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Peer Review Report for 10.1105/tpc.16.00600

Arabidopsis TH2 Encodes the Orphan Enzyme Thiamin Monophosphate Phosphatase

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Review timeline:

TPC2016-00600-RA	Submission received:	July 29, 2016
	1st Decision:	Aug. 18, 2016 *revision requested*
TPC2016-00600-RAR1	1st Revision received:	Sept. 19, 2016
	2nd Decision:	Sept. 19, 2016 *accept with minor revision*
TPC2016-00600-RAR2	2nd Revision received:	Sept. 20, 2016
	3rd Decision:	Sept. 20, *acceptance pending, sent to science editor*
	Final acceptance:	Sept. 26, 2016
	Advance publication:	Sept. 27, 2016

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.)

TPC2016-00600-RA 1st Editorial decision – *revision requested* Aug. 18, 2016

We have received reviews of your manuscript entitled "The Arabidopsis TH2 Gene Encodes the Orphan Enzyme Thiamin Monophosphate Phosphatase." 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.

My own reading of the manuscript and the reviews suggest that attention to the following comments might be useful for the reader.

1. A reviewer brought up the following legitimate question: "One major issue is the fact that th2 shows a strong phenotype (typical of thiamin mutants) but the T-DNA insertion mutant in At5g32470 does not.

The T-DNA insertion mutant has the same metabolic phenotype (i.e. halved ThDP levels) but shows no visual phenotype according to what is written in the manuscript (however, this should be shown)."

In the discussion you mention that the T-DNA mutant is "on the verge" of deficiency, but what is the evidence for that? Perhaps it should be re-phrased?

2. I wonder if a diagram (perhaps modification of 1A or a supplement to 1A?) showing thiamin metabolism, pools, transport between compartments, might help to address this issue that the reviewer 2 raises - "Even with supplementation the metabolic phenotype remains the same (i.e. halved ThDP levels, enhanced ThMP (Fig. 1B)), so what is the explanation for growth returning to normal in the context of thiamin?"

I understand that you are saying that the supplementation is minimal, allowing growth but not allowing pools to be filled. Of course this does suggest that there is a hierarchy of thiamin allocation to sites of accumulation, which is

different somehow from thiamin utilization sites? There is a discussion about thiamin deficiency symptoms in animals where half the level is sufficient for phenotype and presumably the same in plants. I can understand the reviewer and reader confusion when supplementation, which alleviates phenotype, does not change the level much from half. Maybe the issue is too complex for a diagram? Additional explanation / discussion perhaps?

3. If relevant, appropriate prior work could be cited.

4. The reader may appreciate an explanation for why the work with maize is included in this story. Is it to make the story more generally applicable (i.e. same pathway in divergent plant species)?

5. The section on location to cytosol vs. mitochondria is quite long. I wonder if it can be trimmed to focus on the take home message. There is much made of the dual targeting, but in the end the message seems to be that the protein is cytosolic. Or is the message that the protein is cytosolic, but there is the potential for some of it to be mitochondrial where it may have a more minor salvage function? Two editors felt that the experimental work was adequate to support your claim of localization.

Figure 3, make all bars equal width.

Figure 5, for structures, perhaps better resolution needed (or thicker lines?)

Naturally, we would also like you to take all reviewer comments seriously and accommodate the reviewers to the extent possible. We appreciate that there are some issues of semantics, and I am sure that appropriate wording can accommodate alternative opinions.

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

Reviewer comments are shown below along with author responses.

TPC2016-00600-RAR1 1st Revision received Sept. 19, 2016

Reviewer and editor comments and author responses:

Editor's Comments

Point 1. A reviewer brought up the following legitimate question: "One major issue is the fact that th2 shows a strong phenotype (typical of thiamin mutants) but the T-DNA insertion mutant in At5g32470 does not. The T-DNA insertion mutant has the same metabolic phenotype (i.e. halved ThDP levels) but shows no visual phenotype according to what is written in the manuscript (however, this should be shown)." In the discussion you mention that the T-DNA mutant is "on the verge" of deficiency, but what is the evidence for that? Perhaps it should be re-phrased?

RESPONSE: We have now measured shoot and root growth of the T-DNA mutant and detected a modest growth defect, which is a priori reasonable and agrees with the mRNA evidence that this mutant is leaky. The new growth data for the T-DNA mutant are given in Supplemental Figure 2 and the Results and Discussion sections have been modified accordingly. We now see that interpreting ThDP levels in the th2-1 mutant and in the T-DNA mutant solely in terms of % reduction relative to wild type is misleading. This is because (i) the absolute level of ThDP is more biologically meaningful, and (ii) wild type ThDP level varies from 1.2 to 1.5 nmol/g in Ler-0 to 2.0 nmol/g in Col-0 (Figs. 1B, 3B, 3C); i.e. it is not a constant value that can be set to 100%. The absolute ThDP level in the th2-1 mutant is 0.6 nmol/g or less (Figs. 1B, 3B), while that in the T- DNA mutant is substantially higher – 1.0 nmol/g (Fig. 3C) consistent with the mild growth phenotype. The Results and Discussion have been rewritten accordingly, and – to facilitate comparisons – a dashed red line has been added to Figures 3B and 3C showing the 0.6 nmol/g ThDP level in the th2- 1 mutant. We are grateful that the review process revealed an inadequate interpretation of the data.

