D experiments using non-tagged CYP40 and the mutant proteins. Consistent with the results with the myc-tagged proteins, only $CYP40^{WT}$ and $CYP40^{R63A}$ facilitated RISC assembly, and the extent of enhancement was larger than that with the myc-tagged $CYP40^{WT}$ and $CYP40^{R63A}$. In the revised manuscript, we showed the results with non-tagged CYP40s (Figure 7C and D), and described these results (main text, page 20, lines 335-343). We also performed statistical analysis and presented the results (Figure 3A, 3B, 7C, 7D, Supplementary figure S2D, S2E, S5B, S5C). We hope that this reviewer will agree with us that the results are convincing and important.
Unfortunately, depletion of endogenous CYP40 from BYL was not successful in our hands.

Minor points:
1. It appears premature to conclude that CYP40 and other TPR-containing proteins do not affect removal of passenger strands, unless the data in Fig. 3C is presented in a more quantitative (and preferably more kinetic) manner. Note that the effects of CYP40 and PP5 in Figs. 3A and B, which the authors rely on to conclude that these proteins do affect duplex loading, are also rather mild.
Response: We examined the time course of siRNA duplex binding to AGO1 and passenger strand removal in the presence and absence of additional TPR proteins (Figure 3C). This experiment clearly showed that CYP40 facilitates small RNA duplex binding to AGO1. We described this result in the revised manuscript (main text, page 11, lines 183-193). As this reviewer pointed out, however, we cannot exclude the possibility that CYP40 has some functions also at later steps such as passenger strand removal. So, we added this comment in the revised manuscript (main text, page 23, lines 387-391).

2. Related to Major point #2: It would be important to confirm that CYP40 can offset the inhibitory effect of CsA (and FK506) in RISC assembly, because specificities of these drugs in this experimental system are unknown.

Response: We examined whether addition of CYP40 protein can offset the inhibitory effect of CsA on RISC assembly, but the offsetting was not clearly observed (Figure A, attached below).

In this experiment, while the concentration of the CYP40 protein in the RISC assembly reaction mixtures with exogenously added CYP40 was approximately 50 nM (the concentration of endogenous CYP40 is less than 5 nM), CsA needs to be added at concentrations higher than a few mM to see the inhibitory effect on RISC assembly. Although CYP40 is not the only protein that binds to CsA (*A. thaliana* genome encodes 29 cyclophilin genes) and we do not know the total concentration of CsA-binding proteins in the reaction mixtures, it is likely that there was excess free CsA even when the drug was added at the concentration of 4 mM (the lowest concentration tested in Figure A). If this is the case, the offset effect should not be detected. Therefore, Figure A result is not necessarily inconsistent with the possibility that CsA inhibit RISC formation through preventing the association of CYP40 with the AGO1-HSP90 complex.

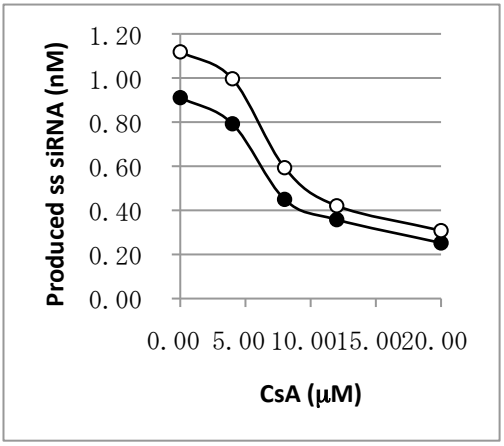

Figure A. Effect of CsA on RISC assembly *AGO1* and *CYP40* mRNAs were separately translated in BYL and mixed (open circles). As a control, *AGO1* mRNA was translated in BYL and mixed with mock-translated BYL (closed circles). The mixtures were incubated with gf698-22 siRNA duplexes (guide strand ^{32}P-labeled) and 0, 4, 8 12, or 20 mM CsA, and the amount of generated ss siRNA was quantified.

Referee #2

Line 220 : that...each protein forms a complex..

Response: Considering this comment, we modified the sentence (main text, page 14, line 235).

Line 255 : were not affected

Response: Because the subject for this verb is 'that (= copurification)', we left the original sentence as it was (main text, page 16, line 271).

Line 340 : replace overexpression by over-accumulation.

Based on the Referee #3's comment, we discussed the previous genetic data on CYP40 in more detail on page 26, lines 441-449 (main text), where we used a description "over-accumulation of the target transcripts…". The original sentence was removed to avoid redundancy.

