The role of Mediator in small and long noncoding RNA production in Arabidopsis thaliana

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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 08 September 2010

Thank you for submitting your manuscript to the EMBO Journal. The manuscript has now been seen by three referees and their comments to the authors are provided below.

While referee #1 is not persuaded that the advance provided is sufficient to consider publication in the EMBO Journal, referees #2 and 3 are much more supportive of the study and find it interesting and suitable for publication in the EMBO Journal pending adequate revisions. Given the interest and the comments provided by referees #2 and 3, I will go with their overall recommendation and ask you to submit a revised manuscript for our consideration. There are a number of different concerns that have to be addressed in a revised version, including the specific ones raised by referee #1.

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 preparing your letter of response to the referees' comments, please bear in mind 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 REPORTS:

Referee #1 (Remarks to the Author):

The authors identified a developmental mutant with fairly low penetrance (reportedly 20%) resulting from a mutation mapping to a putative subunit of the mediator complex, a protein complex that facilitates RNA polymerase II transcription. Insertion mutants of two other mediator subunits reportedly have the same phenotypes. The authors show that many miRNA genes, which are transcribed by RNA polymerase II, are less highly expressed in the mediator mutants, and the expression of a subset of other genes is also affected at least 2-fold. One expects mediator mutants to affect RNA polymerase II transcription, so these results are not too surprising. But it would be interesting to know why only a subset of genes is affected, including miRNA genes. If the authors could provide some mechanistic insights along these lines, it would make the paper more interesting and suitable for EMBO. The most interesting result in the paper may be that several transposon families turn on in the mediator mutants, which is apparently related to a role for RNA polymerase II in making so-called scaffold transcripts needed for RNA silencing. Again, some mechanistic insight into how this occurs would improve the paper. Less than 2-fold affects on RNA polymerase V chromatin IP are shown, but these small effects are of uncertain significance. Overall, the work is publishable, but lacks the depth or insight one expects of an EMBO paper.

Specific comments:
- quantitative data for the phenotype occurrence in the mutants should be provided, as well as data showing the rescue of the phenotypes with the MED20a transgene.
- does the transgene rescue line also rescue the miRNA level reductions?
- quantitative data is needed for the promoter-reporter gene fusion experiments, which the authors use to conclude that miRNA promoter activity is reduced in mediator mutants
- data to show that the different mediator subunits interact directly or indirectly as part of a complex would be useful.
- data to show that they interact with RNA polymerase II would also be useful.
- do double or triple mutants of the mediator subunit mutants have stronger phenotypes than the single mutants?
- can a miRNA decrease explain the developmental phenotypes?

Referee #2 (Remarks to the Author):

Kim et al. isolated a med20a mutant, which showed similar phenotypes to nrpb2-3. MED20a is a component of the Mediator complex and the authors analyzed two other Mediator component mutants, med17 and med18, as well. They showed developmental defects similar to those of med20a. Then the authors showed that MED20a (and the Mediator) is involved in the miRNA biogenesis at the pri-miRNA transcriptional step and some siRNA production.

The content is attractive and informative and deserves to be published in EMBO Journal, as long as the following comments are carefully considered.

Major points

Fig.1 data: regarding to phenotypes, analysis of other alleles is necessary to make statements more conclusive. Or cannot the authors show phenotypes of the complemented plants which are described in Supplemental information?

Fig. 2A: The blot patterns are not as clear as Fig.2B or 2C. Most of all, U6 bands are not consistent between lanes. Also describe some statistical analysis of Fig.2 data.

Fig.3A: The authors discussed the pri-miRNA accumulation. How did the authors consider the
existence of multiple alleles corresponding to some miRNAs? miR 159, 167 and 171 have three, four, and three independent alleles, respectively. If only one species of pri-miRNA was analyzed, can we discuss overall aspects of Mediator contribution in miRNA biosynthesis? For example, in Fig.3A, pri-miR166a showed only 30% reduction. How do the authors explain this? The authors should describe the details on the regions which were amplified in real time PCR, for example, in supplementary information.

