An m^{6}A-YTH Module Controls Developmental Timing and Morphogenesis in Arabidopsis

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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.)

TPC2017-00608-BR 1st Editorial decision – *declined* Aug. 18, 2017

One particular concern that we discussed was that the YTH domain protein function is already known in animal and yeast systems, and you demonstrate (with some additional insights) that it also has the same function in plants. The editors felt that your work would indeed benefit from having some evidence of functional significance (e.g. in the mutants). In its current form, it's a well-written manuscript with interesting observations, but it still seems a bit incomplete relative to what we have in mind for a "Breakthrough Report". Indeed, work developing a better understanding of m6A, including the readers, would be of strong interest to our readers. Thus, if you were able to strengthen the manuscript with additional experiments, we would be interested to see a revised version. Therefore, we feel it best to not send your paper out for review until you have had sufficient time to complete this work. However, we would encourage you to resubmit a revised version of your manuscript to The Plant Cell.

TPC2017-00833-BR Submission received Oct. 26, 2017

TPC2017-00833-BR 1st Editorial decision – *revision requested* Dec. 20, 2017

Please address, to the best of your abilities, all reviewer comments, and please note in a Response to Reviewer comments document also submitted with your revised manuscript exactly how each point was addressed in your updated manuscript version.

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

[Provided below along with author responses]

TPC2017-00833-BR1 1st Revision received March 9, 2018

Reviewer comments on previous submission and **author responses**:

Reviewer #1

Reversible RNA methylation as a regulatory mechanism represents a still emerging but most exciting research area in biology. The currently best characterized of these modifications is methylation of N6 adenosine (m6A) in mRNAs and other RNAs. Here, breakthrough studies are published almost weekly, at least in the animal field. However, research on plants is catching up and since the first comprehensive description of m6A transcriptome pattern in 2014 (Luo et al., 2014 Nature Communications), we have witnessed important progress. For example, the importance of FIP37 as component of the methyltransferase complex for regulating stem cell fate and the comprehensive characterization of the m6A RNA methylation complex in Arabidopsis have been reported this year (Shen et al., 2017 Developmental Cell; Ruzicka et al., 2017 New Phytologist). The basic concept of this epitranscriptomic regulation involves methyltransferase complexes as "writers" which are counteracted by demethylating "erasers". The resulting m6A pattern is decoded by so called "reader" proteins. One defining feature of many reader proteins is the presence of the highly conserved YTH domain.

This is the starting point of the work by Arribas-Hernández et al. The authors performed a phylogenetic analysis of 13 YTH-domain-containing proteins that have been described before by Li et al., 2014. For subsequent analyses, the authors initially focused on ECT2, since it represents the most highly expressed protein of this family. They subsequently used in vitro assays to investigate the RNA binding properties of this protein and provide evidence, that it specifically binds m6A-containing nucleotides. Localization studies of transgenic lines expressing ECT2-mCherry revealed a cytoplasmic localization of this protein. In the context of this study, they observed a potentially drought stress-induced translocation of ECT2 into foci that appear to be distinct from P-bodies. In order to access the in vivo function, the authors isolated and characterized three T-DNA insertion alleles of ECT2. For none of these alleles did they clearly observe discernible phenotypes. Consequently, the authors performed a careful analysis that also involved mutants and mutant combinations of the closely related genes *ECT3* and *ECT4*. In these mutants, the authors detected and characterized defects in leaf initiation and leaf morphology, which importantly could be complemented with functional ECT constructs. Previous studies had revealed that decreased expression of the writer MTA (and thereby reduced RNA methylation) corresponded with an increased number of trichome branches (Bodi et al., 2012). Consequently, the authors also analysed this phenotype. In this regard, the authors observed occasionally four-branched trichomes in *ect2* mutants and also frequently in *ect3* mutants. This phenotype was strongly enhanced. Based on these findings, the authors discuss an important role of m6A modification and its correct decoding by ECT2 (and ECT3 and 4) for appropriate plant development. In this context, they speculate about a potential involvement of, for example, the regulation of class 1 KNOX transcription factors as potential targets of ECT2.

