ELF18-INDUCED LONG NONCODING RNA associates with Mediator to enhance expression of innate immune response genes in Arabidopsis

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Review timeline:

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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.)

TPC2016-00886-RA 1st Editorial decision – *revision requested* Jan. 8, 2017

We have received reviews of your manuscript entitled "Elf18-induced long noncoding RNA associates with Mediator to enhance expression of innate immune response genes in Arabidopsis." Thank you for submitting your best work to *The Plant Cell*. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

Both reviewers were positive in their overall assessment of your work - it's interesting, appropriate for this journal, and novel. Yet, both reviewers also identified numerous issues that need to be addressed, apparent in the figures describing your results. In a number of experiments there are missing controls, mutant phenotypes ascribed based on a single allele, etc. Reviewer #2 found Figure 6 to be particularly problematic, yet those data are important to the story. The revisions suggested by the reviewers are mostly small, although there are many of them, but we believe these are important changes to make. Finally, please be sure to submit your RNA-seq data to Genbank's GEO and obtain accession numbers prior to submission of a revised version.

Note that the sampling and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2016-00886-RAR1 1st Revision received Mar. 6, 2017

Reviewer comments and **author responses**:

Editor's Comments:

Both reviewers were positive in their overall assessment of your work - it's interesting, appropriate for this journal, and novel. Yet, both reviewers also identified numerous issues that need to be addressed, apparent in the figures describing your results. In a number of experiments there are missing controls, mutant phenotypes ascribing based on a single allele, etc. Reviewer #2 found Figure 6 to be particularly problematic, yet those data are important to the story. The revisions suggested by the reviewers are mostly small, although there are many of them, but we believe these are important changes to make. Finally, please be sure to submit your RNA-seq data to Genbank's GEO and obtain accession numbers prior to submission of a revised version.

We have added supporting data to reinforce our interpretation of Figure 6. In addition, we have provided a detailed description of double mutants in the Methods. Our RNA-seq data have been uploaded to Genbank's GEO with the accession number GSE93560.

Note that the sampling and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

Biological replicates in this manuscript mean 3 independent seedling pools. We have so stated in the legend of each figure.

---------- Reviewer comments:

Reviewer #1:

It has been known that non-coding RNAs play diverse roles in animals and human, however, there are few reports on plant non-coding RNAs. This manuscript revealed a role for a long non-coding RNA in plant pathogen resistance. The result is very interesting; however, the following concerns need to be addressed:

1. In Figure 3, the change trends are not very similar between A and B.

In WT, the maximal induction level of *ELENA1* by elf18 is 10-20 times compared to the basal level. But in *ELENA1* OX or mutated *ELENA1* OX plants *ELENA1* expression levels are over 1000 times higher, compared to the basal level. We have analyzed 4 different OX lines with varying *ELENA1* expression levels (1,800 to 3,200 fold) and found that these plants have comparable *PR1* expression levels (new Figure S2). Therefore, different levels of *ELENA1* in the OX plants are not expected to affect *PR1* expression level significantly. We have added *PR1* expression data in different *ELENA1* OX lines (new Figure S2)

2. The author used RNA *in vitro* pull-down assay and RNA immunoprecipitation (RIP) assay to test the binding of *ELENA1* to MED19a. It is also necessary to perform MED19a pull down assays and Northern blots or RT-qPCR to test if *ELENA1* is co-pulled down.

We have done the experiment in the original version of the manuscript. In the RIP experiment we pulled down MED19a protein from *35S:GFP-MED19a* plants using GFP antibody and then performed RT-qPCR with specific primers for *ELENA1*. See Figure 5E.

3. For the competition assay In Figure 5B, a concentration gradient should be set up until all the biotinylated RNA can be completely competed.

We have done this experiment but found that it was technically difficult to reduce the signal further.

4. In Figure 5C, a transgenic line carrying a GFP-vector is necessary to be used as a control.

We have included the GFP-vector negative control in the new Figure 5C.

6. For the triFC assay in Figure 5D, the authors used MED19a fused to nYFP as a negative control, however, the authors used MED19a fused to cYFP to show the results. The conditions are not consistent.

There was a typographical error in the labeling of Figure 5D. We used the same combination (nYFP-MED19a and MSCP-cYFP) for MED19a and MSCP with negative control. We have now corrected this error in the new Figure 5D.

7. In Figure 6C, the double mutant did not shown more serious phenotype than single mutants, the author should try to use plant material at 12 h after elf12 treatment, as the *ELENA1* expression reached a maximum at 12 h of elf18 treatment.

