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Peer Review Report for 10.1105/tpc.16.00554

DNA Methylation Influences the Expression of DICER-LIKE4 Isoforms, Which Encode Proteins of Alternative Localization and Function

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Review timeline:

TPC2016-00554-RA	Submission received:	July 11, 2016
	1st Decision:	Sep. 7, 2016 *revision requested*
TPC2016-00554-RAR1	1st Revision received:	Sep. 30, 2016
	2nd Decision:	Oct. 18, 2016 *accept with minor revision*
TPC2016-00554-RAR2	2nd Revision received:	Oct. 21, 2016
	3rd Decision:	Oct. 26, 2016 *acceptance pending, sent to science editor*
	Final acceptance:	Nov. 12, 2016
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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2016-00554-RA 1st Editorial decision – *revision requested* Sep. 7, 2016

As you will see from the detailed comments of two reviewers, they appreciate your work and attest to its novelty with clear, sufficient data and good documentation. However, there are a few points to be addressed in a revision. The first question of reviewer 1 can hopefully be addressed with bioinformatic tools, not requiring additional experiments. Probably, you can also answer question 2 based on existing data. The concern of reviewer 2 is mainly about some (over)interpretation, so that her/his points can also be considered by modifying the text or the data documentation. Some problems with Figure 2A are mentioned by both reviewers, so please try to improve it.

[Reviewer comments shown below along with author responses]

TPC2016-00554-RAR1 1st Revision received Sep. 30, 2016

Reviewer comments and author responses:

Reviewer #1:

The manuscript by Pumplin et al., entitled "DNA methylation influences expression of DCL4 isoforms, which encode proteins of alternative localization and function" describes the identification of a DCL4 isoform that specifically accumulates in the nucleus in Brassicaceae species. They showed by expression analysis that the expression of the long form of DCL4 is regulated by DNA methylation in the promoter region, and a loss of DNA methylation of the region was induced in the endosperm or by pathogen infection. The long form of DCL4 is responsible for specific 21nt siRNA named disiRNAs, which contributes to silencing of endogenous transposable elements (TEs). Overall, the work has a high scientific quality, with the elegantly performed experiments and the conclusions well supported by a large amount of data. Their finding of an isoform of DCL4 involved in TE silencing is novel, and would further contribute to our understanding of RNAi machinery for TE silencing and gene regulation.

Point 1. I would like to know the nature of the promoter sequence of DCL4, the details of which were not described in the manuscript. Is that related to TE sequence? Are there similar sequences in the genome? Is the sequence conserved among Brassicaceae species, coevolved with the NLS sequence in the 1st exon of DCL4?

RESPONSE: No similarity to known TE sequences was found based on repeat masker searches using default parameters. In addition, the siRNAs arising from this locus are unique matches, meaning they cannot arise from other regions in the genome and are also unlikely to target other sequences. We have indicated this information in the text. The question about coevolution of the promoter sequence with the NLS-encoding CDS is interesting. We do not find any similarity between the methylated region of *Arabidopsis thaliana* and the upstream regions of *A. lyrata*, *Brassica rapa* or *Capsella rubella*. It is entirely possible that promoter methylation was maintained through evolution by chromatin states, rather than sequence conservation. The nature of this regulation in related Brassica species certainly deserves more detailed analysis.

Point 2. Does DCL4 control its own expression, as observed in ROS1? For example, does dcl4 cause hypomethylation and/or reduction of siRNAs in its own promoter?

RESPONSE: DCL4 does not regulate its own expression or methylation status. Specifically, we have tested *dcl4-2* mutant plants and the transcript is expressed similarly to a WT plant. This is an interesting contrast between ROS1. Because ROS1 directly impacts the methylation state of the genome with its function, the autoregulation on its own promoter by this same pathway is a truly clear and elegant example. DCL4 responds to methylation state, but itself does not impact DNA methylation, and thus cannot autoregulate.

Point 3. I was confused with the result in Fig 2C. Even though the DCL4 form with NLS was highly expressed under the Ubq10 promoter, I guess still a large amount of DCL4 without NLS accumulates in the cells, which would result in GFP signals in cytoplasm.

RESPONSE: These constructs with Ubq10 promoter express DCL4 exclusively with the NLS sequence when it is encoded (see qPCR results from Figure 2F). The NLS sequence confers efficient nuclear import of DCL4. In some microscopy experiments, a weak background signal can be observed in the cytoplasm, but the localization of this protein form is consistently strongly nuclear (compare also with Figure 2B and 4D).

Point 5. The figure legend for Fig2A is difficult to follow.

RESPONSE: We have modified Figure 2A and the accompanying legend for clarity.