Most of the work presented here is technically sound and especially the reverse genetics analyses have been performed very carefully with regard to the required controls. However, in principle, this manuscript remains on the level of describing an interesting phenotype. It does not provide mechanistic insights how loss of ECT2 results in the observed phenotype. Consequently, the discussion remains highly speculative. Moreover, this work leaves the impression that it reports several "loose ends" (like the drought stress-induced foci translocation of ECT2) that are not connected to ECT2 function or the phenotypes of the mutant. In the following I provide a list of more specific comments in chronological order (most important comments are labelled with an asterisk *):

Point 1. The first two pages of the Introduction are rather confusing, since the authors fail to clearly formulate if one specific statement relates to mammals or yeast or plants. Sometimes this even leads to incorrect or misleading statements (example: "N6-adenosine methylation is cartelizes by a conserved multicomponent ...mammals and yeast". Moreover, the authors fail to introduce the non-specialized reader into the basic concept of "writers, erasers and readers".

RESPONSE: We have made two clarifications: one on the model system from which knowledge of m^{6}A switches derives, and another on the relevance of m^{6}A for the heat shock response. We could not find more than those

instances in which this type of information was omitted. We have also clarified the sentence that the reviewer considered to be misleading.

Regarding the concept of writers, erasers and readers, we have now defined writers and readers, but do not introduce m^{6}A erasers for a variety of reasons. These include the fact that in contrast to writers, erasers are not mentioned at any subsequent step in the manuscript, making it less relevant to introduce them.

Point 2. Figure 1 is largely redundant with previously published data in Ok et al., 2005 and especially with Li et al., 2014. However, Figure 1a may be informative for the orientation of the reader and may remain in the manuscript. Figure 1d is only needed to illustrate why the authors hone in on ECT2 and could be presented in the supplements.

RESPONSE: We agree that the schematic diagrams of YTH domain proteins are redundant with Li et al. (2014), and we have, consequently, removed panel 1B. However, the alignments and phylogenetic trees published in Ok et al. (2005) and Li et al. (2014) are not redundant with ours, because

1) **they do not include mammalian YTHDC and YTHDF clades as references**
2) **they do not highlight conserved amino acid residues involved in m^{6}A and RNA binding.**

These are two important points of the paper, and we have retained the corresponding figure panels.
It is always difficult to decide what should be in supplementary information and what should be in main figures. As a broad matter, we try to put as little information as possible in Supplementary Material, especially in a Breakthrough Reports format like the present one. We think that Figure 1D (now Figure 1B) is useful for the reader, and prefer to keep it in the main figure, but this is a point we can change if other reviewers and editors also request it.

Point 3. The authors perform in vitro binding studies with more or less arbitrary used oligonucleotides that correspond with previously used m6A consensus sites. The authors conclude that ECT2 does not distinguish between different m6A consensus sites. This is clearly overstated and not justified by the provided data. What these data suggest is that ECT2 appears to distinguish in vitro between methylated and non-methylated oligonucleotides. This experiment does not tell anything about the specificity of ECT2 in vivo. To this end, the authors could have for example pursued an approach in which functional and non-functional tagged ECT2 would be precipitated from plants and the bound RNA would be analysed by sequencing. In principle, the authors have generated the required plant materials. However, they do not attempt to provide these most important sets of data. Moreover, the data presented in Figure 2 have been generated with an isolated YTH domain and not with a full-length protein, which largely restricts their conclusiveness. Finally, the description of the experimental conditions is insufficient for an informed evaluation of the data.

RESPONSE: The in vitro binding data have been removed from the revised manuscript. Please see a full explanation below in the paragraph with the heading "Revision related to *in vitro* binding experiments by microscale thermophoresis".

Nonetheless, we agree with the reviewer that it is an important point to prove the relevance of m^{6}A binding *in vivo*. As important as knowledge of individual target mRNAs will be for further understanding of the function of m^{6}A-YTH modules, it is our judgement that this level of detail is not required to prove the importance of m^{6}A binding *in vivo*. This is because it is possible to design surgical point mutants specifically defective in m^{6}A recognition. In our revised version, we include 3D models of the structures of the YTH domains of ECT2 and ECT3 generated by homology modelling with human YTHDF1 as a template. These models show that the m^{6}A binding site in ECT2 and ECT3 almost certainly is identical to that in YTHDF1, with nearly all amino acid residues implicated in binding invariant between the three proteins. Thus, it is meaningful to mutate residues equivalent to the ones demonstrated to be necessary for m^{6}A binding in YTHDF1. We have taken this approach, and now show convincingly that such point mutants of ECT2 and ECT3 completely fail to rescue the leaf developmental phenotypes, despite the fact that they are expressed at levels comparable to wild type and are expressed in the same tissues as wild type. These data indicate that m^{6}A binding sites in ECT2 and ECT3 indeed are required for their *in vivo* functions.

