Enhanced microRNA accumulation through stemloop-adjacent introns

Rebecca Schwab, Corinna Speth, Sascha Laubinger and Olivier Voinnet

Corresponding author: Olivier Voinnet, ETH Zurich

Review timeline:

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Editor: Esther Schnapp

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 26 November 2012

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

As you will see, all referees acknowledge that the findings are potentially interesting and suitable for publication in EMBO reports. However, all referees also point out that the data need to be quantified and that statistical analyses need to be performed in order to strengthen the findings. Referee 3 further indicates that a more detailed description of the experiments is required. Referees 2 and 3 also note the inconsistency between your study and the one from Jarmolowski’s lab regarding the requirement of the 5’ splice site of introns for the enhanced levels of mature miRNAs. This inconsistency must be addressed and at least a possible explanation should be provided. Referee 2 also suggests in the comments to the other manuscript that the constructs used in both studies should be compared in order to address the opposing results.

Given these referee comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive
outcome of a second round of review and I should also remind you that it is EMBO reports 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.

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 the corresponding main figure. Please also include the number (n) of experiments and please specify the error bars and statistical tests used to calculate p-values for all quantifications in the corresponding figure legends.

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 this manuscript, Schwab et al. demonstrated that introns located at 3’ end of the stem-loop in plant miRNA precursors can promote the accumulation of the mature miRNA. In transient or stable transgenic plants expressing variant forms of MIR172a and MIR163, the presence of a 3’ intron was critical for mature miRNA accumulation regardless of intron sequences and whether splicing occurred. The authors also investigated the correlation between miRNA processing and splicing by expressing the variant forms of MI7RNA in dcl1, a mutant impaired in miRNA processing, and speculated the positive intron effect on miRNA accumulation was prior to or during spliceosome recruitment.

This study was quite interesting as the authors investigated the roles of introns in intron-containing plant MIRNA genes. However, the execution of data presentation and interpretation should be improved. The authors may want to present RT-PCR or northern blot results in a quantitative manner so the abundances could be readily compared. Proper replication should also be included to present the data with statistical significance. There were interesting findings, but the data should be presented more convincingly and the data interpretation performed more substantially and critically.

Below is the summary of my comments/concerns regarding the manuscript.

1. The authors should include a more clear explanation of their experimental design and details. For example, were those MIR163 and MIR173 variant constructs driven by their native promoters or 35S promoter in transient or stable transgenic experiments? Why was miR173 used for control in
small RNA northern blot? Were the RT-PCR experiments done in semi-quantitative or quantitative ways?

2. Regarding intron characteristics and miRNA accumulation: On page 6-7, the authors compared 11 introns and their characteristics as they relate to miRNA accumulation. They concluded that "The levels of mature miRNA were neither correlated with intron length nor with the AT content of the engineered piece of DNA". However, I argue that there may be a relationship between miRNA levels and AT content. As shown in Figure 1D and Table S1, miR163 were higher in panel i163, iGUS, and i172b3. And these three actually had higher AT content (69.9%, 62%, and 65.5%) compared with the panels that had lower miR163 levels (39.3% in i172b5, and 55.1% in GFP). This is worth consideration.

3. Regarding 5' intron and miRNA accumulation: On Page 7, the authors claim that the 5' intron did not alter mature miRNA accumulation. However, in Figure 2 Panel A, I do see a gradual drop in miRNA level from 172b-1 to 172b-2 (lacking 3' intron), and 172b-2 to 172b-3 (lacking both 5' and 3' Introns), which suggests that there might be some effect of a 5' intron on miR172b accumulation. Further experiments with placement of iCHS and iGUS on the 5' end also slightly increased miR163 accumulation compared with the no-intron version 163-4. Thus it may be possible that 5' introns also play a role, albeit not too significant, in miRNA accumulation.

4. Regarding tests of the dicing effect on splicing: I really like this experimental design, that after testing the 3'-adjacent introns can affect miRNA accumulation, the authors tested the opposite effect: miRNA accumulation also affected intron splicing. Nicely done!

