

A Role for TIC55 as a Hydroxylase of Phyllobilins, the Products of Chlorophyll Breakdown During Plant Senescence

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Review timeline:

TPC2016-00630-RA	Submission received:	Aug. 8, 2016	
	1 st Decision:	Sept. 4, 2016	<i>accept with minor revisions</i>
TPC2016-00630-RAR1	1 st Revision received:	Sept. 8, 2016	
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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.)

TPC2016-00630-RA 1st Editorial decision – *accept with minor revisions* Sept. 4, 2016

We have received reviews of your manuscript entitled "A novel role for TIC55 as a hydroxylase of phyllobilins, the products of chlorophyll breakdown during plant senescence." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in *The Plant Cell*. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please consider the following:

As noted by Reviewer 3, your assignment of TIC55 enzymatic function is predominantly based on metabolic analysis of the *tic55* mutant and not *in vitro* characterization of the enzyme. We realize that *in vitro* characterization of a membrane bound, co-factor containing enzyme might not be a straightforward task and is hence subject of future work. It would thus be appropriate to clearly discuss this caveat and the potential limitation, such as definition of the main substrates, in your manuscript. Reviewer 3 also mentions the lack of any photographs showing the mutant phenotype. Here we tend to side with your choice not to show such data as apparently no significant differences have been observed between mutant and wild type. However, if you have noted any phenotypic differences, for example during certain life stages of the plant, such as senescence, it would be appropriate to show the data.

It is *Plant Cell* policy that Supplementary Figures should support figures in the main text. As noted by Reviewer 3, your Supplementary Figure 4 shows important primary data in support of your conclusions; hence you might want to include this data as a main figure in the text.

Reviewer 2 is concerned about citing the Reinbothe et al 2004 *PNAS* paper, the findings of which in the meantime have been shown to be based on second site mutations and thus no longer considered to be reliable. Hence you might want to remove this reference or replace it with a more recent appropriate one.

----- Reviewer comments:

[Reviewer comments shown below along with author responses]

Reviewer comments and **author responses**:

Decision letter:

As noted by Reviewer 3, your assignment of TIC55 enzymatic function is predominantly based on metabolic analysis of the *tic55* mutant and not *in vitro* characterization of the enzyme. We realize that *in vitro* characterization of a membrane bound, co-factor containing enzyme might not be a straightforward task and is hence subject of future work. It would thus be appropriate to clearly discuss this caveat and the potential limitation, such as definition of the main substrates, in your manuscript.

Indeed, like PAO, which we unsuccessfully tried to express heterologously with different systems for many years, biochemical investigation of TIC55 is “difficult”. In the frame of this work, we tried to express TIC55 in *E. coli* but were unsuccessful and therefore are unable to provide solid biochemical data on the enzyme. Although, we could determine epi-pFCC hydroxylating activity with protein fractions isolated from bell pepper chromoplasts, we did not try to analyze in detail the substrate-specificity for this activity, because in our opinion, such analyses reasonably is only done with a purified enzyme fraction or with recombinant protein. Instead, and to clarify the confusion raised by Reviewer 3, we argue in the Discussion (lines 278-281) about pFCC being the sole substrate *in vivo*. To address the (justified) notion of Reviewer 3 about missing clarity about what phyllobilins are, we now detail that each linear tetrapyrrole derived from chlorophyll is considered as a phyllobilin. For this, we changed the text of the manuscript at the following positions: line 59 (naming pFCC as a primary phyllobilin), line 73 (clarifying that not only NCCs and DNCCs are phyllobilins), line 78 (clarifying that nonfluorescent phyllobilins arise from the isomerization of respective fluorescent phyllobilins). Finally, we specified in the title of line 208 that TIC55 hydroxylates pFCC.

Reviewer 3 also mentions the lack of any photographs showing the mutant phenotype. Here we tend to side with your choice not to show such data as apparently no significant differences have been observed between mutant and wild type. However, if you have noted any phenotypic differences, for example during certain life stages of the plant, such as senescence, it would be appropriate to show the data.