Reviewer #2:

The manuscript by Pumplin et al. describes two isoforms of DCL4 and their regulation and specific biological functions. It starts with showing that DCL4 has two alternative TSSs, and the choice between those sites is controlled by an RdDM target site within the gene's promoter. This is an interesting and very well documented finding. This is also a particularly strong part of the manuscript, which could be a worthy publishable unit on its own. The manuscript then shows that the DCL4 isoforms have different subcellular localizations and shows two mechanisms responsible for their localizations. Although not as elegant as the first part of the manuscript, this is well documented and important because localization differences are interpreted as a basis for functional differences. Finally, a large body of work is devoted to showing unique siRNA products of the isoforms and their specific biological functions. Some functional specificity is shown, however the manuscript does not fully convince about the functional importance of the isoforms.

Overall, this is a very strong manuscript, which describes an interesting phenomenon and presents a huge amount of high quality data. It significantly exceeds the standards of the field in the strength of proof (at least in its first half). It is also very well written. Criticism is focused on some over interpretations, which should be easy to correct in revision and do not reduce the enthusiasm for this work.

Point 1. There are 118 loci dependent on the longer isoform of DCL4. The manuscript would benefit from a more thorough explanation of the stringency of calling/validation and the extent of sRNA changes compared to other mutants in DCLs. This would help the readers better understand the importance of DCL4 isoforms for sRNA biogenesis. Also, reported changes in gene expression are very small, which further indicates that the role of DCL4 isoforms may be overestimated. A conclusion that this role is very minor would not decrease the overall impact of this work.

RESPONSE: We had previously outlined the analysis criteria and thresholds applied in the methods sections, and we also highlighted the fact that our sRNA sequencing experiment did not include biological replicates. This aspect limits the power to make significant statements about whole-genome effects.

To clarify these points for the reader, we have updated the figure 6 legend with the specific threshold used for calling candidates and updated the results section to state that we sought to identify candidates for manual validation. We have also inserted the comparison that approximately 5% of DCL4-dependent loci are candidate disiRNAs. We have inserted language into abstract, results and discussion sections to explicitly state that the changes are minor, and that DCL4 does not play a major role in seed development. We agree that the changes to gene expression are limited and subtle. This aspect of the story was presented as a minor aspect of the work; however, the impact on gene expression remains significant and an important sign that the silencing pathway is functioning during seed development. We also make the point in the manuscript that miRNA-mediated regulation can also manifest itself in minor effects to gene expression, particularly when whole-tissues are analyzed if silencing effects are restricted to certain cell types.

Point 2. The order of subsections in the results could be reconsidered, as data addressing similar questions appear to be split into non-consecutive subsections. "The DCL4 promoter methylation state directly influences TSS usage" is separated from the first subsection by localization data. Analysis of DCL4 orthologs also appears out of place.

RESPONSE: We agree that the focus jumps back and forth, and we have experimented with different arrangements of the sections. The questioned section on methylation/expression of individual transgenic lines follows the presentation of the basic conclusions from these lines. Moving the methylation section to an earlier place would require substantial reorganization of the other sections and of Figure 2. We also believe that discussing orthologs of Arabidopsis DCL4 is placed best following the description of what we learned about localization from Arabidopsis. If the editors feel that the current order impedes the clarity of the results, we are willing to work together on a reorganization of the sections.

Point 3. Data about relative abundance of the isoforms is shown as ratio of long vs. total. It is unclear how this number can be greater than 1. It would be better to show percentages of long and short and if needed also the total amount (which may require redoing the qPCR assays with standard curves) or at least better explain the existing assay.

RESPONSE: The qPCR measurement testing the relative abundance of long vs. total DCL4 is used widely in this work, and we added an additional sentence in the results to make the approach clear. This comment refers to the analysis in Figure 2F, which uses the same approach as the other measurements but expressed as a percentage (compare for instance 3B. Measurements in Figure 1 are shown in proportion to the control, set to 1). However, this is not an exact measurement because slight differences in primer efficiency (which are similar between pairs but not exact) can skew the results, and the reviewer is correct that expressing the number as a percent can be misleading. We do not attempt to measure exactly the percentage of Long DCL4 expressed, but rather measure the changes between samples. Thus, we have changed Figure 2F to relative expression, as shown in 3B, and adapted the text accordingly in addition to the added description.

Point. 4. Data about Pseudomonas infection appear only remotely relevant and could be easily removed from the manuscript. If the authors want to keep these data, I would suggest a more thorough explanation.

RESPONSE: While we do not further develop the topic of bacterial pathogens in this work, it serves as an additional piece of evidence correlating promoter methylation changes to isoform expression changes. It is particularly useful because WT plants are used and the same tissue is compared between mock and infected samples, and we thus

believe that it strengthens our general argument that DCL4 isoform expression is influenced by methylation. The purpose of including these data to highlight a further example of correlation has been added to the text.

Point 5. Fig. 2A is not as clear as most other figures in the manuscript. I would suggest showing a slightly expanded diagram in the main figure and moving the nucleotide sequence to the supplement.

RESPONSE: We have followed this suggestion and rearranged Figure 2A, moving the sequence information to a supplemental figure.