Fig.5: The authors should make these data and description in text (P,13) easier for readers to understand. They can split the 5C result into regions A and B data or those of RNA involving PolIV or involving PolV, Pol II.

P,11, L.15: None of the known genes in miRNA biogenesis were affected in med20a. Which genes have the authors analyzed? Some details should appear in Supplementary information. I imagine that a dozen of genes are involved in miRNA biosynthesis and was rather surprised that no effects were observed in the analysis. Then, can the authors state that Mediator is authentic general transcriptional factor?

Minor points

Order of samples in blot analysis and graph: I felt strongly uneasy since the miR designations appear seemingly at random. Why cannot they show them in the order of number?

Referee #3 (Remarks to the Author):

This is an important paper describing the role of a Mediator complex component - MED20a - in transcription of both protein-coding and non-coding RNAs. The analysis of protein-coding RNAs is the introduction to the system and indicates that med20a and a previously characterised Pol II mutant nrpb2-3 have a similar effect on mRNA accumulation. It would be interesting perhaps to know whether the effect on these protein-coding mRNAs is associated with basal transcription (as with non-coding RNA) or inducible expression. It would also be good to have confirmation that the effects on protein-coding mRNAs is affected by multiple mediator subunits - MED17 and 18 - as has been done with the non-coding RNA analysis.

The miRNA analysis is novel and will stimulate parallel assays with animal systems to find out whether mediator is also involved in biogenesis of miRNAs across the different kingdoms. However the most interesting part of the whole paper to my mind is the part in which non-coding transcripts associated with heterochromatin formation are assayed.

The data with these heterochromatic systems indicates that the Mediator complex is required for silencing of these loci although not necessarily for siRNA production. The data indicate, at least for type II loci, that mediator is involved in production of scaffold transcripts required for siRNA mediated targeting. For the type I loci at which pol V is associated with targeting the production of scaffold RNA is not affected by the Mediator mutations although the silencing process was and the situation is more complex. Pol V occupancy is affected at these loci.

Overall the paper is very good and suitable for publication with only minor revision. My minor comments are:

i That the gus histochemistry in figure 3 is not convincing and should be quantified if possible.

ii The reasoning in paragraph 2 of page 11 - Although we showed that the ..... - is weak and the paragraph could be omitted. Data in the next paragraph also address the likely action of Mediator at the level of transcription.

iii The pol II occupancy difference at AtSN1B in the med20a mutant is not convincing. Please include statistical analysis.
Referee #1 (Remarks to the Author): The authors identified a developmental mutant with fairly low penetrance (reportedly 20%) resulting from a mutation mapping to a putative subunit of the mediator complex, a protein complex that facilitates RNA polymerase II transcription. Insertion mutants of two other mediator subunits reportedly have the same phenotypes. The authors show that many miRNA genes, which are transcribed by RNA polymerase II, are less highly expressed in the mediator mutants, and the expression of a subset of other genes is also affected at least 2-fold. One expects mediator mutants to affect RNA polymerase II transcription, so these results are not too surprising. But it would be interesting to know why only a subset of genes is affected, including miRNA genes. If the authors could provide some mechanistic insights along these lines, it would make the paper more interesting and suitable for EMBO. The most interesting result in the paper may be that several transposon families turn on in the mediator mutants, which is apparently related to a role for RNA polymerase II in making so-called scaffold transcripts needed for RNA silencing. Again, some mechanistic insight into how this occurs would improve the paper. Less than 2-fold affects on RNA polymerase V chromatin IP are shown, but these small effects are of uncertain significance. Overall, the work is publishable, but lacks the depth or insight one expects of an EMBO paper.