We extended the description of a missing phenotypes and added references that support our findings (lines 230-232). The request of Reviewer 3 to analyze stress conditions (such as high light) to investigate the impact of TIC55 absence, is fair. However, we believe that if phenotype effects would occur in relation to the absence of TIC55 as a phyllobilin-modifying enzyme, these effects might even be enhanced when looking at a multiple mutant that is abolished in ALL phyllobilin-modifying reactions. Since we propose to work on such a mutant in the future, we added the aim to also investigate the impact that the entire absence of phyllobilin modification might have in response to stress conditions (line 395-298).

It is *Plant Cell* policy that Supplementary Figures should support figures in the main text. As noted by reviewer 3, your supplementary figure 4 shows important primary data in support of your conclusions, hence you might want to include this data as a main figure in the text.

As proposed, we added the data of the old Supplementary Figure 4 as new Figure 7 in the main part of the paper.

Reviewer 2 is concerned about citing the Reinbothe et al 2004 *PNAS* paper, the findings of which in the meantime have been shown to be based on second site mutations and thus no longer considered to be reliable. Hence you might want to remove this reference or replace it with a more recent appropriate one.

We removed this citation, re-wrote the respective text (lines 220-222) and provide a new reference (Kim and Apel 2004) that supports the added notion that the proposed role of PTC52 has been questioned.

Reviewer #1:

In this paper, Hauenstein et al. identify TIC55 as the enzyme responsible for hydroxylation of phyllobilins, the end products of chlorophyll degradation occurring during senescence. This is a very surprising and interesting finding since it revisits the function of TIC55, a Rieske-type oxygenase previously described as a sensor of the chloroplast redox status and a regulator of chloroplast protein import.

This finding is supported by strong experimental evidence using *Arabidopsis thaliana* as the model system. The authors first carefully describe the biochemical approaches leading to the characterization of the hydroxylase as an intraplastidial, membrane-localized, ferredoxin and oxygen-dependent activity. These features lead the authors to conclude that the hydroxylase activity depends on a Rieske-type enzyme, limiting the potential candidates to 3 proteins. Analysis of phyllobilin hydroxylation in corresponding T-DNA insertion lines clearly indicated that expression of TIC55 is essential for this modification to occur.

Hydroxylation of phyllobilins, is a ubiquitous process in the land plants. Although the physiological function of this modification remains undescribed, the discovery of the hydroxylase is of great interest in plant biology.

In summary, this paper makes an important step forward in the understanding of chlorophyll catabolism. In addition, it puts an end to the controversy about the role of "TIC55" in chloroplast protein import.

This is a high quality manuscript that is well written and contains nice figures.

Minor Revisions:

1) Page 5. The description and relevance of the isomerization experiment (acid isomerization) is not clear. It could need some additional information.

We provide a short rationale for performing the isomerization experiment and added a corresponding reference (lines 138-140). We hope that the reason for doing this experiment, i.e. to confirm that FCCs fragment differently in LC-MS compared to respective NCCs, is now clearer.

2) Figure 4: although the size of the peak corresponding to hydroxy-epi-pFCC formation indeed appears smaller under oxygen-depleted conditions, it would be nice to get some relative values to compare the different conditions and ratios between hydroxylated versus non-hydroxylated epi-pFCC. Normalization using epi-pFCC would be excellent.

We analyzed the data of these experiments and now plot the relative abundance of formed hydroxy epi-pFCC (ratio of hydroxy epi-pFCC to epi-pFCC) as an inset in Figure 4A. We also changed the legend accordingly.

3) In the legend of Figure 4, "Note that absence of oxygen largely inhibits epi-pFCC formation" should be "epi-pFCC hydroxylation" (we think).

We are very thankful to the reviewer for spotting this mistake. We corrected the legend of Figure 4.

4) In Material and Methods the authors should give more details concerning the experiment using MES16: what were concentrations of the MES16 enzyme and buffer conditions

We describe this method in more detail (line 463-464).

5) p8, 248: add a reference for "because phyllobilin glucosylation depends on prior C32 hydroxylation"

Again, we are very thankful for this comment and we changed the text related to this notion (line 239-240). Actually, up to now, it was only considered that hydroxylation precedes glucosylation. With the data presented in Figure 6, we are now able to experimentally show that, indeed, there is no glucosylation in the absence of hydroxylation.

