Cytochrome b_5 is an Obligate Electron Shuttle Protein for Syringyl Lignin Biosynthesis in Arabidopsis

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Review timeline:

TPC2018-00778-RA	Submission received:	Oct. 15, 2018
	1st Decision:	Nov. 22, 2018 *revision requested*
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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.)

TPC2018-00778-RA 1st Editorial decision – *revision requested* Nov. 22, 2018

We ask you to pay attention to the following points in preparing your revision. Specifically, there were four common themes in the reviews and the reviewer discussion that should be addressed:

1. Improved phylogenetic analysis.
2. Improved information on all genetic lesions in the manuscript.
3. All experiments must have independent biological replications.
4. An appropriate negative control for in planta interaction studies.

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

[Reviewer comments shown below along with author responses]

TPC2018-00778-RAR1 1st Revision received Jan. 30, 2019

Reviewer comments and **author responses**:

Reviewer #1:

Gou et al provide an in-depth analysis of where p450s in lignin biosynthesis obtain their reducing equivalents required for catalysis in the model plant Arabidopsis. While it has long been known that plant p450s require redox active partner proteins for turnover, limited studies have investigated the specificity of individual p450:reducatse relationships, one of which has been considered generally non-specific. Gou et al. provide compelling evidence that particular plant p450s have a higher affinity for certain electron providers over others, adding another level of complexity to in vivo p450 metabolism. Specifically, the authors show that amongst the three critical p450s involved in lignin biosynthesis, F5H shows significant dependency upon a single cytochrome b5 (CB5D) for its activity while the other two p450s are unaffected by its absence. Quantification of S lignin content of WT and transgenic plants,

combined with microsomal activity assays, provide clear indication that F5H preferentially draws its reducing equivalents from CB5D in addition to the more universal NADPH-reductases. These findings would be of high interest to the plant and synthetic biology community, as they have important implications for regulation of plant p450 activity and replication of plant biosynthetic pathways. However, the manuscript raises additional questions that could be addressed within the article. We recommend publication after minor edits.

Point 1. The primary question leading from this study is how specific is this CB5D dependency? That is, do other plant p450s display unique redox-partner preferences and where/how did this come to be for F5H? Obviously the former point is beyond the scope of this paper, but the phylogenetic tree provided in SFig2 should be expanded to include additional CB5s from related and distant species. Does a CB5D clade evolve with F5H appearance? More could be inferred from additional analysis to add depth to the discussion.

RESPONSE: Thanks for the comments. In fact, we had thought about the same intriguing questions and planned on performing follow-up work. As suggested by the reviewer, we have now expanded the phylogenetic analysis by including CB5 family (primarily CB5D)-related orthologs across land plant species. We present the expanded phylogenetic tree in a separate Supplement Figure 11.

Briefly, BLAST searching using the CB5D sequence and combining the documented least divergent orthologs of CB5D in the database of the PANTHER classification system, over 80 putative orthologs were initially identified and used in the alignment and preliminary phylogenetic analysis. We then chose the closer orthologs specific to CB5D with amino acid sequence identity over 60% followed by removing the CB5D clade orthologs that appeared in the replicate trees with percentage less than 50% in the bootstrap test (1000 replicates) to ensure that we obtained a reliable phylogenetic tree. Eventually, with the inclusion of other Arabidopsis CB5 family members and the orthologs in distant plant species, we finally obtained a phylogenetic tree composed of 29 members to estimate the evolutionary relationship of CB5D across land plant species.

We found that close and reliable CB5D orthologs were primarily present in angiosperms and were especially overrepresented in the Brassicaceae family (under the genera Arabidopsis, Camelina, Brassica and Capsella), as well as the Brassicaceae sister family, the Cleomaceae. There were also two close orthologs found in *Zea mays* but there was no homolog member with reasonable sequence similarity found in gymnosperms. Interestingly, we did identify three distant CB5 homologous sequences from the lycophyte *Selaginella moellendorffii*, which formed a unique clade that is evolutionarily connected to the CB5D clade of angiosperms. Considering the occurrence of S-lignin synthesis and the convergent evolution of F5H in the genus of Selaginella and in angiosperms, our data suggest that 1) CB5D may have co-evolved with F5H for S-lignin synthesis, and 2) after the emergence of CB5D, the redox donor and acceptor partnership may have been further specified within a few lineages of angiosperms.