Finally, we note that Plant Cell editors have assured us that identification of target mRNAs of the ECT proteins – as interesting as it is – would not be required for this manuscript.

Point 4. Figure 3. The authors provide evidence that ECT2 appears to accumulate in stress-induced foci. However, this work falls short of providing clues about the physiological significance of this observation, and they do not

attempt to provide a connection between mutant phenotypes and this observation. Would *ect* mutants be impaired in drought adaptation?

RESPONSE: We may not have been clear enough on this in the original version. The point here is not to test whether ECT proteins play a physiologically relevant role in stress adaptation. The point we wish to make is that ECT2 is able to relocate to foci, and that this property *in vivo* coincides with the *in vitro* property of the pure protein to form regularly sized and shaped aggregates. This is relevant information, even outside of plant research, because although YTHDF proteins have been studied much more extensively in animals and have been found to localize to cytoplasmic foci in mammalian cells, properties similar to the ones we describe here for ECT2 have not been reported for any of the mammalian homologues to our knowledge.

Point 5. Figure 5: A careful characterization of the trichome phenotype would have required a quantitative analysis of the different mutants and mutant combinations.

RESPONSE: We agree with the reviewer. We now include a careful quantification with branch counts of a total of more than 12,000 trichomes across all genotypes. We also apply rigorous statistical analysis by fitting to a proportional odds model for ordinal regression. These analyses show that single mutations of *ECT2* or *ECT3* cause increased trichome branching, and that this effect is dramatically enhanced in double mutants. ECT4, on the other hand, has no observable effect on trichome branching. Please note that we now also include two independent alleles of *ect2*, two independent alleles of ect3, and, importantly, two *ect2/3* double mutants generated with different *ect2* and *ect3* alleles. In this way, conclusions on causality between *ect* mutation and defective trichome morphogenesis can be drawn. Please note also that we now include fluorescence microscopy analysis of expression in trichomes of ECT2 and ECT3, and find, most interestingly, that these proteins are highly expressed at an early stage in trichome development in which the branching pattern is determined.

Point 6. Figure 4: The characterization of the leaf formation phenotype would strongly benefit from a more detailed investigation of the meristematic structure and gene expression. This could easily be achieved by electron microscopy or in situ hybridisation or analysis of fluorescent marker genes.

RESPONSE: This is a good point, and clearly one that we will follow up on in future work. We did do preliminary histological analysis of the meristem structure comparing wild type and *ect2/3/4* mutants at 9 days post germination, and did not find striking differences. We now believe that the way to do it, is to follow meristem structure and generation of leaf primordia over time – a relevant, but considerable piece of work that we could not accommodate in the revision period. We are holding a little bit back on in situ hybridizations until we know the direct targets of the ECT proteins, as these will be obvious candidates to check, particularly if known regulators of meristem function and formation of leaf primordia come up in the analyses. Please note also that we now include expression analyses not only of *ECT2*, but of all three ECT genes. These analyses show high expression of all three genes at sites of leaf formation (new Figure 1).

Point 7. Discussion: The discussion remains largely speculative. In this regard, for example none of the subheadings provide useful information or clear statements. Also, it would have been required that the authors clearly put their findings in the context of what has recently been reported about the function and consequences of m6A modifications in plants. The authors can only speculate about potential consequences for mRNA regulation and about the identity of potential target genes. However, considering the clear phenotype of the mutants, it would have been relatively simple to study at least the accumulation or stability of the mRNA of candidate genes (like class 1 KNOX) which the authors discuss here. This shortage is even acknowledged by the authors ("Clear answers...must await...")

RESPONSE: The two other reviewers have not raised criticisms on the way the Discussion was structured. In fact, they both note that the paper as a whole was well written, which we assume also applies to the discussion. We do not agree fully with the reviewer on these points, but have, nonetheless, attempted to improve the Discussion along the lines of criticism raised here.

Reviewer #2:

Secondary modifications of RNA molecules are widespread and important for posttranscriptional mechanisms. Among these, m6A methylation is the most abundant, with N6-methyladenosine methyltransferases (writers), demethylases (erasers) and reader proteins being implicated in this process. In Arabidopsis, large-scale analyses have confirmed the presence of m6A methylation in the transcriptome and identified the location of modifications in

numerous specific transcripts. While components of the N6-methyladenosine methyltransferase complex have been studied in detail and identified as crucial regulators of plant development, the biological relevance of m6A methylation readers has not been addressed so far.

