Transcriptome-wide Mapping of RNA 5-Methylcytosine in *Arabidopsis* mRNAs and non-coding RNAs

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Review timeline:

TPC2016-00751-RA	Submission received:	Sep. 29, 2016
	1st Decision:	Nov. 16, 2016 *revision requested*
TPC2016-00751-RAR1	1st Revision received:	Dec. 6, 2016
	2nd Decision:	Dec. 15, 2016 *acceptance pending, sent to science editor*
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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.)

TPC2016-00751-RA 1st Editorial decision – *revision requested* **Nov. 16, 2016**

The three reviewers were consistently positive about your work. In fact, we think it's unlikely that you would need to do additional experimental work to address the concerns of the reviewers. One topic of interest to all three reviewers is the extent of the phenotype, and particularly whether you're claiming the phenotype of the mutant is root-specific, or was only observed in roots, or you only measured the roots. If you could clarify this, preferably by describing the extent to which you observed phenotypes in other tissues, or the basis for believing the phenotype is more pronounced in the roots, supported by data, this would be best.

Reviewer #1 had a number of more specific questions, and it would be useful if you could address these points in your revised manuscript.

TPC2016-00751-RAR1 1st Revision received **Dec. 6, 2016**

Reviewer comments and **author responses**:

Reviewer #1:

The study by David et al. demonstrated the function of RNA m5C in Arabidopsis by transcriptome-wide mapping of this RNA modification. In this study, the authors used RNA bisulfite sequencing to identify sites of the m5C modifications on different classes of RNAs. They observed that m5C modification is tissue-specific, indicating that the regulation by m5C is also likely tissue-specific. The authors further identified a RNA methyltransferase, TRM4B, which is responsible for RNA m5C modifications in Arabidopsis. Loss of m5C on mRNAs and noncoding RNAs was observed in the trm4b mutant, which showed root developmental phenotypes and increased sensitivity to oxidative stresses. Furthermore, the authors found that the 50 nt sequence surrounding the 5mC sites was sufficient to recruit TRM4B for m5C modifications.

Lots of studies on RNA modifications have been done in animals; this study provides evidences to reveal the potential regulatory roles of RNA modifications in plant development and stress responses. The data presented in

this study are convincing and the manuscript is generally well organized. However, a few concerns remain to be addressed:

Point 1. One topic of interest is the extent of the phenotype, and particularly whether you're claiming the phenotype of the mutant is root-specific, or was only observed in roots, or you only measured the roots. If you could clarify this, preferably by describing the extent to which you observed phenotypes in other tissues, or the basis for believing the phenotype is more pronounced in the roots, supported by data, this would be best.

RESPONSE: 1. We thank the reviewers for their question regarding the extent that we observed other phenotypes, apart from the short-root, in the *trm4b* mutant when compared to wild type. We observed no differences in shoot or inflorescence growth phenotypes that we measured of *trm4b* plants when compared to wild type. This detailed data is presented in a new supplementary figure 6 and consolidates our previous observations in Burgess et al., (2015).

In supplementary figure 6, we present the following data. We weighed *trm4b* mutant and WT plants (without roots) at inflorescence emergence and no difference in shoot mass was observed. We also counted the days to inflorescence emergence and rosette leaves at flowering and no difference was observed. If a slower cell division rate occurred in the *trm4b* shoot meristem, we may have expected fewer rosette leaves and subsequently a reduced shoot mass at flowering when compared to wild type. During the reproductive stage, inflorescence length was measured 16 days after inflorescence emergence and no difference in inflorescence length was observed between wild type and the mutant. This data suggests that the inflorescence meristem cell division rate is the same in the mutant and WT. All together, this data suggests *trm4b* mutant phenotypes are either root specific or are more pronounced in the root, as other phenotypes were not observed.

Point 2. Do the TRM4B overexpression lines show enhanced resistance to the oxidative stress? The authors revealed that m5C might be involved in oxidative stress resistance. Does plants have the mechanism to increase m5C on tRNAs to increase oxidative stresses? In other words, is the gene TRM4B responsive to H2O2 treatment in wild-type Arabidopsis?

RESPONSE: We agree that this is an interesting question but is outside the scope of the current study. This could be answered by performing qRT-PCR to measure TRM4B mRNA abundance on RNA from seedlings grown under control and oxidative stress conditions.

Point 3. In Figure 4, a 50 nt sequence context was sufficient to confer the methylation. The authors thus speculated that this secondary structure of mRNAs might be involved in m5C. It should be easy to do a secondary structure prediction for those sequences around m5C sites on mRNAs to test this hypothesis.

RESPONSE: Reviewer #1 noted that we speculated a RNA structure is recognized by TRM4B and then a cytosine is methylated. We agree with reviewer 1 that this speculation could be bolstered by performing a computational RNA structure prediction on a large number of RNA sequences flanking control and methylated cytosines; however, we have not done this for the following reasons. First, computational versus *in vivo* RNA structures often are very different and second, comparing computational predictions of hundreds of predicted RNA structures is challenging to do in a meaningful way.

Point 4. The mRNA expression showed negative correlation with m5C levels, while tRNA is stabilized by m5C. The authors need to discuss possible factors contributing this difference, because this is important to understand the function of m5C in regulating RNA stability.

RESPONSE: In our manuscript we have not laboured this point, as a large number of possible factors may contribute and may lead to extensive speculation. We have a brief statement is in the second paragraph of the discussion and do not feel that this should be extensively laboured in the manuscript.

Point 5. 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. Similarly, in the histograms shown in Figure 4, it is not clear whether biological replicates were used and this should be clearly stated.

RESPONSE: Reviewer #1 asked that we clearly report the number of biological replicates used in our experiments and we have done so by adding text to supplemental figure 1 and the Materials and Methods.