We discussed the observation in the Discussion section of the revised manuscript and presented the data in Supplemental Figure 11.

Point 2. It is clear that CB5D is important for S lignin biosynthesis, but could it also be utilized by other p450s? It would be interesting for the authors to see if the closest F5H relatives also display a CB5D dependency. Are C4H and C3'H the most similar?

RESPONSE: Arabidopsis F5H (CYP84A1) has a closest paralog, CYP84A4, that was identified to be the key enzyme for α-pyrone biosynthesis. Meanwhile, CYP84As are evolutionarily close to CYP75A1, the flavonoid 3'-hydroxylase (F3'H). Therefore, to address the reviewer's question, we analyzed the accumulation levels of α-pyrones and 3'-hydroxylated flavonol, quercetin in cb5d, atr-2 and wild-type plants. We found that disruption of *CB5D* significantly depleted the formation of arbidopyrones but did not affect flavonol quercetin accumulation. These data suggest that CB5D, besides functionally associating with F5H (CYP84A1), is also required by the closet paralog CYP84A4 but has no functional interaction with F3'H (CYP75A1).

We presented the data in Results, together with a new Supplemental Figure 5. We also discussed the data in the revised Discussion.

Point 3. In Figure 3, the *ref3-3* line shows a significant decrease in sinapoyl ester and G lignin content but limited affect upon S lignin content. Can the authors explain this considering that the *ref3-3* mutation is in the *C4H* gene? Wouldn't all three be expected to decrease based upon the model provided in SFig1?

RESPONSE: The reviewer pointed out an interesting question that is actually worthy of future exploration. In the previous study from Prof. Chapple's group and in our current study, the data indeed showed that mutation of *C4H* (*ref3-3*) resulted in the significant reduction of G-lignin and sinapoyl ester content, but did not substantially impair S-lignin synthesis. Considering the well accepted linear monolignol biosynthetic pathway in Arabidopsis (which was the model presented in SFig1) and that only one *C4H* gene for phenylpropanoid biosynthesis is present in the pathway, the results for *ref3-3* appears confusing. However, one of the explanations for this result is the potential existence of alternative lignin biosynthetic pathway(s) at the tissue and/or cellular level in Arabidopsis that preferentially leads to S-lignin synthesis. This notion was well discussed in a recent excellent review article by Renault et al. (Curr. Opin. Biotech. 2019, 56:105-111). The key evidence supporting this hypothesis is the identification of a bifunctional Phe and Tyr ammonia-lyase (PTAL) in *Brachypodium distachyon*. The activity of PTAL could bypass C4H step and lead to the synthesis of about half the amount of lignin in the plant. Moreover, feeding isotope tyrosine, the labels were preferentially traced into S-lignin and cell wall p-coumarate ester.

Although currently PTAL and the potential parallel lignin biosynthetic pathway have not been reported in Arabidopsis, it is of high interest to explore such possibility in the future. Considering the information in grass, we added PTAL in the schematic pathway in SFig.1 and, in the Figure legend we revised the title as "The 'simplified' scheme of phenylpropanoid-lignin biosynthetic pathway, illustrating three P450 enzyme-catalyzed hydroxylation reactions in monolignol biosynthesis."

Point 4. In SFig3, panel A shows a slight decrease in Sinapoyl malate in the *cb5c-1* line but the lignin content is not shown in panel D. Does *cb5c* change the S-lignin content slightly?

RESPONSE: In our previous submission, we had presented histochemical detection data on S-lignin content in the *cb5c* mutant and now, we additionally present the data on chemical composition analysis of lignin in *cb5c* plants in SFig 3. The data clearly confirm that the disruption of *CB5C* did not alter lignin content (both G- and S-lignin) at all.

Point 5. The pull-down assays for all three p450s revealed associations with all CB5C-D, ATR1/2, and CBR1, yet only dependencies on CB5D and ATR2 were shown for F5H. Can the authors comment more on what is known regarding the physical interactions of p450s with its partner redox proteins? Do other plant p450s also pull-down all the same proteins?

