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A novel plant protein interacts with eIF3 and 60S to enhance virus-activated translation reinitiation

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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 May 2009

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to authors are provided below. As you can see, the referees appreciate the analysis and find the identification of RISK and the characterization of its role in TAV-dependent reinitiation interesting. However, they also raise a number of different issues with the manuscript that would have to be resolved before further consideration here. In particular referee #3 raises concerns regarding the quality of some of the data including the immunofluorescense images, immunoprecipitation and pull down analysis. Also further physiological data in support of the significance of RISP for CaMV replication is needed. As you can see there are many issues that would have to be resolved, however should you be able to address the concerns raised in full then we would be willing to look at revised version. Please note that some of the issues brought up by referee #3 are listed in the general comments and these will have to be addressed. 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.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In this manuscript Thiebeauld et al. present identification of a novel component of the translational machinery in plants designated RISP that is required for TAV-mediated activation of reinitiation on polycistronic mRNA of Cauliflower mosaic virus (CaMV) and some related pararetroviruses. This study clearly demonstrates that RISP may serve as a scaffold protein capable of interacting with eIF3a and c, RPL24 and TAV via separate well-defined binding domains and thus bridge contacts between various factors involved in this type of a reinitiation mechanism. Most of the findings are well documented and the proposed model provides a new interesting insight into the TAV-mediated reinitiation mechanism. Moreover, existence of a novel translational component of unknown cellular function opens up a new avenue for the future research. Overall, this paper makes an impression of a good quality work and would be of specific interest to those in the field of eukaryotic translational control.

Nevertheless, this reviewer finds several concerns, as follows, which, if addressed, would strengthen the entire story considerably.

Major points:
1. Abstract is somewhat vague and does not clearly emphasize the major achievements of this study in the context of the proposed model. In particular, the forth sentence is a bit confusing in terms of the 40S-involvement in the entire mechanism.

2. Page 14, last line. It is suggested that RISP enters the translation machinery pre-bound with eIF3 at the 43 PIC-formation step but TAV comes to the picture later, upon subunit joining. Now, L24 of the 60S is engaged in bridging the contact between 40S and 60S subunits and thus would not be accessible for making contacts with both RISP and TAV. Also, neither RISP nor TAV can directly interact with the 40S. Hence it is unclear to this reviewer what stabilizes eIF3 on the ribosome during elongation? The authors propose that it would be TAV (page 17, line 2), but how if it does not interact with the 40S where the whole complex was placed in Fig, 7B? In the original model proposed by these authors in Cell 2001 (also in EMBO 2004), it was the L18-TAV-eIF3g contact that was assumed to serve this purpose (eIF3 was believed to translocate onto the 60S subunit). The new model presented in Fig. 7 provides a reasonable explanation for the TAV-mediated reinitiation mechanism per se, however, does not offer any alternative option addressing the latter important question.

3. Based on Fig. 3B, the authors suggested that the presence of the 40S subunit may preclude loading of RISP onto the 60S subunit of the complete ribosome. That could be explained by the proposed competition between L24 for helix 44 of 16S rRNA and RISP (page 15, line 8 from bottom). But then it is also unclear what makes RISP to co-purify with 80S ribosomes (Fig. 3A).

4. It is assumed that in healthy cells most ribosomal subunits are engaged in translating polyribosomes. Given the aforementioned L24 competition, one would not expect to find the majority of RISP particles co-localizing with 60S ribosomal subunits in healthy cells as shown in Fig. 3C. In this regard, Figure 5A should show a control probing for a 40S subunit marker to demonstrate unambiguously that the 60S-TAV-RISP complex free of 40S subunits is analyzed (despite the fact that 300 mM NaCl was used in the extraction buffer). A presence of TAV promotes RISP-association with polysomes (Fig. 2A), so could it be proposed that TAV somehow eliminates this competition? In opinion of this reviewer, more careful interpretations of all these observations will certainly improve clarity of this work.