Point 6. The claimed direct causal role of promoter methylation in determining TSS choice (page 11) appears to be an over interpretation. The evidence is pretty strong but more careful phrasing would be more appropriate as alternative explanations are not impossible.

RESPONSE: We have clarified the argument in the section to specify that this result likely rules out a major contribution from other regions of the genome, which could influence DCL4 TSS selection during times of whole-genome hypomethylation.

Point 7. The authors came up with a new named class of small RNA, "disiRNA". Given the small number of identified loci and their unclear role, I would suggest rethinking if naming these RNAs is appropriate at this time. Proliferation of named small RNA classes creates a lot of confusion and should probably be avoided.

RESPONSE: We agree that adding new classes of siRNAs is done too often these days. In spite of the limited number and impact of these small RNAs, we believe that the biosynthesis pathway (PolIV/RDR2 production of precursors and dicing by DCL4^{NLS}) is an important distinction that warrants a new name for simplicity of future discussions.

Point 8. The overlap between 21 and 24 nt sRNAs is interpreted as evidence of a common precursor but this could also be explained by technical issues like sequencing bias or truncation of 24 nt sRNAs.

RESPONSE: We believe that the data clearly show the 21nt siRNA products to be genuine and not artifacts. Northern data presented clearly preclude sequence bias as an explanation. Truncation of 24nt sRNAs would likely result in intermediate 23- and 22nt long species, which were never observed. Furthermore, the fact that DCL4 produces 21nt siRNAs is extremely well documented throughout the literature.

Point 9. PTGS from Pol IV/RDR2-produced precursors is an interesting finding and could be further discussed.

RESPONSE: We agree! We believe that the extensive discussion is appropriate however, considering the limited biological function we can currently establish for disiRNAs. It would be premature to speculate too wildly about the potential for PTGS derived from this pathway before its biological relevance is more broadly demonstrated. We do hope that the key concept is clearly presented in the discussion, namely that PTGS arises from PolIV/RDR2 when the genome is hypomethylated and thus during a time when the RdDM pathway is less effective and PTGS can provide a substitute to buffer gene expression.

TPC2016-00554-RAR1 2nd Editorial decision – *accept with minor revision* Oct. 18, 2016

As you will see from the comments, your documentation of data and the responses to the reviewers' questions were appreciated, and both reviewers want to see your work published. However, we agree with reviewer 2 that your argumentation, addressing the concern from the first round of review, should have been better and more included in the manuscript, so that the statements and explanations are accessible to the future readers, not only to the reviewers. Therefore, we kindly ask you for another round of revision, incorporating some points into the manuscript.

[Reviewer comments shown below along with author responses]

TPC2016-00554-RAR2 2nd Revision received Oct. 21, 2016

Reviewer comments and author responses:

Reviewer #1:

The authors addressed all points I raised in the revised manuscript, and I do not have further comments.

Reviewer #2:

The authors have been surprisingly reluctant addressing reviewers' comments in the revised manuscript text. Although the Response document explains their reasoning, only isolated small changes made it to the manuscript. This is unfortunate since the readers, not the reviewers, need to see the authors' reasoning explained.
My sole major comment has been addressed in the text (although very briefly), so I do not see any outstanding major issues with the manuscript, however I am disappointed that the review process made so little impact on the explanations and interpretations presented in the manuscript.

RESPONSE: Following the reviewer's reasonable request that the questions raised during review be used to inform the manuscript, we have updated the manuscript to:

Explain that the methylated region of DCL4 is a unique sequence in the *Arabidopsis thaliana* genome. We have also explained that DCL4 promoters of *A. lyrata*, *B. rapa* and *C. rubella* do not contain a homologous sequence with the methylated region of *A. thaliana*, but that sequence conservation is not a prerequisite for maintenance of methylation.

Expand the rationale for including data about Pseudomonas infection in the results and discussion.

Clarify that the DCL4 NLS confers efficient nuclear import, thus resulting in minimal cytoplasmic signal in fluorescent reporter fusions. Also pointed out the measurement in qPCR that the Ubq10 promoter makes NLS-encoding transcript.

Include an additional summary of why we conclude that the same dsRNA precursor gives rise to overlapping disiRNAs and 24nt of discrete sizes, stressing that disiRNAs are only a small proportion of DCL4-dependent products and not likely due to experimental artifacts.

Explain that DCL4 does not autoregulate in the manner of ROS1, and referred to data in Supplemental Figure S10 which shows this.

We believe that these expanded explanations and interpretations sufficiently reflect the information brought to light during the review process and that the current manuscript can be interpreted by readers in a more complete manner. Finally, if the manuscript is accepted for publication, we would like to discuss options for publishing the raw data files, originally intended to support the review process, as additional supplementary material available to the broad readership.

TPC2016-00554-RAR2 3rd Editorial decision – *acceptance pending* Oct. 26, 2016

We are pleased to inform you that your paper entitled "DNA methylation influences expression of DCL4 isoforms, which encode proteins of alternative localization and function" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor Nov. 12, 2016