Substrate Specificity of LACCASE8 Facilitates Polymerization of Caffeyl Alcohol for C-Lignin Biosynthesis in the Seed Coat of Cleome hassleriana

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Review timeline:

| Date                     | Event Description                                           |
|--------------------------|-------------------------------------------------------------|
| TPC2020-00598-RA         | Submission received: July 28, 2020                          |
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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.)

TPC2020-00598-RA 1st Editorial decision – accept with minor revision Sept. 1, 2020

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. All three reviewers have provided enthusiastic and detailed reviews, but the comments of Reviewers 1 and 3 will be particularly important to address. Specifically, the statements about the relative role of laccases and peroxidases in initiating lignin polymerization and extension appear to be an over-interpretation and are not supported particularly well by your data. Furthermore, please focus on Figures 2 through 7, their legends, and the manuscript text that accompanies them (particularly the text on the monolignol feeding experiments) to improve their clarity.

RESPONSE: Please see responses below. We have carefully re-written the sections that implied differences between the functions of laccases and peroxidases in initiation and “bulk synthesis” of lignin, and have provided clarification of the monolignol feeding experiments and the details in the figure legends.

Reviewer comments and author responses:

Reviewer #1:

The study by Wang et al. entitled "Substrate-Specificity of LACCASE 8 Facilitates Polymerization of Caffeyl Alcohol for C-Lignin Biosynthesis in the Seed Coat of Cleome hassleriana" investigates the specificity of a laccase (Laccase 8) paralog for the oxidation of caffeyl alcohol residues in cleome. The study is coherent and well written, but several points listed below need to be addressed to clarify the findings of the study.

Point 1. The transcriptomic analysis focuses on fold changes, thereby neglecting other laccase paralogs during seed coat lignification that could be regulated post-transcriptionally (moreover that the authors show that lignification progresses even if their candidate laccase is silenced) - the different levels of expression of the different paralogs should also be discussed (as provided in the supplemental table and figures - i.e. LAC15 is highly expressed).
RESPONSE: The other laccases with expression in the seed coat are LAC5 and LAC15 (ortholog of Arabidopsis TT10). We now provide more discussion of why we concentrated on LAC8 in the context of the expression pattern of these other laccases.

Point 2. More bioinformatic analysis of the candidate laccase needs to be provided, including the presence and localization of its peptide signal as well as potential N-glycosylation sites (glycan normally required for the activity and stability of eukaryotic laccases) - as lignification occurs in the cell walls and external feeding experiments are performed, the authors must ensure that their candidate laccase has the proper localization and structure.

RESPONSE: Thanks for this suggestion. We have now indicated the signal peptide, protein localization and the predicted N-glycosylation sites of ChLAC8. The signal peptide is consistent with those in plant cell wall laccases, and the cleavage site is predicted to occur between amino acids 25 and 26. The predicted subcellular localization of ChLAC8 is extracellular. This is now mentioned, and the positions used indicated and cited. The positions of the 10 predicted N-glycosylation sites are now listed in new Supplemental Table 1.

Point 3. Recombinant laccases are generally not produced in E. coli (the authors cite an article that recently produced recombinant laccase in Pichia, which still allow some N-glycosylation), have the authors performed any codon optimization and/or truncation of the ORF (is the peptide signal still present)?

RESPONSE: There are in fact a number of reports of expression of recombinant laccases in E. coli (for example Salony et al, BBA- Proteins and Proteomics, 1784, 259-268, 2008; Ihssen et al., Sci Rep, 5:10465 | DOI: 10.1038/srep10465, 2015). However, these are mainly fungal and bacterial enzymes, but do include proteins that are normally glycosylated in vivo. Studies with both plant and fungal laccases have shown that removal of the glycan portion can have significant to no effect on the turnover rate and stability of the enzyme. We have added a section on this issue to make it clear that obtaining an active laccase in E. coli is not necessarily surprising, and to further discuss the position of the glycan substitution sites; three additional references have been added.

We did not perform any codon optimization and used the full-length coding sequence (ORF) for expression of ChLAC8 in E. coli. The peptide signal of the recombinant ChLAC8 was still present (now indicated).

Point 4. What is the rational behind performing the in vitro activity essays at pH 6.8, which is closer to cytoplasm than cell wall pH (the candidate laccase could moreover have different pH optimum for different substrate, as has been shown for other enzymes) - this should be checked? Additionally, more information needs to be provided on the linkages types in the dimers and trimers that are produced during the in vitro activity essays.