5. Fig. 4D is of a poor quality and co-purification of RISP with eIF3g (row 1, lane 4) does not seem to be statistically significant. Perhaps using antibodies against eIF3a or c, if available, might improve the outcome.

Minor points:

6. Character count is not shown.
7. Page 3, line 9. It is eIF5B but not eIF5 that promotes subunit joining.
8. Page 3, line 12. A corresponding reference is missing.
9. Page 3, line 3 from bottom. De novo recruitment of TC to the 40S prior to reinitiation should be referred by Dever et al., Cell 1992.
10. Page 5, line 2 from bottom. "is" is missing before "required".
11. Page 6. I would suggest using RISPa and RISPb instead of repeating accession numbers.
12. Figure 5D is not discussed in the corresponding section on page 11.
13. Fig. 1D. Is the different shape of the cells in first three rows vs. the last one somewhat significant?
14. Figure 5E. Binding between GST-L24 and eIF3 should be carried out and shown here to prove the point that RISP is required to mediate a contact between L24 and eIF3.
15. Fig. S3 seems to be an important culmination of the entire story and should be shown as Fig. 6C.
16. Assuming that a cellular role of RISP is unknown, the authors might consider renaming RISP for "TAV-specific ReInitiation Supporting Protein" (T-RISP). Since it is not known whether or not this protein also participates in reinitiation on endogenous mRNAs, besides its specific involvement in the TAV-mediated reinitiation mechanism, the current name might be somewhat misleading.
17. Page 15, last line. A wrong reference is used. Povry et al., 2007 should be replaced with Szamecz at el., 2008.
18. Page 16, line 3 from bottom. The statement: "Thus, RISP can ensure TAV binding to L24 and the 40S-bound eIF3 complex" is unclear. Perhaps this line would be a little bit easier to follow: "Thus, RISP strengthens TAV binding to L24 and at the same time bridges the relaxed 40S-60S interaction via its direct binding to the eIF3 complex"
19. Fig. 7. The authors should use short thick black lines (or some other symbols) to indicate direct interactions between individual factors. The way this figure appears now, one may get an impression that both TAV and RISP make contacts also with the 40S subunit. Also, Fig. 7B should clearly show that not only TAV but also RISP no longer interacts with L24; perhaps by moving RISP in green further down onto the 40S body.

Referee #2 (Remarks to the Author):

The authors employed a yeast two-hybrid system to identify a protein, called RISP, that interacts with the plant transactivator, TAV, involved in reinitiation. They show conclusively that RISP also binds to eIF3 through its a and c subunits, and to the 60S ribosomal protein, L24. A 40S initiation complex containing eIF3 together with RISP, TAV and eIF2 was detected by co-immunoprecipitation. Similarly, 60S immunoprecipitation brought down RISP (and TAV if cells were infected with CaMV). Thus, RISP, together with TAV, may contribute to linking 60S subunits with eIF3. The various interaction domains were identified by deletion analysis or mutagenesis. When domains normally responsible for the RISP-TAV or RISP-L24 interactions were made non-functional by mutation, reinitiation/trans-activation was impaired in vivo, thus indicating that these interactions are required.
The identification of RISP as an important component of the TAV-mediated transactivation/reinitiation pathway is of great interest and significance. The experiments are well conceived and executed, and the results are compelling and appropriately interpreted. Although a detailed molecular explanation for how RISP and TAV function is not fully attained, the advance is sufficient to warrant publication at this time. I find few problems with the text, except as follows.
1) The citation of Figure 7 on pages 16 and 17 is wrongly indicated as Figure 6.
2) On page 17, the top paragraph is confusing. One sentence states that "TAV-activated polyscistronic translation is not dependent on the distance between two ORFs". The next sentence states "close spacing between the stop and the start codons was shown to be essential". The two phrases appear to contradict each other. If the authors mean that two different reinitiation mechanisms are involved, this should be said more explicitly.

