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Yet another twist in lignin biosynthesis: Is there a specific alcohol dehydrogenase for H-lignin production?

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Lignin is an aromatic biopolymer deposited in secondary cell walls of specialized cell types and synthesized upon polymerization of monolignols, which are hydroxycinnamyl alcohols produced from the phenylpropanoid pathway. This polymer plays important roles in plant growth and development and in the responses of plants to their surrounding environment. Lignin not only imparts structural fibers with mechanical strength, allowing plants to stand upright, but it also waterproofs xylem cells, enabling long-distance water transport (Barros et al., 2015). Additionally, lignin deposition is triggered by various biotic and abiotic stresses (Cesarino, 2019). However, the same physicochemical properties that allow lignin to perform its biological functions constitute a major hurdle in the processing of plant biomass into downstream products in biorefineries. Engineering plants to synthesize lower lignin contents or altered lignin composition/structure can optimize feedstocks for the bioeconomy (Mottiar et al., 2016).

Polymerization of the canonical monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol results in the deposition of the lignin units *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively (Figure 1). H units are a minor component of lignins, rarely exceeding 5% of the total monomeric content (Ralph et al., 2019). In terms of biomass utilization, lignins enriched in H units are desirable because they have a lower degree of polymerization, resulting in improved biomass digestibility (Mottiar et al., 2016). H-rich lignins can be produced by repressing genes encoding the enzymes acting immediately downstream of the branch-point between H- and G/S-units, but the resulting plants present considerable developmental abnormalities (Li et al., 2010; Vanholme et al., 2013; Bonawitz et al., 2014). For instance, the *reduced epidermal fluorescence 8-1* (*ref8–1*) mutant in Arabidopsis (*Arabidopsis thaliana*), harboring a missense mutation in *p-COUMAROYL SHIKIMATE 3′-HYDROXYLASE* (*C3′H*), is severely dwarfed and sterile (Figure 1; Franke et al., 2002). However, disruption of the MED5a/5b subunits of the MEDIATOR transcriptional co-regulator complex in the *ref8–1* mutant background blocks the transcriptional reprogramming that leads to dwarfing, enabling the plants to recover to near-normal stature while retaining their H-enriched lignin structure (Figure 1; Bonawitz et al., 2014). Consequently, these plants also exhibit substantially facilitated biomass digestibility.

Another bioengineering strategy toward higher biomass digestibility involves generating aldehyde-enriched lignins via down-regulation/loss-of-function of *CINNAMYL ALCOHOL DEHYDROGENASE* (*CAD*). CAD catalyzes the reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols, the last step in monolignol biosynthesis (Figure 1). Aldehyde incorporation is thought to make lignin more hydrophobic and reduce its association with hemicellulose, improving cell wall processability (Carmona et al., 2014). Indeed, the biomass of Arabidopsis plants with mutations in *CADC* and *CADD*, the two major lignin-related CADs in wild-type mature stems, releases double the amount of glucose when compared with that of the wild type with minimal effects on plant yield (Figure 1; Anderson et al., 2015).

In this issue of *Plant Physiology*, Muro-Villanueva et al. reported on the attempt to stack the double mutations *cadc cadd* (aldehyde-rich trait) on the *med5a/5b ref8–1* genetic background in Arabidopsis to block the synthesis of

