Point-by-point responses to the reviewers' comments (Schenck et al.)

Editor comments:

This manuscript demonstrates the role of prephenate dehydrogenase in the alternative Tyr biosynthesis pathway in Medicago. The reviewers raised some minor concerns, such as (1) justification on not exploring the metabolite feeding experiments further; (2) discussion on the interpretation of why Tyr accumulates after feeding shikimate, but less so in the pdh mutant; (3) inconsistencies in the amounts of tocopherol and Tyr and the effects of senescence on PDH enzymatic activity by senescence. Please revise accordingly.

Response: Thank you very much for handling our manuscript. Here we addressed all of the individual comments and revised the manuscript accordingly. Please see below our point-by-point responses.

Reviewer #1:

>This manuscript is a solid piece of research demonstrating to role of Prephenate Dehydrogenase in the alternative tyrosine biosynthesis pathway in Medicago. Two gene insertion mutant alleles were isolated that lacked PDH mRNA and enzyme activity, convincingly showing the mutants are gene knockouts. The mutant alelles showed little differences from wild type in a range of different phenotypic assessments, including association with rhizobia. However, when plants were fed shikimate, a precuror of PDH, Tyr accumulated, but it accumulated much less so in the pdh mutants.
There is little that can be suggested to improve the manuscript. The conclusions is in good alignment with the evidence. The manuscript is clearly and succinctly written. There are copious supplementary data to support the conclusions of the manuscript.

Response: Thank you for positive comments on our manuscript.

>I do wonder why the authors did not explore the metabolite feeding experiments a bit more, given that this was the one clear difference the pdh mutants showed. For example, it would have been informative to feed different metabolites, like chorismate and prephenate. How did the authors choose to feed shikimate?

Response: We did try to pursue the metabolic phenotype associated with shikimate feeding such as performing non-targeted metabolomics in these plants. However, these additional experiments did not result in obvious differences and were run on sub-optimal equipment, which cannot identify low abundance metabolites. Since these experiments were performed when the first author was finishing his PhD, we were unable to pursue additional follow up experiments.

We chose to feed shikimate as it is a relatively stable upstream metabolite of all AAAs, allowing us to assess feeding efficiency by measuring other AAAs. Also, shikimate was readily available from Sigma at reasonable costs for feeding to *Medicago* leaves with multiple replications. On the other hand, chorismate and prephenate were much more expensive and had concern on stability during the feeding experiments.

>Also, added confidence in the shikimate feeding result would have resulted if the authors showed a time and dose relationship of Tyr accumulation after shikimate feeding. The same would be important for feeding other metabolites.

Response: We indeed performed an initial trial experiment in a time-course and concentration-dependent manner to determine the optimal time and concentration of shikimate feeding. We did observe that Tyr accumulates over time and with increased concentration of shikimate. However, longer labeling times and higher shikimate concentrations resulted in aberrant leaf phenotypes (curling and photobleaching). Based on these experiments, we chose 8 hours of feeding with 25 mM shikimate. We are now briefly describing the findings from these preparatory experiments in the result section.

>Lastly, I would have liked to see discussion on the authors interpretation of why Tyr accumulates after feeding shikimate, but less so in the pdh mutant. This important observations seems to have not been addressed in the discussion in favor of the other phenotypes that the pdh mutants were unaffected in.

Response: We have now added further discussion about altered Tyr levels following shikimate labeling to the discussion. See pg. 15-16 lines 370-374 "Furthermore, after the shikimate precursor feeding, Wt accumulated more Tyr than mutants (Fig. 4), suggesting that the PDH enzyme is indeed involved in synthesizing Tyr when the shikimate pathway flux is increased. Therefore, the alternative PDH pathway could be used under certain conditions (e.g., stress) to support production of Tyr/HPP-derived specialized metabolites."