5. I'm wondering if these examples are peculiar cases or a representative phenomenon?: The authors have shown that introns 3' of miR163 and miR172 could increase level of mature miRNA. It will be really nice to test how commonly miRNAs have 3' introns, not only in Arabidopsis. It will be more interesting to see which versions of poly-A site are more preferable in nature - those with or without introns.

Some minor suggestions/edits/comments:
Page 6, "variant (164-4)" should be "variant (163-4)".
Page 14, Figure 2 legend B: should explain each lane. It is a little bit confusing (which lanes are 5' introns and which lanes represent 3' intron).
Figure 1 bottom panel: should also include wildtype miR163 levels.
Figure 2 C: The authors claimed that they did not find a positive effect of splicing on miRNA accumulation (Page 11, 2nd paragraph). However, in this figure, they did show an increase in miR172 levels in the 172aA mutant in both transient assay and transgenic lines, which might suggested that unspliced miRNA precursors actually increased dicing. This needs further explain or study.

Referee #2:
The manuscript convincingly shows that introns in miRNA precursors correlate with higher levels of mature miRNAs.
miR163 accumulates to a higher level from the MIR163-1 primary transcript with spliceable intron than from the MIR163-2 precursor without intron both in transiently transformed Nicotiana benthamina leaves and stable transformed Arabidopsis miR163 T-DNA line. Also for MIR172 the presence of introns correlates with increased miR172 accumulation.

To begin to understand which features of introns/intron processing are responsible for this effect, the authors tested several unrelated introns. They found that the chalcone synthase A intron from petunia and the intron of a leaf-specific gene from potato, originally used by the Willmitzer group, as well as intron 3 of MIR172b stimulates mature miRNA accumulation but intron 5 of MIR172b does not. The effect on miRNA levels does not correlate with the length of the introns or their U content and is not specific for introns normally present in miRNA precursors.
In figure 1D it should be explained more clearly that "div" relates to the introns of the various sources (point 1).
Further hints to a possible mechanism came from the observation that introns located 5´ of the stem-loop in the miRNA precursor did not affect the accumulation of miRNAs in contrast to introns located 3´ of the stem-loop. Moreover, mutation of the 5´ splice site in MIR163 and MIR172a as well as mutation of 5´ splice site, 3´ splice site and branch point sequence simultaneously. Thus, the splicing reaction per se is not crucial for the observed positive effects of introns on miRNA accumulation. Furthermore, the authors showed that the effect does not correlate to intron-mediated enhancement: Both UBQ with strong IME and TCH with weak IME boost miR163 accumulation whereas TRP1 with moderate IME does not boost miR163 accumulation. Figure 3C is not referred to in the text (point 2).

Then the opposite question was addressed: Can miRNA precursor processing affect splicing of MIR introns? In dcl1 mutants which do not process the precursors, splicing of 3´ introns are stimulated. This is convincingly shown for MIR172a and b where a clear effect can be seen in semiquantitative RT-PCR shown in figure 3D, suggesting that processing of miRNA precursors negatively influence splicing of introns located downstream. For MIR163, only a very small effect is seen. This needs confirmation in independent replicates and statistical assessment of quantification (point 3).

The manuscript provides novel insights into a connection between splicing of miRNA precursor transcripts and miRNA biogenesis. The conclusion that specific locations in some but not all introns located 3´ of stem-loop structure in priMIRs can increase levels of the corresponding mature miRNAs contrasts with the conclusion of the accompanying paper that the 5´ splice site of introns in priMIRs makes a major contribution to increased levels of the corresponding mature miRNAs. This point should be addressed for the readers (point 4).