Response: It should be clarified that the med20a mutant has fully penetrant, pleiotropic phenotypes (such as dwarfism, late flowering and reduced fertility, which are documented in the revised manuscript by Figures 1A, 1B, and S1B). The one aspect of the phenotypes that is not fully penetrant is the presence of three, instead of two, cotyledons and first true leaves (Figure S1A). The text was modified to prevent misunderstanding on this issue.

specific comments:
- quantitative data for the phenotype occurrence in the mutants should be provided, as well as data showing the rescue of the phenotypes with the MED20a transgene.
Response: Although this comment was likely related to the misunderstanding of the penetrance of the various aspects of the phenotypes, we provided quantitative data for the reduced fertility of the mutants (Figure S1D). We also provided data showing the rescue of the phenotypes by the MED20a transgene (Figure S1C).

- does the transgene rescue line also rescue the miRNA level reductions?
Response: Yes. Data supporting this conclusion are now included in Figure S3B.

- quantitative data is needed for the promoter-reporter gene fusion experiments, which the authors use to conclude that miRNA promoter activity is reduced in mediator mutants
Response: We performed realtime RT-PCR to determine the levels of the GUS reporter mRNA in wild type and med20a and showed that the reporter gene was expressed at much lower levels in med20a than in wild type. The results are now in Figure 3C.

- data to show that the different mediator subunits interact directly or indirectly as part of a complex would be useful.
- data to show that they interact with RNA polymerase II would also be useful.

Response: We did not explicitly mention this in the manuscript, but the Arabidopsis Mediator was biochemically characterized by a previous study (Backstrom et al. Molecular Cell 26, 717-729), which demonstrated that the subunits form a complex and that Mediator co-purified with Pol II.
-do double or triple mutants of the mediator subunit mutants have stronger phenotypes than the single mutants?

Response: We did not generate the double or triple mutants because it would not be easy to interpret the phenotypes. MED20a has two other paralogs, and if they are functional, we would expect all mutant combinations involving med20a (such as med20a med17 or med20a med18) to be more severe than med20a in phenotypes. Similarly, med17 and med18 may not be null alleles such that when they are combined with other med mutations, phenotypic enhancement is expected and, if observed, does not lead to insights into the relationship among the Mediator subunits.

-can a miRNA decrease explain the developmental phenotypes?

Response: Given the pleiotropic nature of the phenotypes, it is unlikely that reduced accumulation of a single miRNA is responsible for the phenotypes.

Referee #2 (Remarks to the Author): Kim et al. isolated a med20a mutant, which showed similar phenotypes to nrpb2-3. MED20a is a component of the Mediator complex and the authors analyzed two other Mediator component mutants, med17 and med18, as well. They showed developmental defects similar to those of med20a. Then the authors showed that MED20a (and the Mediator) is involved in the miRNA biogenesis at the pri-miRNA transcriptional step and some siRNA production. The content is attractive and informative and deserves to be published in EMBO Journal, as long as the following comments are carefully considered.

Major points
Fig.1 data: regarding to phenotypes, analysis of other alleles is necessary to make statements more conclusive. Or cannot the authors show phenotypes of the rescued plants which are described in Supplemental information?

Response: We included the phenotypes of the rescued plants in Figure S1C.

Fig. 2A: The blot patterns are not as clear as Fig.2B or 2C. Most of all, U6 bands are not consistent between lanes. Also describe some statistical analysis of Fig.2 data.

Response: We re-did all the northern blots in the original Figure 2A as well as northern blots in the same genotypes for more miRNAs. The new data, which are of higher quality, are now in Figure 2A and Figure S3A. The observed reduction in miRNA levels in Fig. 2 was confirmed by 2-3 biological replicates.

Fig.3A: The authors discussed the pri-miRNA accumulation. How did the authors consider the existence of multiple alleles corresponding to some miRNAs? miR 159, 167 and 171 have three, four, and three independent alleles, respectively. If only one species of pri-miRNA was analyzed, can we discuss overall aspects of Mediator contribution in miRNA biosynthesis? For example, in Fig.3A, pri-miR166a showed only 30% reduction. How do the authors explain this? The authors should describe the details on the regions which were amplified in real time PCR, for example, in supplementary information.