Referee #3 (Remarks to the Author):

Using the Cauliflower Mosaic virus (CaMV) TAV protein as bait in a yeast 2-hybrid screen, Thiebeauld et al isolate and describe a novel plant factor they term RISP, reinitiation supporting protein. RISP binds TAV in vitro and in vivo, associates with polysomes in infected plants, and the authors claim that RISP also specifically associates with the a/c subunits of eIF3. While RISP is not shown to "support" translation reinitiation, the data clearly show that it stimulates TAV-dependent reinitiation in protoplasts transiently transfected with TAV and a reporter plasmid.

While the general subject of cellular proteins that stimulate translation reinitiation is exciting and potentially of broad interest, the quality of some of the data needs to be addressed to firm up the authors conclusions. In particular, the immunofluorescence images are poor and lack quantification. In addition, all the immunoprecipitations and pull downs lack critical controls to rigorously establish a specific physical interaction between RISP, L24, and eIF3 (intact and subunits). Physiologic data regarding the importance of RISP to CaMV replication is likewise omitted (ie RNAi directed against RISP and its consequence on CaMV replication and on reporter gene translation). Finally, speculative aspects of the discussion are a bit overly mechanistic, lacking in biological data, and somewhat overstated with respect to the data. Even the title appears to overstate the findings, suggesting that the "cellular protein" mediates an interaction or stimulates joining between 40S-bound eIF3 and the 60S subunit. In fact, this data is never presented, but instead is inferred from RISP binding to both L24 and eIF3. Just because RISP could (pending the completion of the proper specificity controls) associate with eIF3 subunits and L24 does not necessarily mean that RISP is the mediator of eIF3 and 60S interactions. While speculating that RISP mediates this interaction is perfectly fine for a model or as speculation in the discussion, it may not be best as the manuscript title, as provocative as it may be. The running title likewise overstates that RISP mediates TAV-activated reinitiation - whereas the data clearly show that it stimulates. Without establishing that TAV-dependent reinitiation requires RISP, it remains a hypothesis that RISP mediates the TAV-dependent stimulation.

Additional specific points:

Page 6, line 16. it is difficult to appreciate any discrete subcellular localization from the data shown

Figs 1B, 2D, 4A, 4C, 4D, 5A, 5E: all the immunoprecipitations (IPs) and GST pull-downs require additional controls. For the IPs, "beads alone" is not the correct control. There needs to be a relevant control antibody bound to the beads. In the case of rabbit polyclonal primaries, this could either be pre-immune, or if that is limiting, normal rabbit serum would work fine. It would also be nice if the authors could blot for something excluded from the immune complex, because at this point it looks like everything they look for is present in the immune complex. Similarly, for the GST pull-downs, practically all the input test proteins associate with the GST-fusions examined. Specificity needs to be established - namely, a protein that does not interact with the GST-fusion protein of interest needs to be included. Unfused GST is not sufficient to establish this.

Are the protein-protein interactions the authors present RNA-dependent or are they RNAse sensitive? Can the authors rule out RNA as a cofactor in these interactions?

Fig 1 D. how do the authors know that the colocalization of the two aggregated proteins is not the result of their over-expression?

Figure 2 B how do the authors distinguish endogenous RISP from recombinant - it is not stated.

The immunofluorescence in 3C needs to be quantified. What fraction of RISP- positive aggregates colocalize with 60S?

Fig 4A, lanes 11 and 12. Authors conclude that RISP binds purified eIF3, but does not interact with 40S-bound eIF3. It looks to me that it does interact with eIF3, although with reduced efficiency. This could be measured and quantified.
P14, line 2 "..in plant protoplasts RISP supports the function of a reinitiation factor of viral origin - TAV-..." Perhaps stimulates is a better term, given the data presented.

1st Revision - authors' response 05 July 2009

Thank you for inviting us to resubmit our paper to EMBO J with additional data. We found the comments of the reviewers to be very constructive and we appreciate their fair evaluation. We have performed several new experiments and now submit an improved version of the manuscript taking into account the reviewer's suggestions.