Point 2. I wonder if a diagram (perhaps modification of 1A or a supplement to 1A?) showing thiamin metabolism, pools, transport between compartments, might help to address this issue that the reviewer 2 raises - "Even with supplementation the metabolic phenotype remains the same (i.e. halved ThDP levels, enhanced ThMP (Fig. 1B)), so what is the explanation for growth returning to normal in the context of thiamin?" I understand that you are saying that the supplementation is minimal, allowing growth but not allowing pools to be filled. Of course this does suggest that

there is a hierarchy of thiamin allocation to sites of accumulation, which is different somehow from thiamin utilization sites? There is a discussion about thiamin deficiency symptoms in animals where half the level is sufficient for phenotype and presumably the same in plants. I can understand the reviewer and reader confusion when supplementation, which alleviates phenotype, does not change the level much from half. Maybe the issue is too complex for a diagram? Additional explanation / discussion perhaps?

RESPONSE: Re-examination of the 30 nM thiamin supplementation experiment originally included in Figure 1B showed that the th2-1 plants had, by the time of harvest, used up the thiamin dose supplied in the medium, and so had become deficient. The data for supplemented plants are thus uninformatively redundant with the data for unsupplemented plants and have been deleted. Results and Discussion sections have been changed accordingly. Again, we are grateful that reviewing uncovered this issue.

Point 3. If relevant, appropriate prior work could be cited.

RESPONSE: We now cite the Ajjawi et al. 2007 study in the Discussion. The other suggested references are not cited because they do not report ThDP levels.

Point 4. The reader may appreciate an explanation for why the work with maize is included in this story. Is it to make the story more generally applicable (i.e. same pathway in divergent plant species)?

RESPONSE: Wording has been added to the Introduction and Results section to indicate that maize proteins were included in the study to confirm that the findings for Arabidopsis apply to other plant species. It is worth adding that the NSF award that funded the study has a strong maize focus.

Point 5. The section on location to cytosol vs. mitochondria is quite long. I wonder if it can be trimmed to focus on the take home message. There is much made of the dual targeting, but in the end the message seems to be that the protein is cytosolic. Or is the message that the protein is cytosolic, but there is the potential for some of it to be mitochondrial where it may have a more minor salvage function? Two editors felt that the experimental work was adequate to support your claim of localization.

RESPONSE: The Results section has been shortened by 40% and refocused to make the main point that the protein is cytosolic and the subsidiary point that some mitochondrial targeting is possible. The Discussion has been revised to match.

Reviewer 1

The work described in this manuscript by Mimura et al. identifies the gene encoding th2, one of the earliest auxotrophic mutants of Arabidopsis; and exhaustively investigates function of the TH2 enzyme, a thiamin monophosphate phosphatase; and demonstrates that alternate translational initiation sites target the enzyme to mitochondria and cytosol. The manuscript bears obvious novelty and significance in that the report adds the last "orphan" step in thiamin biosynthesis in plants, now completing knowledge of the entire pathway. The identification of a phosphatase that specifically acts on thiamin monophosphate refutes the prior hypothesis that a general-specificity phosphatase catalyzes this reaction. Moreover, it is demonstrated that the gene, At5g32470, encodes a bi-domain enzyme including the phosphatase and a TenA domain that functions in thiamin salvage. And the subcellular localization data clarifies the compartmentation of the thiamin biosynthesis pathway.

The manuscript is elegantly written and illustrated with high quality figures.

My concerns are generally nitpicking, but they are the following:

Point 1. Figure 3 legend "Only th2-1 transformants with a normal phenotype were analyzed." One wonders why the null segregants weren't analyzed since they would seem to be the best control for complementation because their Th profile should be like th2 and the plants are siblings of the complemented plants. In addition, were the healthy looking plants shown to carry the transgene?

RESPONSE: Given the expected 3:1 ratio (noted in Fig. 3A legend), we judged that further analysis of segregants was inessential. Also, thiamin data for non-growing segregants would be redundant with Figure 1B.

Point 2. Page 16 "Thiamin Vitamer Analysis" the definition of vitamer is "A vitamer of a particular vitamin is any of a number of chemical compounds, generally having a similar molecular structure, each of which shows vitamin-activity in a vitamin-deficient

biological system." Do all the compounds analyzed have B1 activity?