RESPONSE: In yeast and mammal studies, P450 enzymes are well proven to interact with their redox partners, CPR and CB5, via electrostatic interactions to form functional 1:1 complexes. However, the stoichiometry of P450s and their redox donor proteins is substantially different. The concentration of redox partners such as CPR has been estimated to be 10 to 100 times lower than that of total P450s in human and mammalian cells (Watanabe et al. 1993). Since there are multiple P450s in excess of their redox partners in the same membrane, it is impossible for redox proteins to interact with all P450s at the same time. Therefore, either the P450s organize in complexes to facilitate the interactions with a limited number of redox carriers or the monomeric P450s and their electron carriers must interact stochastically and dynamically.

Co-IP or pull-down assays not only precipitate the physically direct interaction partners of the bait but also the proximal proteins at a certain distance. Therefore, the fact that pull-down with three P450s revealed a common set of electron transfer components may provide further evidence that monolignol biosynthetic P450s as well as their related donor proteins are spatially organized together or located in close proximity on the ER membrane. However, within the complex, different redox donors may be specified for particular P450s, which explains why three P450s pull-down CB5D but only F5H depends on it.

On the other hand, this does not mean that any other P450 proteins could pull-down the same suite of electron partners, since the interactions between redox proteins and P450s are stochastic and dynamic. The pull-down also depends on whether or not the P450s are organized together, and different experimental conditions, such as the harshness of membrane protein solubilization, and inclusion of fixation regents etc. We added the above comments to the Discussion of the revised paper.

Reviewer #2:

This is an interesting and important paper documenting the overlooked role of cytochrome b5 isoform D (CB5D), an electron shuttle intermediate in S-lignin and sinapoyl ester biosynthesis. The work seems to have been rigorously performed, although a few essential details could be added (see comments below). The text could be improved to be

a little more concise and clear; there are many long sentences where the syntax, tense or other aspect of grammar gets lost. The quality of the English could also be improved, for example by correct inclusion of 'the', 'an' or 'a'.

Point 1. Complementation assays, Section 263-278 and legend to Figure 4: It is important for the authors to state that the original *cbd-1* mutant was homozygous and/or to say that they confirmed the continued presence of the original *cbd-1* mutation in the transgenic CB5D/*cb5d-1/2* lines analysed. Without knowing that the mutation is still present, these lines are not 'complemented'.

RESPONSE: Examining and confirming homozygosity of the mutant lines are always the first jobs that we conduct in our genetic analysis. All those reported mutant lines and the mutant background for transgenic study had been thoroughly screened and verified for homozygosity in this study. The genotyping primers for all the mutant lines actually had been presented in Supplemental Table 1 in our original submission. We now more specifically stated such information in a consolidated paragraph in the Methods and repeated this in each related section and/or the figure legends.

Point 2. Line 316-318: While I agree that the data are convincing in showing that the CB5D mutant variants are expressed but do not restore the production of sinapoyl esters or S lignin biosynthesis, I wonder if the authors can be completely confident that this is because the electron transfer property of CB5D is compromised. I appreciate that it would be difficult to demonstrate that the protein is properly folded, but the use of protein structural predictions, and a comment in the text on the result, could at least suggest that the mutations made should have little effect on protein folding.

RESPONSE: Thanks for the comment. Following the reviewer's suggestion, we conducted protein homology model analysis on the mutant variants, which was performed with intensive structural simulation and energy minimization. The model with two His amino acid substitutions showed a nearly identical tertiary structure to that of parental protein, implying that substitutions have no substantial effect on protein folding and overall structure. We present the data in the Results, and showed the model of the variant in Figure 6.

Point 3. Lines 172-182: It is impossible to evaluate the data on CB5C and CB5D without knowing whether or not the Arabidopsis mutants used are knock-outs or likely to be just knocked-down in gene activity. The authors should comment on this in lines 172-182. The data should not eliminate CB5C and CB5D from further consideration unless they are knock-outs or severe knock-down mutants.