Our response to each of the specific points raised by the reviewers have been addressed as detailed below. In addition, the following amendments have been made to the figures: we have removed the original Figure S3, since these data are now presented in the new Figure 6B, and have replaced the IP gels (e.g., Figure 4D) with a new experimental set up including controls suggested by the reviewers. Additional appropriate controls are now included in the GST pull-down interactions between TAV and RISP (Fig. 1C), and L24 and RISP (Fig. 3D). We have included data on GST pull-down interactions between L24 and eIF3 or eIF3/ RISP as supplementary Figure S3.
We also analyzed the effect of RISP on CaMV replication and TAV function in planta; these data are now presented in Fig. 6 as parts C-E.

Detailed response to Reviewers:

Reviewer 1

Major points:
1. ´Abstract is somewhat vague and does not clearly emphasize the major achievements of this study in the context of the proposed model. In particular, the forth sentence is a bit confusing in terms of the 40S-involvement in the entire mechanism.'

The abstract has been rewritten to better reflect the findings of this study in the context of the proposed model.

2. ´Page 14, last line. It is suggested that RISP enters the translation machinery pre-bound with eIF3 at the 43 PIC-formation step but TAV comes to the picture later, upon subunit joining. Now, L24 of the 60S is engaged in bridging the contact between 40S and 60S subunits and thus would not be accessible for making contacts with both RISP and TAV. Also, neither RISP nor TAV can directly interact with the 40S. Hence it is unclear to this reviewer what stabilizes eIF3 on the ribosome during elongation? The authors propose that it would be TAV (page 17, line 2), but how if it does not interact with the 40S where the whole complex was placed in Fig, 7B? In the original model proposed by these authors in Cell 2001 (also in EMBO 2004), it was the L18-TAV-eIF3g contact that was assumed to serve this purpose (eIF3 was believed to translocate onto the 60S subunit). The new model presented in Fig. 7 provides a reasonable explanation for the TAV-mediated reinitiation mechanism per se, however, does not offer any alternative option addressing the latter important question.'

Our earlier model stated that TAV/eIF3 can travel with 80S during the elongation step through relocation of TAV/eIF3 to the back of the 60S subunit via interaction between TAV and L18. Now, we have found that TAV also promotes association of RISP with polysomes. This allows us to suggest that RISP can travel with 80S as a part of the TAV/eIF3/60S complex since it enters the 43S PIC together with eIF3. At the termination step, TAV/RISP/eIF3 moves back to 40S via the eIF3/40S interaction. Here we propose that 40S/eIF3-bound TAV/RISP can restore or make new contacts with 60S via interaction with L24. This has now been clearly explained in the text of the discussion.

3. ´Based on Fig. 3B, the authors suggested that the presence of the 40S subunit may preclude loading of RISP onto the 60S subunit of the complete ribosome. That could be explained by the proposed competition between L24 for helix 44 of 16S rRNA and RISP (page 15, line 8 from bottom). But then it is also unclear what makes RISP to co-purify with 80S ribosomes (Fig. 3A). '

Association between wheat germ RISP and 80S does not necessarily mean that these ribosomes are

active in translation. Such an association could also result from re-association between 60S-RISP and 40S. It seems unlikely that RISP-bound 40S will be active in elongation. We propose a role for RISP and TAV interactions with 60S-bound L24 during the scanning step of translation reinitiation.

4. ´It is assumed that in healthy cells most ribosomal subunits are engaged in translating polyribosomes. Given the aforementioned L24 competition, one would not expect to find the majority of RISP particles co-localizing with 60S ribosomal subunits in healthy cells as shown in Fig. 3C. In this regard, Figure 5A should show a control probing for a 40S subunit marker to demonstrate unambiguously that the 60S-TAV-RISP complex free of 40S subunits is analyzed (despite the fact that 300 mM NaCl was used in the extraction buffer). A presence of TAV promotes RISP-association with polysomes (Fig. 2A), so could it be proposed that TAV somehow eliminates this competition? In opinion of this reviewer, more careful interpretations of all these observations will certainly improve clarity of this work. ‘

60S was precipitated from healthy and infected plants and assayed for TAV and RISP. Now, we show that 60S ribosomes pull-down efficiently 40S and apparently other components of polysomes, and RISP and TAV (new Fig. 5A). This suggests that RISP together with TAV is present in some 60S-containing complexes. 60S-TAV-RISP complex formation we showed in vitro using purified components.