Reviewer #2:

The field of "epitranscriptomics" is rapidly expanding and is revealing unexpected layers of post-transcriptional regulation. Model plant systems have been at the forefront of this revolution and the manuscript by Burgess et al makes an important new contribution to our understanding of one of the less studied mRNA modifications.

The authors identify a number of m5C sites in tRNA, ncRNA and mRNA species and importantly they verify these by both amplicon sequencing and anti-m5C immunoprecipitation. They demonstrate that TRM4B is the major enzyme responsible for these methylations and they show that the identified methylation sites are largely lost in a TRM4B mutant. This provides a very high level of confidence in the methylated sites that the authors have mapped.

Unlike loss of writer function for other more common "epitranscriptomic" marks, mutation of TRM4B and global loss/reduction in m5C gives relatively mild phenotypes. The most obvious of these are impaired root meristem development and sensitivity to oxidative stress. As the authors point out, these phenotypes could be due to altered m5C levels in tRNAs rather than the identified mRNAs and this will be a productive area for future research.

Overall, the manuscript is very well written and the conclusions fully supported. At this stage it is not yet clear if 5C methylation of the mRNAs or the ncRNAs other than the tRNAs has a biological consequence. However, it seems likely that this will be the case and the identification of the methylase and methylated transcripts provides the necessary tools for addressing this. It will be interesting to see if there are endogenous proteins that specifically recognise m5C modified RNAs, but that is outside the scope of the current manuscript.

Point 1. Line 476 "...In addition, no correlations were observed for mRNA abundance and m5C levels in mammals, as no global changes in mRNA abundance were observed in mouse or human nsun2 mutants (Tuorto et al. 2012; Hussain et al. 2013b; Hussain et al. 475 2013c), suggesting that m5C may play alternate roles in specific mRNAs and may affect the translation of transcripts..." The last part of this sentence is rather too strong; there could be several alternatives e.g., no function, altered sub cellular targeting, altered translation.

RESPONSE: Reviewer 2 highlighted that the last part of the sentence (starting at line 476) was too strongly worded. This has been accordingly modified.

Point 2. Line 539 Should this be "..intracellular.." ?

RESPONSE: Thank you; this has been corrected.

Reviewer #3:

The manuscript of Burgess et al. examines the transcriptomic prevalence of m5C in three organs of Arabidopsis: siliques, and seedling roots and shoots, thereby extending their recent work surveying m5C of rRNA and tRNA in nucleus, chloroplast, and mitochondria across several plant taxa. They find organ-specific differences in m5C patterns at nucleotide resolution, and show that the RNA methyltransferase TRM4B is responsible for specific methylation patterns on many mRNAs and non-coding RNAs, including tRNA(asp) stability. Furthermore, the trm4b mutation is shown to have root growth defects related to root meristem deficiencies which can be exacerbated by stress. Finally, they show that a 50nt sequence can confer TRM4B-dependent methylation of a reporter RNA in tobacco.

The work delves into a relatively unknown area of plant RNA metabolism, which potentially influences post-transcriptional and translational regulation on a large-scale. The experiments seem careful and well-conceived, leaving little doubt as to the veracity of their data. Still, while the results of differences in methylation occupancy of specific nucleotide sites in mRNAs from root, shoots, and siliques is intriguing, there are limitations as to how we can interpret them in a larger biological context. For example, should we ascribe differences in root/shoot and silique methylation patterns to organ-specific differences, or to differences in juvenile versus mature life stages? In general, the lack of longitudinal study along a developmental axis, the focus on one ecotype of many, and one "environment" of many provides only a narrow window for understanding the m5C phenomenon. This isn't meant to be strong criticism of this work per se, but instead begs interesting questions or follow-up experiments of the dynamics of m5C over time, and as a function of genetic variation and environment. As a reader, I would appreciate some treatment of this reality in the discussion.

Similarly, there is a high-bar to say much about the role of 5mC and especially TRM4B in specific aspects of plant development such as root growth. The primary roots are obviously shorter in the trm4b mutants, and seem much thinner based on images presented in the ms (ex Fig 5C, S8a). But given the demonstrated negative effects on tRNA(asp) stability, are the authors sure that the root development effects are root-specific rather than due to reduced translational capacity/efficiency? TRM4B is stated to control meristem proliferation, when instead it may simply be a cog in the wheel of basic cell metabolism, defects in which seems likely to slow cell division and plant growth in a very general sense (an example are phenotypes of TOR mutant that inhibit ribosome function).

Overall, the manuscript breaks new ground on a potentially important aspect of plant growth regulation via post-transcriptional modifications of RNA. As with many such foundational works, many more questions are generated than are answered, but a framework for study is appearing.

Point 1. A special role for TRM4B would be more convincing if evidence were provided that caused allometric changes in root growth relative to shoots. The authors do make a glancing reference to their previous work that shoot growth appeared normal in trm4b plants, and after some digging in the supplements, I found a picture of a rosette that could be taken for qualitative evidence in this regard... but the burden of proof should be higher to effectively convince the readership that these are root-specific phenotypes, such as comparative plant/shoot biomass, leaf size/ shape, and perhaps flower or silique morphology.

RESPONSE: Reviewer 3 requested that we perform additional measurements on the shoots of wild type and *trm4b* mutants. This was addressed above (response to reviewers #1), and the data showing no differences between wild type and mutant shoots is shown in supplementary figure 6.

TPC2016-00751-RAR1 2nd Editorial decision – *acceptance pending* Dec. 15, 2016

We are pleased to inform you that your paper entitled "Transcriptome-wide mapping of RNA 5-methylcytosine in Arabidopsis mRNAs and ncRNAs" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor Jan. 2, 2017