RESPONSE: The diagram of the T-DNA insertions in *cb5c* and *cb5e,* and the qRT-PCR analysis on the genes expression in two mutant lines are now included in SFigure 3. (The data for *cb5d* had already been presented in the Fig3 in the original submission). The data show that *cb5c* has its T-DNA insertion in the third exon and no transcripts were detected, and therefore it is a null knock-out mutant; *cb5e-1* is a severe knock-down mutant with a T-DNA inserted in the promoter region and the gene expression level was suppressed up to 85% compared to the WT. Both mutations have no significant change in their lignin and phenolic synthesis, and therefore we excluded them from further analysis.

Point 4. Figure 2: While I do not have any reason to doubt this data, it is an extremely poor practice to report comparative expression data without using biological replicates - the legend suggests that only technical replicates have been used. Unless the authors can include biological replicates or can state that the data shown are representative of measurements made on other plants, then I recommend that this figure be move to Supplemental. The same applies to Figure 3 B - why were no biological replicates used? Also Figure 5A - it is crucial to know that all of this expression data are representative, not just the pattern seen in a single plant.

RESPONSE: We are sorry that we did not make the description even clearer, but in our original submission in the Method section on RT-PCR analysis, we had purposely stated that "All experiments were repeated at least twice with different batches of samples, and representative data are presented."

All the q-RT analysis data in Figure 2, 3B and 5A that the reviewer mentioned were the representatives of at least 2–3 independent batches of experiments (i.e. biological repeats), which in most cases were carried out by different individual experimenters. Now with the reviewer's comment, we have re-consolidated or repeated some of the analyses, and presented them with the more detail sampling and replicate information in both the Methods section and the related Figure legends.

Point 5. Figure 5: This data could be removed to Supplemental and perhaps replaced in the text by Supplemental Figure 6, which seems more important.

RESPONSE: Thanks for the comments. We changed the original Supplemental Figure 6 as the main Figure, but we would like to keep Figure 5 as it is, since the Journal editorial policy requires the number of supplemental materials to be reduced. Please note that to better address the reviewer's comment on experimental replicates in Figure 5A, we repeated the analysis once again within biological replicates and replaced the original panel.

Reviewer #3:

The manuscript addresses the role of AtCB5D in lignin biosynthesis. The authors have previously used transgenic Arabidopsis plants with HPB-tagged C4H, C3'H, and F5H to IP microsomal fractions from lignified stems to identify CB5D as the isoform of the CB5s that co-IP'ed the most with all three P450s. In the present study, the authors provide strong genetic and biochemical evidence that CB5D is required for S-lignin biosynthesis and that CB5D is working together with ATR2 to obtain maximum F5H activity.

Hitherto CB5s have been proposed to be associated with electron shuttling during lipid and sterol metabolism. The finding is novel and teaches us that individual CYP450s have different requirements for electron donors, and that activities outside lipid metabolism may require a CB5. The localization of CB5 to the ER was shown by visualizing that GFP-tagged CB5 behaved similarly to SP-GFP-HDEL, and as the respective CYPs it co-IPed with.

Point 1. The BIFC experiment to show protein-protein interaction between CB5 and the respective P450s is less convincing, as the negative control used soluble GFP as the interaction partner. Whether these data truly reflect specificity is an open question, as we are talking about two ER membrane-bound proteins produced from the 35S promoter. Maybe all ER membrane-bound P450s will interact and maybe CB5D will interact with any P450s when both genes are expressed from the 35S promoter in a heterologous host? Is the setup bound to succeed? In short, 35S-driven ER bound proteins may interact in a heterologous host simply due to overproduction and as such an artifact. If protein-protein interaction data should be included, it would be necessary to provide a proper negative control.

RESPONSE: We understand the reviewer's concern. In fact, in our studies, we had paid a great deal of attention to this issue. We now provided two new sets of negative controls to further validate the BiFC assay: one was F5H-YFPn paired with the YFPc fused to the transmembrane domain of calnexin 1 protein (tCNX1), which we had used in our previous study (Guo et al. 2018); CNX1 is an ER-localized chaperon protein. The second set was F5H-YFPn paired with another P450, CYP79B2, involved in auxin/glucosinolate biosynthesis. When they were co-expressed in tobacco leaves, they did not generate any significant fluorescence signals, which clearly contrasts to the results using F5H-YFPn with YFPc-CB5D or Atr2-YFPc. Furthermore, the fusion proteins of F5H, tCNX1 and CYP79B2 in the negative control sets were monitored via western blot analysis to validate their proper protein expression, thus excluding the possibility that the failure in generating chimeric fluorescence signals of the paired proteins was due to the lack of expressed proteins.