Summary:
1. Yes
Introns in miRNA precursors correlate with higher levels of mature miRNAs.
2. Yes
3. Yes
Comparative analysis of miRNA biogenesis and factors affecting processing efficiency in different organisms will provide further insights into a complex process that is not completely understood yet.
4. Yes

Referee #3:
In this manuscript, Schwab et al report the discovery that introns adjacent to the 3´ (3´ intron) of stem-loop of pri-miRNAs promote miRNA accumulation. The authors showed that engineered MIR172/163 with 3´ intron but not 5´ intron results in higher levels of miR172/163 than the ones without intron. The function of 3´ intron appear to independent of splicing as splicing-disabled 3´ introns can increase miRNA accumulation. Furthermore, the authors showed that the reduced processing of pri-miRNAs increases the splicing of 3´ intron, indicating the potential correlation between pri-miRNA processing and 3´ intron splicing. The authors propose that 3´ intron may have roles in pri-miRNA processing or controlling the stability of miRNAs. This is a novel finding and is of general interests in the field. Concerns are:
1, Figure 2, the authors please provide the information on the nature of mutation that abolishes splicing.
2, Please quantify the levels of miRNAs in various northern blot.
3, Please draw a cartoon model for the proposed action of 3´ intron.
4, The font of reference is different with other sections

There is an inconsistence in these two manuscripts. Bielewic et al show that mutated 5´ splicing site
affects miRNA accumulation, while schwab find that mutated 5’ splicing site does not. If possible, the authors shall provide explanation for this inconsistence, which may provides mechanic insight on how 3’ intron affects miRNA accumulation.

Referee #1:

In this manuscript, Schwab et al. demonstrated that introns located at 3’ end of the stem-loop in plant miRNA precursors can promote the accumulation of the mature miRNA. In transient or stable transgenic plants expressing variant forms of MIR172a and MIR163, the presence of a 3’ intron was critical for mature miRNA accumulation regardless of intron sequences and whether splicing occurred. The authors also investigated the correlation between miRNA processing and splicing by expressing the variant forms of MIRNA in dcl1, a mutant impaired in miRNA processing, and speculated the positive intron effect on miRNA accumulation was prior to or during spliceosome recruitment.

This study was quite interesting as the authors investigated the roles of introns in intron-containing plant MIRNA genes. However, the execution of data presentation and interpretation should be improved. The authors may want to present RT-PCR or northern blot results in a quantitative manner so the abundances could be readily compared. Proper replication should also be included to present the data with statistical significance. There were interesting findings, but the data should be presented more convincingly and the data interpretation performed more substantially and critically.

Below is the summary of my comments/concerns regarding the manuscript.

1. The authors should include a more clear explanation of their experimental design and details. For example, were those MIR163 and MIR173 variant constructs driven by their native promoters or 35S promoter in transient or stable transgenic experiments? Why was miR173 used for control in small RNA northern blot? Were the RT-PCR experiments done in semi-quantitative or quantitative ways?

   All these points have now been addressed in the Methods section.

2. Regarding intron characteristics and miRNA accumulation: On page 6-7, the authors compared 11 introns and their characteristics as they relate to miRNA accumulation. They concluded that "The levels of mature miRNA were neither correlated with intron length nor with the AT content of the engineered piece of DNA". However, I argue that there may be a relationship between miRNA levels and AT content. As shown in Figure 1D and Table S1, miR163 were higher in panel i163, iGUS, and i172b3. And these three actually had higher AT content (69.9%, 62%, and 65.5%) compared with the panels that had lower miR163 levels (39.3% in i172b5, and 55.1% in GFP). This is worth consideration.

   We have now incorporated the numbers obtained from the quantification of miRNA levels by qPCR and repeated the analysis shown in Supplemental Figure 1. This new analysis indicates no apparent correlation.

3. Regarding 5’ intron and miRNA accumulation: On Page 7, the authors claim that the 5’ intron did not alter mature miRNA accumulation. However, in Figure 2 Panel A, I do see a gradual drop in miRNA level from 172b-1 to 172b-2 (lacking 3’ intron), and 172b-2 to 172b-3 (lacking both 5’ and 3’ Introns), which suggests that there might be some effect of a 5’ intron on miR172b accumulation. Further experiments with placement of iCHS and iGUS on the 5’ end also slightly increased miR163 accumulation compared with the no-intron version 163-4. Thus it may be possible that 5’ introns also play a role, albeit not too significant, in miRNA accumulation.