RESPONSE: The pH optimum of laccases varies. Fungal laccases have a low pH optimum, whereas the pH optimum of plant laccases has been listed as between 7.0- and 10.0 in a 2015 review article (Wang et al, Biotechnology for Biofuels, 8, 145. https://doi.org/10.1186/s13068-015-0331-yph). The pH optimum of the sycamore cell culture laccase is 6.8 (Sterjiades et al., Plant Physiol, 99, 1162, 1992). The pH optimum of the loblolly pine laccase was 5.9 (Bao et al Science, 1993), whereas in situ assays for AtLAC15 activity are done at pH 6.6 (Pourcel et al., Plant Cell, 2005). We chose pH 6.8 based on the sycamore enzyme.

As for the monomer linkage types, the m/z values of the extracted ions of C-dimers for all three potential bonding modes (benzodioxane, phenylcoumaran, and resinol) are 329. Based on our MS spectra, a fragment of 165 was observed, which could only arise from a C-dimer that features a benzodioxane via 8-O-4 linkage. This is consistent with the NMR results showing that C-lignin is a unique linear polymer featuring benzodioxane units via 8-O-4 linkage (Chen et al, PNAS, 2012). Unfortunately, the amounts of material generated in the in vitro assays are much too small for NMR analysis. The issue of linkage types for the laccase-mediated oxidation products is now mentioned in the text, and the dissociation pathways of the benzodioxane-linked dimer shown in new Supplemental Figure 7 as an explanation for the inset in panel H of Figure 2.

For the S dimers, the m/z values will differ between resinols and β-aryl ethers (417 and 435, respectively). The data in Figure 2 showing the EIC = 417 indicates the S-dimers were resinols joined via 8-8 linkage. This is now mentioned in the text. We could not find the ion at m/z 435, suggesting that the S dimers were not linked as β-aryl ethers.

Point 5. The main method for lignin analysis in the study is thioacidolysis, a method that cleaves 8-O-4 ether bonds and allows discriminating end-residues with free aliphatic as well as internal residues in-between two 8-O-4 ether bonds - the authors need to explain whether the focus is only on the internal residues (which make trithioketal derivatives), and not end-residues (forming either mono or dithioketals)? As feeding experiments are performed and
the authors state that their laccase candidate is implicated in the initiation of lignification, the different position of lignin residue using thioacidolysis should be provided to define how the fed monolignols are incorporated by end-wise polymerisation to the already present lignin.

**RESPONSE:** The focus of the thioacidolysis was on the internal residues. This is the method that has been used throughout our studies on C-lignin (See Chen et al., 2012, 2013 and Tobimatsu et al., 2013 referenced in the present MS.). It is true that it may well underestimate the G-lignin content, though. It is also important to understand that C-lignin is not present at all before around 14 DAP, and that the C-lignin that is made after this time is not linked to G-units. The two lignins are separate polymers. This has been confirmed by NMR studies (Tobimatsu et al., 2013). Please see the response to Reviewer #3 (point 1) regarding the model for lignin “initiation” vs. bulk polymerization. This has now been clarified in the text.

The suggestion of using thioacidolysis to characterize the end-group units for labeled caffeoyl alcohol is at first sight a good one. As the C-lignin polymer is joined almost exclusively by 8-0-4 linkages (in the form of benzodioxane units), thioacidolysis could break the C-lignin polymer and release thithioetals derivatives, however it is still not clear how effectively thioacidolysis can release the end-group units. The peaks for caffeoyl alcohol end-residues (either mono- or di-thioketals) have not been identified by using model compounds. We checked our GCMS chromatograms of our C-lignin samples. The putative peaks of the mono- and di-thioketals derivatives are very small, making it hard to characterize these peaks quantitatively and trace the labelling at the specific end positions in the feeding experiments.

Point 6. For the statistical analysis, please define how the t-test was corrected to enable multiple pairwise comparison encoded by lettering (or use a more appropriate test for multiple comparison such as a 1-way ANOVA)?

**Provide more information on the type of t-test used (non-parametric...)?**

**RESPONSE:** The statistical analysis of data in Figures 7, 8, 9 was done by one-way ANOVA. The different letters above the bars represent statistically significant differences determined by one-way ANOVA (Duncan, p≤0.05) with SPSS Statistics (version 22; IBM). Thanks for pointing out the mistake. The figure legends have been revised. The statistical analysis of data in Figure 4 and Table 1 were performed by unpaired two-tailed students t-test. All statistical analysis results (t-test and one-way ANOVA) are now shown in Supplemental Dataset 6.