Although the BiFC assay could not discriminates whether the co-expressed proteins are in a direct physical interaction or in close proximity, our data validate that this approach is not nonspecific for revealing protein associations on the same membrane. We presented the new data in the revised manuscript.

TPC2018-00778-RAR1 2nd Editorial decision – *revision requested* **Feb. 21, 2019**

Editor comments and **author responses**:

Reviewer 2 had some key albeit minor editorial observations that should be addressed. Additionally, there are the following statistical concerns that became apparent in the second round of review that need fixing.

Point 1. In Figure 2A and 7A, the error bars are from technical replicates, but this is not valid. Instead, technical replicates are averaged and then this is used as a single value in combination with the independent biological

replicates. Further, whenever technical replicates were conducted, it has to be clear how these were utilized in the analysis.

RESPONSE: Considering the comments of the reviewers on independent batches of experiments, and to unify our studies, we completely re-conducted the related qRT-PCR analysis presented in the previous Figure 2A and 7A. We performed them with three biological replicates, each replicate was composed of three technical repeats. The data of technical repeats were then respectively averaged as a single value for each biological replicate. Statistical analysis (when applicable) was conducted based on data from three biological replicates. The data are presented now in the re-ordered Figure 4A and Figure 9A. The results are consistent with our previous conclusion.

Point 2. In Figure 7B, it appears that there is no replication. If this is the case, then the average across the independent T1 lines should be presented, as this provides the information about what is or is not due to the transgene vs. position effect variation. Additionally appropriate statistics should be provided. The independent lines per construct provide the replication to allow this. This is equally true in Supplemental Figure 6, 7 and 8A.

RESPONSE: Since the Arabidopsis T1 transgenic line is a single plant, in most analytic cases, it is impossible to conduct biological replication. As suggested, we averaged the data derived from all the independent T1 lines (around 7–15 lines) per genotype as the single value, which represents 7–15 replicates. Correspondingly, the statistical analysis was performed with one-way ANOVA, Tukey's HSD test. The revised data are presented in the re-ordered Figure 9B (original Figure 7B) and Supplemental Figure 4,5 and 6A (for original Supplemental Figure 6, 7 and 8A).

Point 3. Sampling methods 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.), along with a clear description of and rationale for any statistical analyses conducted. 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.

RESPONSE: Since the sampling methods vary in different experiments, we had described the related information in the corresponding Figure legends in our previous submission. Now we described them more explicitly in both the Methods section (on qRT-PCR analysis and Phenolic quantification) and the Figure legends.

TPC2018-00778-RAR2 2nd Revision received March 14, 2019

Reviewer comments and **author responses**:

Reviewer #1:

The authors have addressed all my comments in a satisfactory manner.

Reviewer #3:

The authors have satisfactorily addressed my comments and also the other reviewers' comments.

Point 1. Line 460-462. this sentence should be stated more cautiously, e.g. start with 'This suggests that either ..... or that the monomeric P450 and their electron carriers interact....'.

RESPONSE: Revised as suggested.

Point 2. There are still many 'IPed' although reviewer #2 pointed out that this is not a word.

RESPONSE: Corrected them all.

Point 3. Is it an oversight that the title only mentions cytochrome b5 (not the D isoform)?

RESPONSE: As a title general for the broader readers, we prefer using “cytochrome b5” instead of “cytochrome b5 D isoform”. In particular, the classification of cytochrome b5 family members is not so uniform across different reports.

TPC2018-00778-RAR2 3rd Editorial decision – *acceptance pending* **March 14, 2019**

We are pleased to inform you that your paper entitled "Cytochrome b5 is an obligate electron shuttle protein for syringyl lignin biosynthesis in Arabidopsis" 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 2, 2019**