We checked the expression level of *PR1* in double mutants at 12 h after elf18 treatment. Figure 6B shows that in contrast to WT, OX of *MED19a* in the *ELENA1* KD mutant did not increase *PR1* expression. This result shows the effect of MED19a in *PR1* expression is dependent on *ELENA1*. In support of this notion, *PR1* expression in the *ELENA1* KD mutant was not further reduced by a deficiency of MED19a (Figure 6C).

8, In Figure 7B, MED19 enrichment on the *PR1* promoter was highest at 6 h after elf18 treatment, however, the expression of *ELENA1* reaches a maximum at 12 h after elf18 treatment; the authors need to explain this.

The *ELENA1* transcript reached a maximum level at 6 h after elf18 treatment and increased modestly until at least 12 h after treatment (Fig 1B). We checked MED19a protein level in the *35S:GFP-MED19a* transgenic line. We found that the MED19a protein level increased with time of elf18 treatment and reached a maximum at 6 h; after 6 h, MED19a level decreased. A previous paper, Caillaud et al., (2013), showed that MED19a was regulated by ubiquitin-mediated degradation. This suggested that the decrease in MED19a levels 6 hour after elf18 treatment could be due to regulated proteolysis.

9. FISH of *ELENA1* would be helpful to characterize the subcellular/subnuclear localization of the long non-coding RNA.

We have checked the localization of *ELENA1* by TriFC assay. Thank you for your suggestion.

10. To support the authors' claim that *ELENA1* functions upstream of SA signaling, genetic evidence is necessary, such as, generating *EDS16*/*ELENA1* or *NahG* /*ELENA1* double mutants and examining the pathogen resistances of these mutants.

***PR1* and *PR2* are highly responsive to and key marker genes for SA. Our RNA-seq data showed that gene expression levels of most of the SA-responsive genes in *ELENA1* OX were not different compared to WT. Such key SA biosynthesis genes, including *SID2 (EDS16)* and *PAL*, SA conjugation genes, including *NahG*, and signaling TFs, including *WRKY*s and *TGA*s were similar in WT and in *ELENA1* OX plants. Therefore we cannot claim that *ELENA1* functions upstream of SA signaling. Rather we think *ELENA1* may function by an SA-independent signaling mechanism. Therefore we revised the sentence in the Discussion.**

Reviewer #2:

In this manuscript, Seo et al. study the role of a long non-coding RNA, which they name *ELENA1*, in the response to the pathogen elicitor elf18. They find that *ELENA1* is induced by elf18, and that this induction requires the EFR and FLS2 receptors. Using artificial knockdown and overexpression constructs, they show that *ELENA1* plays a functional role in induction of the pathogenesis-related gene *PR1*. They provide evidence that *ELENA1* is indeed a *bona fide* non-coding RNA, and show that *ELENA1* regulates expression of several pathogenesis-related genes. They then perform experiments to determine whether *ELENA1* interacts with the Mediator protein MED19a, and whether this interaction has functional consequences.
This is nice work, and a straightforward story. The most important aspects are identification of the function of a long non-coding RNA, and the demonstration that this lncRNA interacts with a Mediator subunit. Interaction of Mediator (specifically Med12) with a lncRNA had previously been shown in animals, but to my knowledge had not yet been demonstrated in plants, which makes the *ELENA1*-MED19a interaction a significant result.

Despite my overall positive view of this manuscript, there are a number of issues that must be addressed before it can be considered suitable for publication, in particular related to Figure 6, which is sub-optimal in its current form.

1) Abstract: The authors state that *ELENA1* regulates *PR1* expression "through MED19a". This statement refers to

the experiments presented in Figure 6, the weakest part of the paper. Their statement indicates that *ELENA1* requires MED19a to function. But this is not what they show in Figure 6, where *ELENA1* and MED19a appear to act at least partially independently (Figure 6A), or if anything MED19a acts through *ELENA1* (Figure 6C, where the *ELENA1* knockdown is epistatic to *med19a*). It would be better not to make such as strong statement about the functional relationship between *ELENA1* and MED19a (please see my comments on Figure 6 below).

Thank you for pointing this out. We agree that our results show that MED19a acts through *ELENA1*. We have modified the abstract to reflect this point.

Results:
2) First paragraph: the authors should supply more information about how they identified *ELENA1*. By bioinformatics analysis from their previous cited experiment? In a new experiment? What is the sequence of *ELENA1*? No sequence is given- is the sequence in TAIR for AT4G16355 exactly the same as *ELENA1*?