RESPONSE: ThDP and ThMP are thiamin vitamers, see e.g. http://pubs.acs.org/doi/pdf/10.1021/jf202647x /

Point 3. Some part of the body of the manuscript is devoted to the demonstration that HAD Phosphatase At4g29530 IS NOT the identity of the th2 mutation. Wouldn't this data be better located in the supplementary data?

RESPONSE: Perhaps the reviewer overlooked the point that At4g29530 was not tested as a candidate for th2, but rather as a candidate for the residual activity present in the th2-1 mutant. In any case, there is only a short section (137 words) on At4g29530 in the main text, and the accompanying data are already in supplementary data (specifically, Supplemental Figure 2).

Point 4. Can the authors speculate on why mutation of thiamin biosynthesis in plants produces a viable mature embryo wherein thiamin deficiency is observed in the vegetative plant, compared with most other auxotrophic mutants wherein the auxotrophy results in embryo or gametophyte lethality.

RESPONSE: As this question has been extensively discussed by others (e.g. doi:10.1016/0168-9525(86)90190-3) we prefer not to revisit it in our ms.

Reviewer 2

The authors have identified a phosphatase in Arabidopsis, which they convincingly demonstrate to act on ThMP corroborated by the metabolic phenotype of the corresponding Arabidopsis mutants that accumulate ThMP and have a deficit in ThDP. This is an important finding within the specific field but I have a number of major concerns with the manuscript.

Point 1. One major issue is the fact that th2 shows a strong phenotype (typical of thiamin mutants) but the T- DNA insertion mutant in At5g32470 does not. This cannot be explained by the possible residual activity in the latter as the authors suggest (L166-172) because the B1 vitamer profile is the same in both mutants, i.e. ThMP accumulates and ThDP levels are roughly halved. The authors make strong statements throughout the manuscript that "surprisingly modest (50%) ThDP depletion produces severe deficiency symptoms" and that this finding "sheds light on an almost unexplored area: thiamin deficiency in plants". The former statement is not true as the T-DNA insertion mutant has the same metabolic phenotype (i.e. halved ThDP levels) but shows no visual phenotype according to what is written in the manuscript (however, this should be shown).

RESPONSE: See response to Editor's comment # 1.

Point 2. Secondly, thiamin deficiency has been studied rather extensively in plants, a few examples include: Ajjawi et al Plant Mol Biol 2007, Raschke et al PNAS 2007, Bocobza et al Genes and Development 2007, Wachter et al Plant Cell 2007; none of these studies have been cited in the manuscript.

RESPONSE: See response to Editor's comment # 3.

Point 3. Furthermore, the authors have not demonstrated that the mutants can be metabolically rescued with thiamin supplementation, this should be possible as the TPKs (the kinases that pyrophosphorylate thiamin) are presumably still active in both mutants. They state that th2 "grows normally" by supplementation with 30 nM thiamin but do not show it.

RESPONSE: The classical work on the th2-1 mutant (cited in the last para of the Introduction) demonstrated its rescue by thiamin (but not thiamin precursors).

Point 4. However, even with this supplementation the metabolic phenotype remains the same (i.e. halved ThDP levels, enhanced ThMP (Fig. 1B)), so what is the explanation for growth returning to normal in the context of thiamin? In this context also, I do not understand the vague statement given as an explanation on L107-109 that the "thiamin dose was limiting, allowing growth but not "luxury" thiamin or thiamin phosphate accumulation". This does not have any logic and contradicts the authors claims that modest decreases in ThDP levels are harmful for the plant.

RESPONSE: See response to Editor's comment # 2.

Point 5. I also find the conclusions on the whole section elaborating dual targeting of TH2 unsubstantiated (e.g.

strong statements in the section starting from line 354). Aside for the in vitro approach, which may not reflect the in vivo situation, in my opinion the only construct that is relevant in the in vivo approach (albeit still a heterologous system) taken is the full-length construct with its natural KOZAK consensus. All of the others are artificial constructions under strong promoters to show the possible relevance of an opportune second methionine, which is placed under non-physiological conditions and therefore artificially forced to respond. The most natural construct (i.e. full length with native KOZAK, as referred to above) is the only one exclusively found in the cytosol and is the location that is compatible with all that is known about thiamin biosynthesis. Indeed, the authors could not adequately explain the relevance of mitochondrial targeting and according to the techniques used may not be physiologically relevant. If the authors really want to substantiate physiologically relevant dual targeting then an antibody could be used combined with the relevant subcellular fractions from Arabidopsis.

RESPONSE: See response to Editor's comment # 5.