Point 7. The silencing experiments using RNAi in Cleome showed that C-lignin content was reduced initially without affecting the overall lignin amount of the seed coat, these observations appear contradictory and the authors should provide an explanation on which residue(s) compensate for C residue reduction during the initial stage of lignification.

**RESPONSE:** The lignin in Cleome is all G lignin up to around 13 DAP, then a mixture of G homopolymer and C-homopolymer. The changes in C-lignin on knockdown of LAC8 are statistically significant at 20 DAP when less has accumulated, but not at 24 DAP. We interpret this as suggesting that LAC8 is involved more in initiating C-lignin synthesis than in completing C-lignin chains. The data in Figure 4 also show, at least in line 9 with the lowest C-lignin, that G lignin is correspondingly elevated. We believe that the lack of change in overall lignin level is a result of a compensation by increased G accumulation as a result of downregulation of C-polymerization. This is now pointed out.

Point 8. In the hairy root experiment in Medicago and over-expression in Arabidopsis, the authors show that overexpression of their candidate laccase in a comt mutant background leads to an increase in S residues in both species but do not provide any explanation - how are residues, prevented from being synthesized by mutagenesis, increased in lignin by overexpressing the candidate laccase?

**RESPONSE:** We suggested in the original manuscript that this was surprising. In fact, we do have an answer to this question, but we did not want to divulge it at this stage as the story is quite complex. We have identified what we believe to be an enzyme that is critical for maintaining production of coniferyl alcohol that may not be destined for lignin biosynthesis. We have a draft manuscript on this topic that is nearing completion. We now clarify the need for an alternative OMT in the revised manuscript.

**Reviewer #2:**

The manuscript "Substrate-specificity of LACCASE 8 facilitates polymerization of caffeoyl alcohol for C-lignin biosynthesis in the seed coat of Cleome hassleriana" focuses on the characterization of the ChLAC8 enzyme in vitro
and in planta. Enzyme activity and kinetics assays, in conjunction with multiple transgenic plant lines, demonstrate the role of ChLAC8 specifically in C-lignin polymerization. This works represents in important addition to the lignin field and the current understanding of lignin polymerization.

Point 1. Should there be a visible difference in the staining of the condensed tannins in Supplemental Figure 4B? All the images appear the same. Supplemental Figure 4A has ellipsis following the laccase names, which is presumably a typo.

RESPONSE: Condensed tannins are synthesized very early during seed coat development. The earliest we have analyzed seeds for lignin biosynthesis is 8 DAP, when they are still extremely small. Staining seed samples from the same batches that were analyzed for lignin does indeed show a similar level of staining at all times. We have now stained seeds at just 4 DAP, and they also show strong DMACA staining. We have published many papers on condensed tannin biosynthesis (including DMACA staining), and are very confident with the result. However, to provide additional confidence, we now include data in Figure 1A for transcript levels of anthocyanidin reductase (ChANR), the key enzyme for production of flavan 3-ol monomers for condensed tannin biosynthesis. ChANR is most strongly expressed at the earliest time point (8 DAP), and starts to decline around the switch to C-lignin biosynthesis, consistent with the expression pattern of ChLAC15, the homolog of AtTT10/(LAC15), the laccase implicated in oxidation of condensed tannins in the seed coat (Pourcel et al., Plant cell, 2010). Supplemental Figure 4A has been reformatted to correct the labeling of the X-axis.

Point 2. While the activity of ChLAC8 with 5H monomers is not biologically relevant in Cleome seed coats, there are other seed coats that produce 5H lignins with trace or significant amounts of S/G lignin. Can the authors comment on the activity of ChLAC8 with 5H and what it might mean for laccases in other seed coats? Enzyme kinetics of 5H units with ChLAC8 would provide an even more complete picture of the substrate specificity or promiscuity of the enzyme and how the substrates might be interacting within the binding pocket, but these experiments are not necessary for the scope of this manuscript.