***ELENA1* was among the group of elf18-responsive lncRNAs identified in our previous publication using custom arrays (Liu et al., 2012). We examined *ELENA1* full-length transcript with 5' and 3' RACE experiments and found that the transcript is the almost same length as the TAIR-annotated At4g16355 transcript. Therefore, we used the TAIR annotated sequence for further study.**

3) Line 233: What is the effect of the T-DNA insertion corresponding to the *med19a* allele that they used? At present, there is only a vague sentence in the Materials and Methods that 'absence of target gene expression in homozygous plants was further confirmed by RT-PCR'. This is not good enough, especially because the authors use this allele for what is essentially epistasis analysis in Figure 6- epistasis analysis requires the use of null alleles. The authors need to add a supplementary figure with a Northern or RT-PCR analysis showing the effect of the *med19a*-1 T-DNA insertion on the corresponding mRNA. In addition, it is essential that the authors examine the effect of a second mutation in *MED19A*, and perform a genetic complementation test between *med19a*-1 and the second *med19a* allele, to determine whether the phenotypic effects they show for *med19a* are indeed due to the loss. They need not redo all the experiments with this second *med19a* allele, but they need to show that it has a similar phenotypic effect in pathogenesis, and a similar effect on gene expression (for example on the *PR1* gene). Otherwise, it cannot be ruled out that the phenotypes they observe for the *med19a*-1 allele could be due to a second, unrelated mutation.

We obtained seeds of Salk T-DNA insertion mutants in *MED19a* (*med19a-1 and med19a-2*) from the lab of Dr. Jonathan D.G. Jones. In their publication, Caillaud et al. (2013) have shown *med19a-1* to be a null mutant and they have also performed complementation experiments with *med19a-1*. Therefore we did not do further analysis of *med19a-1* and just analyzed its RNA level in the mutant. We have now provided RT-PCR results of *med19a-1* and *med19a-2* and *PR1* expression in *med19a-2* in the new Supplemental Figure 6.

4) Figure 6 and Figure S9:
This figure and supplementary figure stand out from the rest of the paper, in being of lower quality in terms of execution and in what can be concluded from the figures.
It's problematic that, for each of the experiments represented by different panels, all the lines being considered are different transgenic events. For example, in Figure 6A, three different transgenic lines for overexpression of *ELENA1* are being compared. The authors try to remedy this by including gene expression data for the different transgenic events in Figure S9. But even here they are missing the controls for the different transgenic events used in Figure 6C. Also, the labeling is very confusing, for example in Figure 6A, they label columns '19a-1', 'E/19a-7', and 'E/19a-9'. I presume that '19a-1' denotes *med19a-1* (the allele), while the other columns mean 'E transgenic event #7 / in the *med19a-1* background'.

We have changed the nomenclature of mutants and OX plants to provide easy understanding of the labeling. Furthermore, we have replaced previous double transgenic lines with other lines showing similar expression levels as those of single transgenic lines (Figure S9). *E1/m19a* double mutants (Figure 6A) were generated using *med19a-1* for transformation with *35S:ELENA1*. In Figure S9A, we show that *ELENA1* expression levels in the *E1/m19a* lines were similar to those of *ELENA1* OX -16 in WT background. e1#10/M19a (Figure 6B) were generated by using the *ELENA1* KD-10 for overexpression of *UBQ:MED19a* (Figure S1B). We compared *MED19a* expression levels in these e1#10/M19a lines to that of *MED19a* OX-1 in WT background (Figure S9B). *ELENA1* levels in these mutants were the same with *ELENA1* KD-10 (Figure S1B). In Figure 6C, we have carried out genetic crosses between *ELENA1* KD-10

and *med19a-1* and homozygous double mutant plants were used. Therefore the *ELENA1* transcript level in the e1#10/m19a double mutant is the same as that in *ELENA1* KD-10 mutant (Figure S1B). E1#16/M19a double OX lines (Figure 6C) were generated by using *ELENA1* OX-16 (Figure S2) for a transformation with *UBQ:MED19a*. We compared the *MED19a* expression level in E1#16/M19a plants to *MED19a* OX #1 in WT background (Figure S9C). *ELENA1* levels in these plants were the same as those in *ELENA1* OX-16 in the WT background (Figure S2). We have changed the labeling of mutants (in Figure 6) according to your suggestion and we have also provided more details in the Methods.