The key findings of this study are: i) ECT2, the most highly expressed member of an m6A reader family, binds specifically to m6A-methylated RNA; ii) ECT2 localizes to the cytoplasm and relocates to P-bodies upon drought stress; and iii) *ect2 ect3* double and *ect2 ect3 ect4* triple mutants show delayed development as well as leaf and trichome morphology defects. The manuscript is well written and reports an interesting body of data providing novel insight into the biological roles of m6A methylation in plants. Nevertheless, as outlined below, there are a number of concerns that need to be addressed.

Point 1. The complementation analysis of the timing of leaf formation needs to be extended to the leaf shape/trichome phenotypes if the authors want to claim involvement of the ECT genes in the latter processes.

RESPONSE: We thank the reviewer for raising this important point. To prove the involvement of the ECT genes in all the processes described in the paper, we have done the following: First, we have done analyses of two independent alleles of *ect2* and *ect3*, including double mutants obtained with the different allele combinations. This analysis of independent alleles benefits the whole study, but in particular the section on trichome branching phenotypes that was not backed up by complementation in the originally submitted version. Second, we have generated transgenic lines expressing wild-type ECT4 in *ect2/3/4* triple mutants to provide stringent proof that the enhancement of leaf initiation and morphogenesis phenotypes really is due to loss of ECT4 function.

Point 2. In Fig. 3 J/K, why does the VCS p-body marker fail to form the large cytoplasmic bodies in response to stress? Colocalization with ECT2 in the large granules is also not very clear.

RESPONSE: Here, there has been a misunderstanding. We do conclude, as does the reviewer, that ECT2 does not colocalize with the VCS-GFP marker in most cases, and hence that ECT2 foci are not P-bodies.

Point 3. The drought stress assay employed is rather unconventional and the subcellular localization results would better be reproduced with established assays under more controlled conditions (e.g. using an osmotic stress treatment).

RESPONSE: We agree with the reviewer. We have now done confocal microscopy observations following an established protocol for osmotic stress treatment: 30% polyethylene glycol for one hour. The results show clear relocalization to foci of ECT2 and ECT4, and are shown in the new Figure 5.

Point 4. It would also be important to quantify the trichome phenotype (as in e.g. Plant Cell 2009, 201: 2307-22 or Front Plant Sci 2012 3: 48).

RESPONSE: We agree that this is an important point and have now done careful quantitative analyses, as described in the response to reviewer 1's comment 5.

Point 5. It would be interesting if the microscale thermophoresis experiment shown in Fig. 2C would also include negative controls to address whether ECT2 is specific for the previously identified consensus sites or binds any m6A methylated RNA sequence.

RESPONSE: This is a good suggestion. Unfortunately, we have had to remove the results on *in vitro* binding of the YTH domain of ECT2 to m^{6}A-containing RNA oligonucleotides. Please see a full explanation for this below.

Point 6. Regarding the molecular characterization of the mutant alleles and the western blot analysis (Fig. 4F), the authors should comment on the location of the epitope in the ECT2 protein- because truncated mRNAs appear to be produced in the different alleles, a truncated protein (a therefore some degree of functionality) could still be produced.

RESPONSE: We have indicated the positions of the peptide epitopes used to raise the antibodies in the schematic diagram of ECT2 in the new Figure 1C. In the new Figure 1G (ECT2 western), we now include a larger crop of the gel to show that no truncated protein fragments are detectable in any of the *ect2* insertion mutants in the study.

Point 7. Figure 1 and its legend need to be carefully checked. In panel B, the acronyms for all domains should be defined in the legend. On the other hand, what do pE and poly-Glu, mentioned in the legend, refer to in the Figure? Also, in panel C, it is not very clear what the red background in the alignment means.

RESPONSE: We have now removed the previous panel 1B at the suggestion of reviewer 1. We have now carefully checked the legend describing the alignment (previous Figure 1C, current Figure 2A). We have also made improvements in the clarity and updates to the amino acid residues implicated in binding, paying particular attention to structural data on the YTH-RNA complex of mammalian YTHDF2 and YTHDF1.