6) Abstract: the last sentence does not sound right to me. I would rather write that "chlorophyll degradation co-evolved with land plants".

We changed this sentence as proposed (line 42).

Reviewer #2:

Chlorophyll degradation is an indispensable process for the plants to survive. The chlorophyll degradation pathway can be divided into two parts; one is the core pathway from chlorophyll to FCC, which is catalyzed in the chloroplast and the other is modification processes, which are done outside of the chloroplast. In this report, the authors identified a hydroxylase of phyllobilin, which is the last unidentified enzyme of the core degradation pathway. By combination of mutant analysis and biochemical methods, the authors clearly show that *TIC55* encodes the dehydrogenase. This report is surprising and also reasonable, because *TIC55* was initially proposed as a member of the import machinery, however, this idea was questioned and *TIC55* was deleted from members of import machinery

in some review articles. TIC55 is also proposed as redox regulator, but its idea is also difficult to accept because TIC55 encodes oxygenase, which is not a reversible reaction. The authors solved these questions. This report will contribute not only to chlorophyll metabolism but also to the study of import machinery.

The experimental design is reasonable and the conclusion is well supported by the experiments. This manuscript is well written and can be easily followed. Please consider the following comments.

1. Discussion is interesting but the last discussion part "What is the Biological Role of Phyllobilin Modification" does not discuss the biological role of the modification. This part should be extensively revised.

We are thankful for this notion and the reviewer is absolutely right. We extended the last part of the Discussion (lines 387-395).

2. The authors cited the paper Reinbothe et al. 2004. The paper is not reliable and it is good idea to delete this reference and rewrite this part.

Please see our answer to the respective comment of the editors.

Reviewer #3:

This manuscript is a convincing study on assigning a novel function to TIC55, a protein of the chloroplast envelope, in chlorophyll degradation. This is important in two respects. First, this protein was believed to be part of a redox sensor in protein import and this report provides solid for another role, which is most likely the primary function of TIC55. Second, this is a conserved protein in land plants, pointing to an important contribution of this chlorophyll degradation step in plant evolution.

The experiments are of high quality, well described, and provide convincing evidence of the biochemical activity of TIC55, namely the introduction of a hydroxyl group on the C32 ethyl side chain of primary fluorescent catabolites (pFCCs) of chlorophyll. I would suggest that Supplemental Figure 4 be included as a main figure, because it shows the (presumably) direct consequence of the knocking-out of TIC55, i.e. the absence of hydroxy-pFCC.

The analysis presented in Figure 6 shows a reduction of hydroxylated phyllobilins, which are downstream products of the hydroxylation of pFCC. Since there are no *in vitro* assays on different substrates, one question is what the major substrates of TIC55 are. Based on the localization of TIC55 and the absence of hydroxy-pFCC in *tic55* mutants, one would conclude that TIC55 does indeed hydroxylate primarily pFCC. However the data presented in Figure 6 and the title of the corresponding paragraph present TIC55 as a phyllobilin hydroxylase. This creates some ambiguity regarding the substrate range of TIC55, since phyllobilins are defined in the Introduction as non-fluorescent chlorophyll catabolites, whereas pFCC, presumably the substrate of TIC55 is a fluorescent chlorophyll catabolite. The authors should clarify this issue, by reformulating the text and/or by providing additional evidence regarding the phyllobilin hydroxylase activity.

Please see our answer to the respective comment of the editors.

Beyond the metabolite profiling, the characterization of the mutants is minimal. It is just mentioned that they showed no growth difference or visible phenotype during senescence (page 8, line 239-240), but no data, let alone pictures, are provided. Basic characterization of the mutants, such as biomass, growth characteristics, etc. should be done. Also, because of TIC55 is involved in chlorophyll degradation, actual phenotyping of the mutants with regards to senescence and/or stress situation which impact chlorophyll (for example high light) should be done.

Please see our answer to the respective comment of the editors.

TPC2016-00630-RAR1 2nd Editorial decision – *acceptance pending*

Sept. 8, 2016

We are pleased to inform you that your paper entitled "A novel role for TIC55 as a hydroxylase of phyllobilins, the products of chlorophyll breakdown during plant senescence" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor

Sept. 19, 2016