5. ´ Fig. 4D is of a poor quality and co-purification of RISP with eIF3g (row 1, lane 4) does not seem to be statistically significant. Perhaps using antibodies against eIF3a or c, if available, might improve the outcome’

We provide a new Fig. 4D with all appropriate controls (as suggested by referee 3, see below). Anti-eIF3g antibodies were replaced by anti-eIF3c AB, kindly provided by K. Browning.

The minor points raised by this reviewer have been addressed as follows:

6. Character count is not shown
Character count has now been included on the title page

7. Page 3, line 9. It is eIF5B but not eIF5 that promotes subunit joining
The error has been corrected.

8. Page 3, line 12. A corresponding reference is missing
The reference was cited (Morris and Geballe, 2000)

9. Page 3, line 3 from bottom. De novo recruitment of TC to the 40S prior to reinitiation should be referred by Dever et al., Cell 1992
The suggested reference has been added (Dever TE, Feng L, Wek RC, Cigan AM, Donahue TF, Hinnebusch AG (1992) Phosphorylation of initiation factor 2 by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. Cell 68:585-596)

10. Page 5, line 2 from bottom. "is" is missing before "required"
The error has been corrected.

11. Page 6. I would suggest using rispa and rispb instead of repeating accession numbers.
The referee's suggestion has been adopted

12. Figure 5D is not discussed in the corresponding section on page 11
Discussion of Fig. 5D has been added

13. Fig. 1D. Is the different shape of the cells in first three rows vs. the last one somewhat significant?
In our experience there is no any correlation between cell shape and protein expression pattern.

14. Figure 5E. Binding between GST-L24 and eIF3 should be carried out and shown here to prove the point that RISP is required to mediate a contact between L24 and eIF3.
This control is now included in Supplementary Fig. S3. We now show that GST-L24 does not pull-down eIF3 to any significant extent without RISP or TAV.

15. Fig. S3 seems to be an important culmination of the entire story and should be shown as Fig. 6C.
These data now are shown on Fig. 6B.

16. Assuming that a cellular role of RISP is unknown, the authors might consider renaming RISP for "TAV-specific ReInitiation Supporting Protein" (T-RISP). Since it is not known whether or not this protein also participates in reinitiation on endogenous mRNAs, besides its specific involvement in the TAV-mediated reinitiation mechanism, the current name might be somewhat misleading.
We call RISP a reinitiation supporting protein since it functions in at least one specific case of reinitiation. We do not state that it is a reinitiation factor. Thus we prefer to retain the name RISP.

17. Page 15, last line. A wrong reference is used. Povry et al., 2007 should be replaced with Szamecz at el., 2008.
The reference was replaced

18. Page 16, line 3 from bottom. The statement: "Thus, RISP can ensure TAV binding to L24 and the 40S-bound eIF3 complex" is unclear. Perhaps this line would be a little bit easier to follow: "Thus, RISP strengthens TAV binding to L24 and at the same time bridges the relaxed 40S-60S interaction via its direct binding to the eIF3 complex"
The sentence now reads: " During scanning, RISP strengthens TAV binding to L24 and at the same time bridges the relaxed 40S-60S interaction via direct binding to the eIF3 complex"

19. Fig. 7. The authors should use short thick black lines (or some other symbols) to indicate direct interactions between individual factors. The way this figure appears now, one may get an impression that both TAV and RISP make contacts also with the 40S subunit. Also, Fig. 7B should clearly show that not only TAV but also RISP no longer interacts with L24; perhaps by moving RISP in green further down onto the 40S body.

Double-headed arrows have been added to the figure to clearly indicate proposed interactions.