   We have carefully quantified miRNA levels with qPCR and the newly provided histograms support our notion that 5’ introns have only marginal effects (Fig. 2A). The main text was
adjusted accordingly.

4. Regarding tests of the dicing effect on splicing: I really like this experimental design, that after testing the 3'-adjacent introns can affect miRNA accumulation, the authors tested the opposite effect: miRNA accumulation also affected intron splicing. Nicely done!

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This is a very interesting point, which we would like to address in the future. Unfortunately, there is only limited information available on full-length miRNA precursor transcripts at this point, even in Arabidopsis, so a very labor-intensive process will be required to map transcript variants in different tissues and – most importantly – under different environmental conditions. But this analysis would take a considerable amount of time, and we feel it is outside the primary scope of this first study.

Some minor suggestions/edits/comments:
Page 6, "variant (164-4)" should be "variant (163-4)".

This has been corrected.

Page 14, Figure 2 legend B: should explain each lane. It is a little bit confusing (which lanes are 5' introns and which lanes represent 3' intron).

We improved the figure to make it more obvious which lanes are the ones representing the 3' introns.

Figure 1 bottom panel: should also include wildtype miR163 levels.

The transgenes were transformed in a miR163 KO background, therefore we included this genotype as the 'background control'.

Figure 2 C: The authors claimed that they did not find a positive effect of splicing on miRNA accumulation (Page 11, 2nd paragraph). However, in this figure, they did show an increase in miR172 levels in the 172aA mutant in both transient assay and transgenic lines, which might suggested that unspliced miRNA precursors actually increased dicing. This needs further explain or study.

We have carefully quantified miRNA levels from transient N. benthamiana assays with qPCR and now found a very slight decrease (no increase) in miRNA levels when the donor site was mutated.
In stable lines, only miR172 (not miR163) levels were slightly increased when the donor site was mutated, and the miR172-AP2 module has previously been implicated in feedback regulation. Therefore we limited our statements to ‘mildly affected’ and would like to further investigate miR172 in the future.

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A note was added to the figure legend.

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Furthermore, the authors showed that the effect does not correlate to intron-mediated enhancement: Both UBQ with strong IME and TCH with weak IME boost miR163 accumulation whereas TRP1 with moderate IME does not boost miR163 accumulation.

Figure 3C is not referred to in the text (point 2).

We do refer to figure 3C further towards the end of the manuscript.

Then the opposite question was addressed: Can miRNA precursor processing affect splicing of MIR introns?

In dcl1 mutants which do not process the precursors, splicing of 3’ introns are stimulated. This is convincingly shown for MIR172a and b where a clear effect can be seen in semiquantitative RT-PCR shown in figure 3D, suggesting that processing of miRNA precursors negatively influence splicing of introns located downstream. For MIR163, only a very small effect is seen. This needs confirmation in independent replicates and statistical assessment of quantification (point 3).

We have now analyzed three replicates and quantified band intensities with ImageJ. The results are shown in a newly introduced histogram in Fig. 3D.

The manuscript provides novel insights into a connection between splicing of miRNA precursor transcripts and miRNA biogenesis.

The conclusion that specific locations in some but not all introns located 3’ of stem-loop structure in priMIRs can increase levels of the corresponding mature miRNAs contrasts with the conclusion of the accompanying paper that the 5’ splice site of introns in priMIRs makes a major contribution to increased levels of the corresponding mature miRNAs.

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We have now added a paragraph in our discussion highlighting the differences between the two manuscripts and providing potential explanations.

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1. Figure 2, the authors please provide the information on the nature of mutation that abolishes splicing.

This has been added.

2. Please quantify the levels of miRNAs in various northern blot.

We have now quantified miRNA levels by quantitative RT-PCR from independent experiments (*N. benthamiana* transient assays). The results are shown as histograms throughout the manuscript.

3. Please draw a cartoon model for the proposed action of 3' intron.

A model is now included in the supplemental material.

4. The font of reference is different with other sections

This has been changed.