Determining the functional relationship between *ELENA1* and MED19a is an important part of the story. Can *ELENA1* function in the absence of MED19a activity, and can MED19a function in the absence of *ELENA1*? To determine this, it is necessary to have a CRISPR or T-DNA null allele of *ELENA1*, so as not to depend on artificial miRNA reduction of *ELENA1* activity (which is not fully penetrant). If this is not possible, then the authors should at least use the same transgenic event for each set of experiments.

We did try to generate a knockout mutant of *ELENA1*, but it was impossible to generate *ELENA1* single knockout mutant because *ELENA1* is located inside the *CBL6* promoter region and the location of *ELENA1* is quite close to *CBL6*. There were two T-DNA insertion mutants (salk_050995, GABI_645D07) between *ELENA1* and *CBL6*. In both of mutants, *ELENA1* and *CBL6* were suppressed. We also tried to generate a knockout mutant in the *ELENA1* locus with CRISPR, but the mutants we obtained were deficient in the expression of both *ELENA1* and *CBL6*. In those knockout mutants of *ELENA1*, *PR1* expression was greatly reduced compared to WT like our artificial miRNA *ELENA1* KD mutants. However, in these *ELENA1* mutants, *CBL6* is not induced after elf18 treatment. In WT, *CBL6* is also induced by elf18 treatment and may affect Ca signaling involving in PAMP signaling. Therefore we did not use those KO mutants because we cannot rule out the function of *CBL6* in *PR1* expression.

The three experiments the authors should do are:

a) Compare the effect on *PR1* expression of the *med19a* mutant, the *ELENA1* loss of function, and the *med19a* / *ELENA1* loss of function double mutant. The phenotype of the double mutant line will tell whether MED19a and *ELENA1* entirely depend on each other for their function (the double mutant has the same phenotype as one of the single mutants), or whether they can also act independently of each other (the double mutant phenotype is additive).

Figure 6C. The *ELENA1* KD mutant showed about 30% *PR1* expression level compared to WT. In this mutant background, knockout of *MED19a* had no further effect on *PR1* expression indicating that the effect of MED19a on *PR1* expression was dependent on *ELENA1*. This conclusion is supported by the results of Figure 6B. In the *ELENA1* KD mutant background, OX of MED19a had no significant effect on *PR1* expression whereas in the WT background, OX of *MED19a* increased *PR1* expression by about 2 fold.

b) Compare the phenotype of an *ELENA1* overexpression line in a wt background with this same overexpression line in a *med19a* background. If *ELENA1* depends entirely on MED19a for its function, loss of MED19a will restore the *ELENA1* overexpressor to a wt phenotype.

Figure 6A. In the *med19a-1* background, OX of *ELENA1* led to a small increase of *PR1* expression levels compared to *med19-1* mutant. These results suggest that *ELENA1* interacts with factors other than MED19a to promote *PR1* expression. Candidate factors include MED19b. We have added the possible explanations for this result in the new Discussion.

c) Compare the phenotype of the *MED19a* overexpression line in a wt background with this same overexpression line in an *ELENA1* null allele (this experiment would only be valid with an *ELENA1* complete loss of function, either a T-DNA or a CRISPR allele). If MED19a requires *ELENA1* for its function in regulating *PR1* expression, then the *MED19a* overexpressor in the *ELENA1* mutant will resemble wt.

All *ELENA1* null mutants were also deficient in *CBL6*, which may play a role in *PR1* expression.

Raw data and supplementary data

5) Finally, I don't see any accession numbers for the raw data for the RNA seq experiment, nor any supplementary table with all of the analyzed data for the RNA seq experiment. Both are essential for publication.

We have now added both. The 24 ssRNA-seq data sets generated in this work have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE93560:

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=abehwqeihnsrzoh&acc=GSE93560

In our previous manuscript (Table S1 and Table S2) we had shown mapping statistics and expression levels of all detected protein coding genes. To further clarify the analysis, in this revised version of the manuscript we have now added another 3 supplementary tables: (1) tabulated data of 535 and 603 protein coding genes that were up-regulated at all time points in WT and OX plants (Table S3, related to Figure S4B,C); (2) tabulated data of 251 differentially expressed lincRNAs (Table S4, related to Figure 4A); (3) tabulated data of Group I-IV genes (Table S5, related to Figure 4C-E).

TPC2016-00886-RAR1 2nd Editorial decision – *acceptance pending* **Mar. 31, 2017**

We are pleased to inform you that your paper entitled "Elf18-induced long noncoding RNA associates with Mediator to enhance expression of innate immune response genes in Arabidopsis" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **April 11, 2017**