RESPONSE: By 5H, we assume that you mean 5-hydroxyguaiacyl monomers. We did not perform the experiments with 5-hydroxyconiferyl alcohol because they are not biologically significant as regards Cleome, but we do agree that it is intriguing to know whether formation of 5-OH-G lignins requires a laccase similar to ChLAC8. We are aware of 5-hydroxy-G lignin occurring naturally but rarely in seed coats of some cacti (our earlier work, Chen et al., Plant J, 2013). The problem is that the species that we know to make 5-OH-G lignin (three members of the genus Escobaria in the Cactaceae) grow very slowly, are hard to pollinate, and make very few seeds. There is also no transcriptomic data for these species, so we can’t easily work on the natural production of 5H-lignin. We would love to know more about this, as 5H-lignin is, similar to C-lignin, a linear, benzodioxane-linked homopolymer. At the same time, of course, it is well known that 5H-units accumulate in lignin from COMT down-regulated plants. Vanholme et al (2010) showed that the lignin in Arabidopsis comet mutant plants over-expressing F5H was composed of 92% 5H units. Our present work shows that Arabidopsis does not possess a laccase that can permit polymerization of caffeoyl alcohol, whereas the results of Vanholme et al. clearly indicate that Arabidopsis does possess a laccase that can allow polymerization of 5-hydroxyconiferyl alcohol. In the present Figure 8, loss of function of comet in Arabidopsis results in about 0.5% 5H units in the lignin from the top of the inflorescence stem, but no C lignin. Expression of ChLAC8 increases the 5H-lignin around 3-fold, but still no C-lignin, as the precursor is not there. This suggests that ChLAC8 does work with 5-hydroxyciniferyl alcohol, as well as there being an endogenous system for polymerization of this monolignol in Arabidopsis. Good ideas for further work.

Reviewer #3:

The functions of the enzymes (laccases and peroxidases) that oxidize lignin precursors to trigger lignin polymerization are poorly understood. This study makes a compelling case for the role of the Cleome Laccase8 (ChLAC8) in polymerization of caffeoyl alcohol into catechyl-lignin (C-lignin) in seed coats. Previously, lignin was considered to consist of only H-, G-, or S-units, derived from p-hydroxy, coniferyl, or sinapyl alcohols, respectively. The Dixon group discovered the novel C-lignin, first in vanilla and cacti, then in the seed coats of Cleome. While the developmental basis and underlying transcriptomic mechanisms of C-lignin formation have been described, how the caffeoyl alcohol units were polymerized into C-lignin was unknown. This is important for the goal of engineering plants with high C-lignin content for various bioproduct applications. From a basic biology point of view, this work is also important for providing insight into factors determining lignin composition. The relative roles of monolignol supply compared to substrate specificity of the oxidative enzymes are unknown. The lignin-related laccases and
peroxidases from plants whose substrate specificity have been defined were relatively promiscuous, suggesting that monolignol supply was a key factor. In this study, the ChLAC8 is shown to have activity on caffeyl alcohol and sinapyl alcohol, but not coniferyl alcohol. This aligns with its role in mediating a switch from G-lignin to C-lignin in the seed coat, and it is the first evidence that the substrate specificity of a laccase or peroxidase can contribute to lignin quality.

The quality of the data and breadth of techniques in this work are impressive. The authors use gene expression data to correlate C-lignin with the different laccases and peroxidases expressed at the right place and time. They place these candidates in a larger context with a phylogenetic tree, to permit comparisons with laccases from Arabidopsis. HPLC and LC-MS/MS analyses demonstrate the substrate specificity. Using the recent crystal structure of a maize laccase, molecular modeling and conservation of amino acids provide a hypothesized mechanism to explain the caffeyl alcohol specificity. RNA interference knocking down CsLAC8 in Cleome, combined with extensive thioacidolysis analysis to determine lignin quality, establish that there is decreased C-lignin. In order to track exogenously added 'heavy' monolignols, 13C-caffeoyl and 13C-coniferyl alcohols are synthesized and added to Medicago hairy root cultures or Arabidopsis comt mutants, which produce the substrate. Using these approaches, the engineering of C-lignin into heterologous plants, albeit at low levels, is achieved. As the C-lignin is only detected with both the CsLAC8 expression and appropriate substrate (either engineered in the loss of function COMT plants or added exogenously), the claim that this gene product is responsible for C-lignin polymerization is well supported. I have several concerns and some small suggestions to improve the quality of this manuscript:

Point 1. Throughout the manuscript (lines 108, 138, 439-441), based on their Zhao et al. 2013 triple lac4 lac11 lac17 mutant analysis, the authors state that laccases "are essential for initiating lignin polymerization." I agree that they are essential for lignin polymerization, but do not agree that the full complement of expressed peroxidases genes in the triple mutant indicates that the laccases initiate lignin polymerization. As discussed in lines 428-441, there may be tissue-specific roles for laccases and/or peroxidases. At the end of the day, either class of enzymes produces a monolignol radical that could initiate lignin polymerization. The observation that the peroxidases are unable to compensate for the loss of the three laccase genes may be related to the severe and pleiotropic nature of the triple mutant phenotype. As Chapple et al. have shown, lignin-modification can lead to unexpected growth and metabolic phenotypes. We don't really know why the growth and phenolic profiles of those plants are so strongly affected. An extension of this model (line 494) that "bulk extension of C-lignin is catalyzed by peroxidases" is equally speculative. The gene expression data shows other LAC (as well as PRX) turning on late in seed coat development, so additional laccases turning on as the seed coat develops could also explain this result.

RESPONSE: We understand the reviewer’s objection to such an absolute statement concerning initiation vs. synthesis of “bulk lignin”. It is indeed an inference, and is not directly supported by evidence. There seems to be a similar argument about the absolute role of peroxidases in lignification in some tissues. We proposed an initiation function for laccases based on our studies of the Arabidopsis triple mutant, and it is also consistent with the impact of ChLAC8 down-regulation only at the early times after C-lignin synthesis begins in Cleome. However, far more technically challenging experiments would be necessary to provide absolute confirmation, including determining which parts of the lignin chain become labeled with 13C-caffeoyl alcohol (not sure we know how to do that) in wild-type and knockdown and perhaps CRISPR knockout lines of Cleome. We have revised the manuscript to down-play the idea that laccase is mostly involved with initiation and peroxidase with extension, and present alternative hypothesis that include functions in both for other laccases and peroxidases.

Point 2. To improve the clarity of the manuscript, the figure legends and labels should be more descriptive. Examples: Figure 2: "HPLC chromatograms of reactions of ChLAC8 with caffeoyl alcohol (A, B, peak1)" should indicate the control (A) does not contain ChLAC8 and (B) is the reaction (same throughout this figure). Figure 4F-what do the circles, triangles, and squares indicate? Figures 5 and 7 should say that these are in the comt mutant backgrounds (and indicate the allele for the Arabidopsis stems). The phenotype of the ChLAC8expressing Medicago loss-of-function COMT cultures (lines 287-300) shown in Figure 5A is interesting in its own right, even before the feeding experiments. A more descriptive label than 'blank' would be good. The figure 7 legend (Approach to the 13C isotopic labeling experiments) is particularly cryptic. It is unclear what is hairy root and what is Arabidopsis in this figure. Add arrows to show the M+6 monolignol-derived products.
RESPONSE: Thanks for the suggestions. These have been addressed/incorporated. In Fig 4, the symbols are in panel G, and represent individual measurements. This is now explained. In Figure 5, “blank” is replaced with “no monolignol fed” - we agree that “blank” certainly did not do justice to the experiment. The title of Figure 7 (now Figure 6) has been changed to “Use of thioacidolysis to reveal lignin labeling in the 13C monolignol feeding experiments”, and the label for panel A has been re-written for clarity. The plant species is not relevant for understanding this scheme, but the experiment to which these data refer has been indicated. The remaining panels have also been relabeled to aid clarity. The M+6 monolignol-derived products cannot be labeled in the EICs as they are scanned at the m/z of the labeled peak and run at the same retention time as the unlabeled products. We now explain that the double peaks are due to the presence of threo- and erythro isomers of the tri-etherates.

Point 3. Change the orders of Figure 5 and 6/7 to introduce the synthesis and validation of the 13C-labelled monolignols first, then describe the hairy root and stem experiments.

RESPONSE: Thanks for the suggestion. We have moved the figure with the synthetic scheme to become the new Figure 5, followed by the figure showing how the label incorporation into lignin monomers is determined by thioacidolysis. To accommodate that change, we have introduced a new section into the main text entitled “Synthesis of Labeled Monolignols and Determination of Their Incorporation Into Lignin”.

We are pleased to inform you that your paper entitled "Substrate-Specificity of LACCASE 8 Facilitates Polymerization of Caffeoyl Alcohol for C-Lignin Biosynthesis in the Seed Coat of Cleome hassleriana" 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 its presentation of scientific content, compliance with journal policies, and presentation for a broad readership.

Final acceptance from Science Editor Oct. 6, 2020