>I noted that this manuscript has already been published online as an un-reviewed document on bioRxiv https://www.biorxiv.org/content/10.1101/768317v1. The stated purpose of publishing on bioRxiv is "By posting preprints on bioRxiv, authors are able to make their findings immediately available to the scientific community and receive feedback on draft manuscripts before they are submitted to journals." This is the first that I have seen such a thing and just wanted to point it out.

Response: Yes, we wanted to make the data available while further improving the manuscript during the peer review process.

Please note that there was no comment from Reviewer #2.

Reviewer #3:

>The authors of this article are investigating the role of cytosolic PDH enzyme in Medicago truncatula. They propose 4 non-mutually exclusive in planta functions of the PDH enzyme and strategically investigating those hypotheses. Overall manuscript is written well and clearly. I would suggest a minor change:

1. There is some inconsistency among the amount of tocopherol: According to the methods, the majority of experiments were done with leaves from 6 weeks-old plants. And we would expect similar values for the plants at time 0.

However, in Fig3b, total tocopherol in leaves in Wt is ~ 27 pmol/mg FW and ~ 30 pmol/mg FW in pdh1-1.

In Fig 4 - for H2O treatment total tocopherol in Wt is ~ 18 pmol/mg and ~ 15 pmol/mg in pdh1-1.

In Supplementary Fig 3 - for 0h highlight treatment total tocopherol in Wt and pdh1-1 is ~ 12 pmol/mg FW.

In Supplementary Fig 6b, at day 0, å- tocopherol ~ 26 pmol/mg FW in both Wt and mutant. Similar is with the amount of Tyr, Fig 3a leaves and Supplementary Fig 3a.

Response: For experiments such as feeding with shikimate, time zero indicates leaves that were clipped and then quickly dipped into either water or shikimate solution and then dried and used for metabolite extractions. The differences in tocopherol levels may have something to do with the handling of leaves prior to metabolite extraction. Although we tried our best to collect similar leaves across all experiments, they were conducted over the course of multiple years with plants being grown many times. Therefore, the differences in tocopherol accumulation likely come from differences in the leaf stage and overall health of the particular plants and leaves used across these experiments. Tocopherol levels are more sensitive to variability than the other metabolites measured in these experiments.

>2. Regarding natural leaf senescence experiment- authors stating that "PDH enzymatic activity was not induced upon senescence..." line 204. However, in Supplementary Fig 4c, d amount of PDH mRNA at S2 is almost 60% lower than in S1, and PDH activity is around 17 pKat/mg at S2 compare to 13 pKat/mg at S1. This could mean PDH activity did increase. The total amount of PDH protein in S1 vs S2 could probably help to interpret those results.

Response: Thanks for the thoughtful comment. First, although we do not know the amount of the PDH protein in those extracts, the enzyme activity is normalized by total protein contents in the extracts. Therefore, the slightly elevated PDH activity despite 60% lower mRNA suggests that there may be some unknown post-transcriptional regulation of PDH, which is now briefly discussed in the discussion.

3. Shikimate feeding experiment (Fig4) and the Senescence experiment (Supplementary Fig 6) done with the excised leaves. Both showed slight changes in metabolites in corresponding control, which may point to the involvement of PDH in wounding response.

Response: We agree and included discussion of potential roles of the PDH pathway in response to stress (e.g. wounding) that may increase flux through the shikimate pathway (as shown by prior studies).

4. Lines 222-225 - describing the extended dark treatment. The authors use (Xing and Last, 2017) reference to support the text and describe the experiment. However, the Xing and Last talking about branched-chain amino acids and not aromatic amino-acids and does not have any direct description of the treatment. I would suggest including another reference that more directly relate to the text. And add a few sentences in the method section describing the conditions of this experiment.

Response: Additional experimental details were added into the methods section and the reference by Xing and Last 2017 was replaced by Peng et al., 2015.

5. Lines 212 -215 - ...The expression of genes...was, (not were...)
Response: Corrected. Thank you.