Line 365-372 : the single sentence is very long and needs to be broken down.

Response: We broke down this sentence as suggested (main text, page 22, lines 379-386).

Referee #3

A major weakness is the lack of in vivo biological relevance or lack of discussion on the in vivo biological relevance. For CYC40, a previous study using mutants in this gene has documented the role of this gene in miRNA biogenesis and function. It was shown that cyc40 mutants have reduced accumulation of miRNAs, which is consistent with the proposed role of CYC40 in RISC assembly. It is unclear why the authors do not discuss their biochemical findings in the context of the previous genetic studies. For PP5, there have been no prior genetic studies, and this reviewer wonders whether the authors have examined mutants in this gene in terms of miRNA or siRNA accumulation.

The prediction based on their biochemical findings would be that pp5 mutants accumulate more miRNAs or siRNAs.
Response: Considering this comment, we added sentences discussing our biochemical results in the context of previous genetic findings about *cyp40* plants (main text, page 26, lines 441-449).

With regard to PP5, we agree with the reviewer that analyses with *pp5* mutant plants are very interesting. Unfortunately, however, we have not at present analyzed *pp5* mutant plants in terms of miRNA or siRNA accumulation, and we added sentences describing that further analyses are required to show the involvement of PP5 in PTGS *in planta* (main text, page 27, lines 468-469).

Two minor issues: One is that there is no description of the anti-CYC40, anti-HSP90, and anti-AGO1 antibodies that were used extensively in the study. It is not known how the specificities of the antibodies were determined.
Response: Anti-CYP40 antibody was raised in a rabbit against synthetic peptide corresponding to amino acid residues 45-63 of tomato CYP40. Anti-HSP90 antibody was raised in a rabbit against a full-length recombinant protein expressed in E. coli. In the previous version of the manuscript, this information was in the Supplementary Information. To let readers access this information more easily, we moved this part to the "Immunoblot analysis" section of Materials and Methods. With regard to specificity, these antibodies detect respective target proteins as main bands on Western blots of total BYL proteins. We described this in the revised manuscript (main text, page 31, lines 531-548).

The other is that the conclusion that CYC40 does not associate with mature RISC containing a single-stranded siRNA guide strand is not justified by the experiments in Figure 4A. In this experiment, the presence of ATPγS would inhibit the production of mature RISC. The lack of association of single-stranded guide RNA with CYC40 could simply be due to low levels of mature RISC.
Response: From the RISC assembly reaction mixture to which ATPgS was added, low but significant amount of single-stranded (ss) small RNA (sRNA) was copurified with FLAG-myc-AGO1 (the amount of ss sRNA was compared to that copurified with FLAG-myc-AGO1 from the RISC assembly reaction mixture without ATPgS). This result indicates that, even in the presence of ATPgS, RISC is slowly formed. However, from the same RISC assembly reaction mixture to which ATPgS was added, sRNA duplexes but not ss sRNAs were copurified with myc-CYP40 (Figure 4A and B). From these results, we concluded that CYP40 is not contained in mature RISCs. Since the explanation was not clear in the original manuscript, we rewrote the relevant part (main text, page 13, lines 208-212).

Other changes
1) A paper entitled "Binding of SQUINT to Hsp90 is Required for the Function of this Cyclophilin 40-related Protein in Arabidopsis" was published during this revision. We referred to this paper (main text, page 8, lines 128-130).

2) We added "regardless of small RNA addition" and referred to Figure 4C (main text, page 27, line 463).

3) We rewrote how to calculate the ss siRNA generation activity for accuracy (main text, page 43, lines 820-822).

4) We described the method of plasmid constructions for non-tagged CYP40 mutants and for *N. tabacum* TPR proteins (supplementary information, page 5, lines 75-98).

5) Several other minor corrections were made for accuracy and/or consistency (main text, page 3, line 48; page 10, line 163; page 17, lines 278 and 282; page 17, line 289; page 21, line 352; page 23, lines 398-399).

2nd Editorial Decision 06 October 2011

Thank you for submitting your revised manuscript to The EMBO Journal. It has been re-evaluated by one of the original referees who finds that you have satisfactorily addressed all the initial

concerns raised. I am therefore happy to accept the manuscript for publication in The EMBO Journal, where I believe it will make a good contribution to the journal. You will receive the official acceptance letter in the next day or so.

Yours Sincerely,

Editor
The EMBO Journal

REFEREE REPORT

Referee #1

The authors have adequately addresses my previous concerns, and the manuscript is now ready for publication.