Reviewer #2
1. ´ The citation of Figure 7 on pages 16 and 17 is wrongly indicated as Figure 6 ‘
The mistake was corrected

2. ´ On page 17, the top paragraph is confusing. One sentence states that "TAV-activated polyscistronic translation is not dependent on the distance between two ORFs". The next sentence states "close spacing between the stop and the start codons was shown to be essential". The two phrases appear to contradict each other. If the authors mean that two different reinitiation mechanisms are involved, this should be said more explicitly. ‘
This contradiction in the text has been removed during the reworking of the Discussion.

Reviewer #3

1. ′ ... immunofluorescence images are poor and lack quantification. ‘
We now provide quantification analysis for Fig 3C

2. ′... immunoprecipitations and pull downs lack critical controls to rigorously establish a specific physical interaction between RISP, L24, and eIF3 (intact and subunits).’
These experiments were repeated with appropriate controls as suggested (for immunoprecipitations, new results are shown in Figs. 4D and 5A; and for GST pull-downs Figs. 1C and 3D). We repeated the most important experiments showing the basic interactions between RISP and TAV, or L24. Also, our data suggest that their binding to RISP is not mediated by RNA. Purified conalbumin was used as a specificity control.

3. ′ Physiologic data regarding the importance of RISP to CaMV replication is likewise omitted (ie RNAi directed against RISP and its consequence on CaMV replication and on reporter gene translation)’
We now include data obtained with RISP knockout mutant plants, which show a delay in CP and TAV accumulation as well as a lower level of transactivation when infected with CaMV. We speculate that the protein encoded by rispb may act to support CaMV infection (albeit less efficiently) and the residual level of transactivation. Double knockdown (rispa and rispb) was lethal.

′... speculative aspects of the discussion are a bit overly mechanistic, lacking in biological data, and somewhat overstated with respect to the data. Even the title appears to overstate the findings...’
The title and running title have been changed, and parts of the discussion have been reworded bearing in mind the reviewer's concerns.

Specific points

1. ′Page 6, line 16. it is difficult to appreciate any discrete subcellular localization from the data shown’
We now include a new Fig. 3C (left panels) clearly demonstrating cytoplasmic localization of RISP.

2) ′ Figs 1B, 2D, 4A, 4C, 4D, 5A, 5E: all the immunoprecipitations (IPs) and GST pull-downs require additional controls. For the IPs, "beads alone" is not the correct control. There needs to be a relevant control antibody bound to the beads. In the case of rabbit polyclonal primaries, this could either be pre-immune, or if that is limiting, normal rabbit serum would work fine. It would also be nice if the authors could blot for something excluded from the immune complex, because at this point it looks like everything they look for is present in the immune complex. Similarly, for the GST pull-downs, practically all the input test proteins associate with the GST-fusions examined. Specificity needs to be established - namely, a protein that does not interact with the GST-fusion protein of interest needs to be included. Unfused GST is not sufficient to establish this. Are the protein-protein interactions the authors present RNA-dependent or are they RNAse sensitive? Can the authors rule out RNA as a cofactor in these interactions? ‘
IP controls. Controls were done using normal rabbit serum (4D) and normal human serum (5A) and control anti-katanine antibodies. The IP extraction buffer contains some RNase A to disrupt RNA (see Supplementary Materials and Methods).

3) ′ Fig 1 D. how do the authors know that the colocalization of the two aggregated proteins is not the result of their over-expression? ‘
Fig. 1D. TAV/ RISP co-overexpression results in a perfect co-localization, where RISP signals take on the shape and size of TAV aggregates. In contrast, a small deletion within the MAV domain of TAV that did not affect over-expression of TAV aggregates, disrupts its colocalization with RISP.

4) ′ Figure 2 B how do the authors distinguish endogenous RISP from recombinant - it is not stated.
We think that referee's question is about Fig.3B.
We agree that it is impossible to distinguish endogenous from recombinant RISP. However, we do not claim to do so, and this was not the aim of the experiment.