Reviewer #3:

The authors investigate a potential function of *Arabidopsis thaliana* YTH domain proteins in "reading" m6A RNA modification. They show that ETC2, the most abundant YTH domain protein, binds synthetic m6A containing sequences upon purification of recombinantly expressed protein. Furthermore, they show that the sequence context around the m6A nucleotide has little influence on in vitro binding. In vivo, ETC2 localizes to the cytoplasm and appears to assemble in cytoplasmic foci in response to drought stress. An in vivo function of ETC2 in timing of the appearance of the first leaf pair was shown. In this process, ETC2 acts redundantly with ETC3 and ETC4. Furthermore, a role in trichome branching was found although the investigated ETCs appear not to be essential for this developmental process. The manuscript is very well written and all data are clearly presented.

Point 1. m^6A modification is well known to be able to influence local RNA structure. This may not be reproduced with synthetic oligonucleotides in vitro. Therefore it would be desirable to expand the in vitro binding studies to plants and correlate in vivo binding of ETCs with the m6A status of targets. Along the same lines, the manuscript would greatly benefit from the identification of in vivo target transcripts involved in the physiological processes that the authors investigated.

RESPONSE: For reasons explained in detail below, we have had to withdraw the *in vitro* binding results from the manuscript. The experiments suggested by the reviewer are indeed interesting, but obviously require knowledge of targets, a requirement that the editors have assured us we do not have to meet for this paper. In our revised version, we now include a series of new experiments to demonstrate the importance of m^6A binding of ECT proteins in vivo. Please see the response to reviewer 1, point 3 for details on these important new data.

GENERAL COMMENT BY THE AUTHORS: Revision related to *in vitro* binding experiments by microscale thermophoresis

Unfortunately, we have had to remove the data on specific *in vitro* binding of the YTH domain of ECT2 to m^6A-containing RNA oligonucleotides. This has been a painful process: When we attempted to address the point of reviewer 2 to test additional RNA oligonucleotides in the microscale thermophoresis assay, we also decided to include an additional control: a point mutant of the ECT2 YTH domain with defective m^6A-binding specificity. In the course of repeating experiments similar to the originally reported ones, we could not measure any difference in binding affinity between the wild type and mutant YTH domain. Of note, these experiments used a protein that we assigned to a truncation fragment without the disordered extension C-terminal to the YTH domain on the basis of two observations:

(i) Its molecular weight as determined by MALDI-TOF mass spectrometry fit exactly the size of a minimal YTH domain without the disordered C-terminal extension included in the expression construct

(ii) The protein reacted with His_6-antibodies, suggesting that it retained the N-terminal His-tag.

Our new results with the ECT2 point mutant made us revisit this conclusion. We purified the band and had it identified by electrospray-MS/MS analysis. This analysis showed that what we had taken to be a C-terminal truncation of ECT2 was in fact two *E. coli* proteins: One known nucleic acid binding protein that we now know has specific m^6A-binding activity, and another protein, SlyD, of the same size that is naturally His-tagged, and therefore reacted with our His-antibodies. In short, the results shown in the originally submitted version were obtained with an *E. coli* contamination, and, consequently, cannot be used to back any conclusion on binding of ECT2 to m^6A-containing RNA. We are extremely sorry about this error. We do wish to make clear that we have not had any intention to mislead editors or reviewers: the purifications used in the original manuscript reproducibly give the binding curves to m^6A-containing oligonucleotides that we showed – they just do not mean what we thought they meant four months ago. We also wish to make clear that the error did not happen as a consequence of wanting to

move too fast: we obtained the first binding results in March 2015, and repeated it many times and with different oligonucleotide sequences since then. We just did not do the additional control that made us realize that something was wrong until very recently. Finally, we wish to emphasize that the case for m^{6}A-dependence of the observed phenotypes of *ect* mutants is still strong: We now also include models of 3D structures of ECT2 and ECT3 obtained by unbiased homology-modelling. These structural models clearly indicate that m^{6}A recognition pockets are intact and available for m^{6}A binding in ECT2 and ECT3. Combined with our black-and-white *in vivo* results obtained with transgenic lines expressing aromatic cage mutants of ECT2 and ECT3, it is fair to conclude, in our view, that m^{6}A binding sites of ECT2 and ECT3 are required for their biological functions.

TPC2017-00833-BR1 2nd Editorial decision – *acceptance pending* **March 24, 2018**

We are pleased to inform you that your paper entitled "Control of developmental timing and morphogenesis in Arabidopsis by an m6A-YTH module" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor **April 10, 2018**