Point 6. The reason for linkage of the phosphatase to the TENA is not sufficiently addressed, although in saying this, this is the least major issue of the manuscript. For example, the authors did not test the thiaminase activity of the TH2 (only the amino-HMP hydrolase activity was tested, the physiological relevance of which is unknown). If the protein does indeed cleave a thiamin vitamer (i.e. TMP was not tested), then this may help to explain the association with the phosphatase and should be accounted for in the discussion on the rationale of the TenA-HAD fusion. The explanation given for oxidative damage in mitochondria could be tenable but this would also be required in the plastid and should be taken into account in the discussion.

RESPONSE: The activity of the TH2 protein against thiamin and ThDP was tested and found to be undetectable, as stated in the original ms (line 227). For completeness, have now tested for activity against ThMP, which was also undetectable. This new negative result is included in the revised ms. The detection limit for the assays (6 pmol min^{-1} mg^{-1} protein) is given in the Methods section.

Point 7. What is the relevance of including the maize orthologs? There appear to be two of these in maize but they are not discussed or put into context with the study in Arabidopsis, e.g. where are they localized in maize at the subcellular and tissue levels?

RESPONSE: See response to Editor's comment # 4.

Point 8. In line with the above comments, I find the discussion needs clarification and more rational construction of argumentation. Also L313, that "the way is thus now clear to engineering very nearly all the steps of the thiamin pathway" is inaccurate. There are several steps of this pathway missing, not least of which from an engineering perspective are the transporters required to get the vitamers out of the green tissue, where they are predominantly made, to sink tissues, which are the major organs of consumption. With the exception of the mitochondrial ThDP transporters and a reported long distance transporter, nothing is known of this steps. This needs to be acknowledged, as from an engineering perspective its importance should be highlighted.

RESPONSE: The phrase "the way is thus now clear to engineering very nearly all the steps of the thiamin pathway" has been qualified by inserting the word "enzymatic" before "steps".

Point 9. The authors do not address or comment on the severe reduction in thiamin levels (only the changes in TMP and TDP are discussed), e.g. Fig. 3B?

RESPONSE: These differences were statistically non-significant and so do not merit discussion.

Point 10. Quantitative PCR should be used to assess the levels of TH2 expression in the mutants characterized, especially to distinguish levels in th2-1 from the T-DNA insertion mutant.

RESPONSE: The th2-1 mutation is a deletion and there was no detectable message by RNA-seq (as stated in the text). Therefore qRT-PCR comparisons are not possible.

TPC2016-00600-RAR1 2nd Editorial decision – *accept with minor revision* Sept. 19, 2016

Editor comments are shown below along with author responses.

TPC2016-00600-RAR2 2nd Revision received Sept. 20, 2016

Editor's Comments**

Point 1. "Fig. 3A: The empty vector plants shown look very heterogeneous, do the authors have an explanation for this?"

The seeds from which the empty vector plants grew came from soil-grown mother plants irrigated as needed (i.e. at irregular intervals based on water use) with a 0.1 mM thiamin solution. As thiamin is unstable in soil, the thiamin status of the plants would not have been constant, and nor would the amount of thiamin available for developing seeds. We therefore attribute the observed heterogeneity to variation in thiamin endowments among seeds developing in different siliques at different times.

** I can imagine that readers may have this question so if it is possible to work this into the methods or legend, that would be acceptable. Or do you think that it is not necessary at all?

RESPONSE: The proposed explanation has been incorporated into the methods section (p. 20).

Point 2. "Can the authors speculate on why mutation of thiamin biosynthesis in plants produces a viable mature embryo wherein thiamin deficiency is observed in the vegetative plant, compared with most other auxotrophic mutants wherein the auxotrophy results in embryo or gametophyte lethality."

As this question has been extensively discussed by others (e.g. doi:10.1016/0168-9525(86)90190-3) we prefer not to revisit it in our ms.

**I can understand that one would not want to revisit this discussion, but perhaps a statement in the introduction to indicate that unlike other auxotrophic mutants, thiamin mutants exhibit deficiency only in the vegetative plant (citation). Would that be appropriate? Or is it unnecessary? I leave this to you.

RESPONSE: Wording about the special status of the thiamin pathway as one in which auxotrophic mutants can be recovered in plants has been added to the last paragraph of the introduction (p. 4).

Point 3. **Finally, I noticed that the type of bulbs used to grow plants (i.e. quality of light) is not indicated in the methods (beyond saying that the Arabidopsis plants were grown in fluorescent lights with PFD indicated). Sorry, I did not catch that earlier. The number of the bulb is quite adequate (e.g. Sylvania FT xyz...) or a link to the spectrum. This change can also be made at the science editor stage.

RESPONSE: The fluorescent light manufacturer and product number have been added to the methods (p. 20).

TPC2016-00600-RAR2 3rd Editorial decision – *acceptance pending* Sept. 20, 2016

We are pleased to inform you that your paper entitled "The Arabidopsis TH2 Gene Encodes the Orphan Enzyme Thiamin Monophosphate Phosphatase" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor Sept. 26, 2016