5) ′ The immunofluorescence in 3C needs to be quantified. What fraction of RISP- positive aggregates colocalize with 60S? ‘Fig. 3C

The immunofluorescence was quantified and data are now included in the manuscript

6) ' Fig 4A, lanes 11 and 12. Authors conclude that RISP binds purified eIF3, but does not interact with 40S-bound eIF3. It looks to me that it does interact with eIF3, although with reduced efficiency. This could be measured and quantified. '
Comparison of lanes 11 and 7 strongly suggest that the presence of 40S reduced significantly eIF3 binding to RISP, suggesting that 40S out-competes RISP for eIF3. Our quantication analysis of eIF3 distribution between B and U fractions suggested that max 5% of eIF3 was present in the GST-RISP B fraction.

8) ' P14, line 2 "..in plant protoplasts RISP supports the function of a reinitiation factor of viral origin - TAV-..." Perhaps stimulates is a better term, given the data presented 'p14, lane 2
The reviewer's suggestion was adopted

2nd Editorial Decision 04 August 2009

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee #3 to review the revised version and we have now received the comments from this referee. As you can see below, the referee really appreciate the carried out revisions and supports publication here. S/he also has a few minor comments that should be attended to before acceptance here in a final revision. We will accept the manuscript as soon as we receive the revised version. When you send us your revision, please include a cover letter with an itemised list of all changes made.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #3 (Remarks to the Author):

The authors have done a commendable job of putting together this revised manuscript. In particular, the demonstration that RISP contributes wild-type levels of viral protein accumulation in infected cells is compelling.

At this stage, I have only relatively minor comments:

p.8, line 17: remove "in the presence of TAV"; should read "Thus, RISP is recruited or stabilized in polysomes in CaMV-infected cells, and may...."

P12, line 7. While I agree that RISP labeling co-localized with large viroplasms, the data in figure 5C concerning the "pearl-necklace like structures" is not particularly convincing. The authors should either provide data of the quality shown in fig 5B, or remove their emphasis on the necklace structure in 5C

P12, line 9 please change "probably large polysomes" to "possibly large polysomes"

P12, third line from bottom: "No significant binding of eIF3 to GST-L24 was detected without RISP." The data in fig S3 shows that similar amounts of eIF3 are in the unbound fraction, and binding of L24 to eIF3 is detectable w/o RISP. Please change to "RISP stimulates binding of eIF3 to L24" or something to that effect. From the data presented, it is not clear what level of binding is

significant and what is not.

p.14, line 3. (Fig S4C) should be FigS4B

p 14, line 6 (Fig S4B) should be fig S4C.

2nd Revision - authors' response 05 August 2009

The minor points raised by reviewer #3 have been addressed as follows:

p.8, line 17: remove "in the presence of TAV"; should read "Thus, RISP is recruited or stabilized in polysomes in CaMV-infected cells, and may...."
The referee's suggestion has been adopted

p12, line 7. While I agree that RISP labeling co-localized with large viroplasms, the data in figure 5C concerning the "pearl-necklace like structures" is not particularly convincing. The authors should either provide data of the quality shown in fig 5B, or remove their emphasis on the necklace structure in 5C
The term ´pearl-necklace like structures' has been replaced by ´round-shaped structures '

p12, line 9 please change "probably large polysomes" to "possibly large polysomes"
The referee's suggestion has been adopted

p12, third line from bottom: "No significant binding of eIF3 to GST-L24 was detected without RISP." The data in fig S3 shows that similar amounts of eIF3 are in the unbound fraction, and binding of L24 to eIF3 is detectable w/o RISP. Please change to "RISP stimulates binding of eIF3 to L24" or something to that effect. From the data presented, it is not clear what level of binding is significant and what is not.
The referee's suggestion has been adopted

p.14, line 3. (Fig S4C) should be FigS4B
The error has been corrected.

p 14, line 6 (Fig S4B) should be fig S4C.
The error has been corrected.