Response: As suggested, we studied the accumulation of pri-miRNAs from each member of an miRNA family (we chose the miR166 family for this purpose). The results are in Figure S5. Also as suggested, we included diagrams of the regions in pri-miRNAs examined in Figure S4.

Fig.5: The authors should make these data and description in text (P,13) easier for readers to understand. They can split the 5C result into regions A and B data or those of RNA involving PolIV or involving PolV, Pol II.
Response: Yes, we included a bit more description in the text. We also boxed the parts on region A and region B separately in Fig. 5C.

P.11, L.15: None of the known genes in miRNA biogenesis were affected in med20a. Which genes have the authors analyzed? Some details should appear in Supplementary information. I imagine that a dozen of genes are involved in miRNA biosynthesis and was rather surprised that no effects were observed in the analysis. Then, can the authors state that Mediator is authentic general transcriptional factor?

Response: We provided a list of genes with known roles in miRNA biogenesis in Table S3. As to the comment on why none of these genes are affected in med20a, this is not surprising since less than 1000 genes are affected in med20a (out of all genes represented on the array). The small effect of med20a is probably because of the existence of other MED20 paralogs.

Minor points Order of samples in blot analysis and graph: I felt strongly uneasy since the miR designations appear seemingly at random. Why cannot they show them in the order of number?

Response: The blots were re-ordered.

Referee #3 (Remarks to the Author): This is an important paper describing the role of a Mediator complex component - MED20a - in transcription of both protein coding and non coding RNAs. The analysis of protein-coding RNAs is the introduction to the system and indicates that med20a and a previously characterised Pol II mutant nrpb2-3 have a similar effect on mRNA accumulation. It would be interesting perhaps to know whether the effect on these protein coding mRNAs is associated with basal transcription (as with non coding RNA) or inducible expression. It would also be good to have confirmation that the effects on protein coding mRNAs is affected by multiple mediator subunits - MED17 and 18 - as has been done with the non coding RNA analysis. The miRNA analysis is novel and will stimulate parallel assays with animal systems to find out whether mediator is also involved in biogenesis of miRNAs across the different kingdoms. However the most interesting part of the whole paper to my mind is the part in which non coding transcripts associated with heterochromatin formation are assayed. The data with these heterochromatic systems indicates that the Mediator complex is required for silencing of these loci although not necessarily for siRNA production. The data indicate, at least for type II loci, that mediator is involved in production of scaffold transcripts required for siRNA mediated targeting. For the type I loci at which pol V is associated with targeting the production of scaffold RNA is not affected by the Mediator mutations although the silencing process was and the situation is more complex. Pol V occupancy is affected at these loci. Overall the paper is very good and suitable for publication with only minor revision.

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- That the gus histochemistry in figure 3 is not convincing and should be quantified if possible.

Response: This was addressed by realtime RT-PCR on GUS mRNAs (Fig 3C).

- The reasoning in paragraph 2 of page 11 - Although we showed that the ..... - is weak and the paragraph could be omitted. Data in the next paragraph also address the likely action of Mediator at the level of transcription.

Response: The paragraph is reduced to two sentences.
- The pol II occupancy difference at AtSN1B in the med20a mutant is not convincing. Please include statistical analysis.

Response: We provided data on all three biological replicates (Fig. 5D and Fig. S8), which showed the same trend.

Acceptance letter

22 December 2010

You revised manuscript has been reviewed once more by one of the original referees, who finds that you have addressed all the concerns raised and recommends publication in The EMBO Journal. I am happy to accept the manuscript for publication, you will receive the official acceptance letter in the next day or so.

Best wishes,

Editor
The EMBO Journal

Referee #2

I am satisfied to see the authors' responses to issues addressed by three referees. Quantification and the quality of gel analysis of the original draft, which was the largest obstacle to appear in EMBO Journal in my opinion, were almost well revised.

Similar results with "general factors" in transcription and translation areas are recently well documented in other areas, thus such Mediator effects should appear as well at this time.