There is an inconsistence in these two manuscripts. Bielewic et al show that mutated 5' splicing site affects miRNA accumulation, while schwab find that mutated 5' splicing site does not. If possible, the authors shall provide explanation for this inconsistence, which may provides mechanic insight on how 3' intron affects miRNA accumulation.

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However, I still believe that some improvements can be made to make the paper clearer and the science more solid.

Page 9: The author stated that there was no effect on the primary transcript level with or without an intron. However, the added claim stating that the "IME-like mechanism is unlikely to account for the increase miRNA levels" seems farfetched and is confusing without a clear explanation of the relationship between the IME-like mechanism and intron-related miRNA accumulation.

Page 11: The author claimed that they did not find evidence for a substantial effect of splicing on miRNA accumulation. They used a mutation of the splicing sites to draw this conclusion. However, have the authors tried to measure miRNA levels in splicing mutants, such as the sr mutants? And how many mutants have they examined, as reproduction of their observations in more than one mutant would be more convincing? Either they should include this, or perhaps modulate their claims.

Some minor edits/suggestions:
Figure 1. Middle panel in B, middle panel in C, and bottom panel in D. The authors did not specify what the histograms represent (not in the figure legend nor the body of the manuscript). Please specify. And please also indicate the level of significance. This also applies to all the histograms in figure 2 and 3.
Supplemental Figure 1. Please specify the A, B, C, and D panels (there are no A, B, C and D listed in the figure).
Last paragraph on Page 8: missing a period.

Referee #2:
In the revised version, the authors have addressed most of the points raised by the reviewers. The new qPCR data are valuable to support their conclusions. The explanations for the discrepancies observed between their work and the accompanying paper are not yet satisfying for the reader.

Referee #3:
The revised manuscript has addressed all my concerns.

2nd Revision - authors' response 09 April 2013

For all the main figures, please specify the error bars and the number of experiments performed (n) in the figure legends.

Done

If less than 3 independent experiments were performed, no error bars can be shown.

*We now only show error bars for the technical replicates (standard errors of the means).*

In this case, please show the actual data points for both experiments.

Done.

If you include p-values, as referee 1 suggests, please also specify the statistical test used to calculate the p-value in the figure legend.

*We prefer not to show p-values as the points we want to make are not about small but statistically*
significant differences, but about those that are larger. We did include an extensive documentation of our qRT-PCT analysis as a Supplemental Methods section.

Referee #1:

In this revised paper by Schwab et al., the authors have performed supplementary experiments and better supported their original hypothesis that introns enhance mature miRNA accumulation. However, I still believe that some improvements can be made to make the paper clearer and the science more solid.

Page 9: The author stated that there was no effect on the primary transcript level with or without an intron. However, the added claim stating that the "IME-like mechanism is unlikely to account for the increase miRNA levels" seems farfetched and is confusing without a clear explanation of the relationship between the IME-like mechanism and intron-related miRNA accumulation.

*We modulated our concluding sentence to make this clearer - in the end the point we want to make is that there is indeed no relationship between the two mechanisms.*

Page 11: The author claimed that they did not find evidence for a substantial effect of splicing on miRNA accumulation. They used a mutation of the splicing sites to draw this conclusion. However, have the authors tried to measure miRNA levels in splicing mutants, such as the sr mutants? And how many mutants have they examined, as reproduction of their observations in more than one mutant would be more convincing? Either they should include this, or perhaps modulate their claims.

*We did modulate those claims and do now no longer generalize on 'splicing' but only refer to the introns we are looking at - the ones adjacent to the microRNA precursors.*

Some minor edits/suggestions:

Figure 1. Middle panel in B, middle panel in C, and bottom panel in D. The authors did not specify what the histograms represent (not in the figure legend nor the body of the manuscript). Please specify. And please also indicate the level of significance. This also applies to all the histograms in figure 2 and 3.

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*fixed*

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In the revised version, the authors have addressed most of the points raised by the reviewers. The new qPCR data are valuable to support their conclusions.

The explanations for the discrepancies observed between their work and the accompanying paper are not yet satisfying for the reader.

*We extended these explanations.*

Referee #3:

The revised manuscript has addressed all my concerns.
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.

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.