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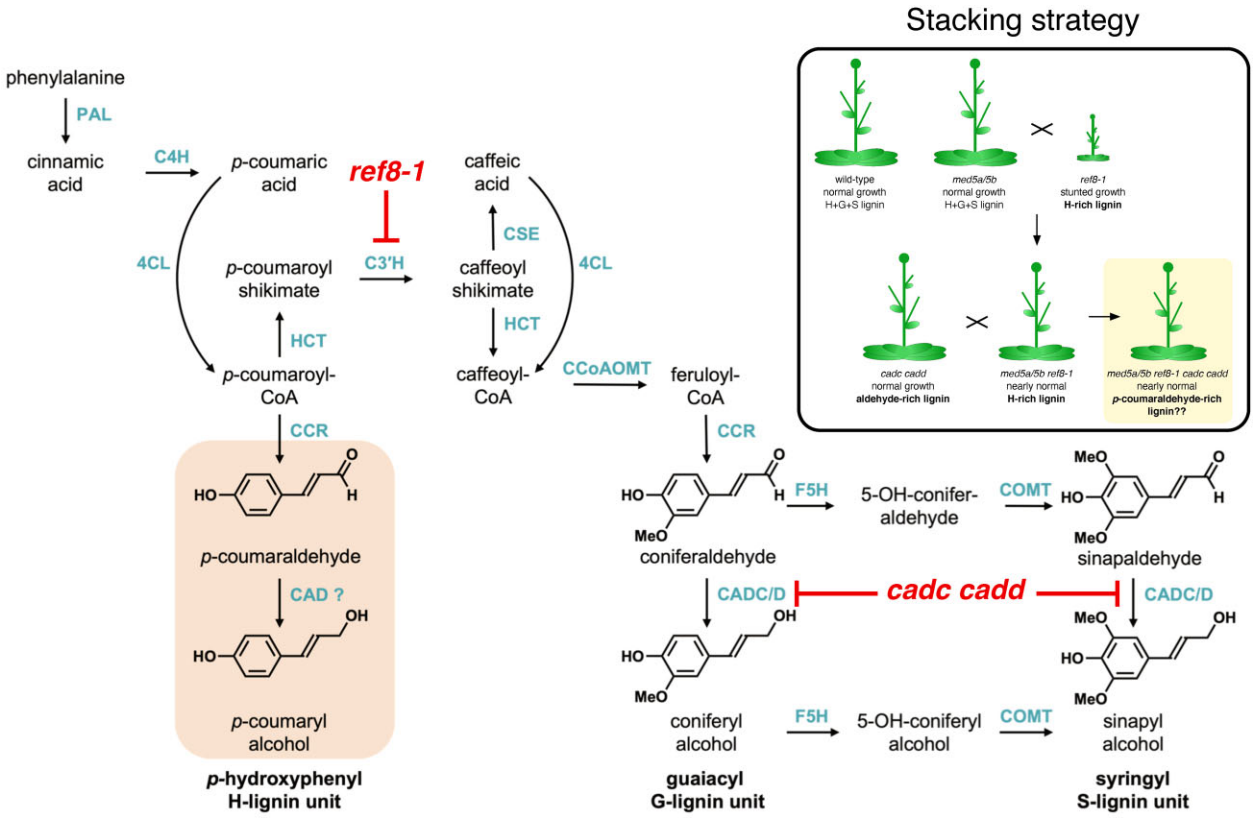

Figure 1 Pathway and strategy toward incorporating *p*-coumaraldehyde into lignin in Arabidopsis. Schematic representation of the monolignol biosynthetic pathway, in which the route toward *p*-coumaryl alcohol is highlighted. The figure of the lignin pathway was taken from Figure 1 of Muro-Villanueva et al. (2022). The stacking strategy is shown in the upper right panel: the H-enriched lignin genotype (*med5a/5b ref8–1*) was crossed with the aldehyde-rich lignin genotype (*cadc cadd*) to generate the quintuple mutant *med5a/5b ref8–1 cadc cadd*. Legend for the mutants: *med5a/5b*, double mutant for the *MEDIATOR subunits 5a* and *5b*; *ref8–1*, single mutant for the lignin biosynthetic gene *C3′H*; *cadc cadd*, double mutant for the lignin biosynthetic genes *CAD*. Legend for the enzymes in the pathway: PAL, PHENYLALANINE AMMONIA LYASE; C4H, CINNAMATE 4-HYDROXYLASE; 4CL, 4-COUMARATE:COENZYME A LIGASE; HCT, *p*-HYDROXYCINNAMOYL-COENZYME A:QUINATE/SHIKIMATE *p*-HYDROXYCINNAMOYLTRANSFERASE; CSE, CAFFEOYL SHIKIMATE ESTERASE; CCoAOMT, CAFFEOYL-COENZYME A *O*-METHYLTRANSFERASE; F5H, FERULATE 5-HYDROXYLASE; and COMT, CAFFEIC ACID *O*-METHYLTRANSFERASE.

p-coumaryl alcohol and therefore generate a different type of lignin built exclusively with *p*-coumaraldehyde units (Figure 1). The combination of these two strategies could further increase gains in biomass digestibility beyond those achieved with each individual manipulation. The resulting quintuple mutant was identified among the segregating population and showed similar growth and lignin content when compared with *med5a/5b ref8–1* mutants. Surprisingly, compositional analyses revealed that the lignin synthesized by the quintuple mutant was indistinguishable from that of *med5a/5b ref8–1*, being exclusively comprised of *p*-coumaryl alcohol units. These results led the authors to conclude that *cadc cadd* mutations stacked on *med5a/5b ref8–1* have no effect on lignin content and composition.

The incorporation of H units into the lignin of *med5a/5b ref8–1 cadc cadd* plants suggests that other enzyme(s) catalyze the reduction of *p*-coumaraldehyde to *p*-coumaryl alcohol. To test this hypothesis, the authors first analyzed CAD activity in stem protein extracts of *cadc cadd* and *med5a/5b ref8–1 cadc cadd* plants and found that they still exhibit substantial CAD activity despite their high aldehyde lignin phenotype. Given that the Arabidopsis genome harbors an additional seven *CAD* paralogs, the authors hypothesized that another CAD enzyme might be responsible for such activity. To identify the candidate CAD(s) involved in the synthesis of H units, the authors mined transcriptomic data generated for *ref8–1* and *med5a/5b ref8–1* rosettes, searching for genes upregulated in genetic backgrounds that synthesized high levels of *p*-coumaryl alcohol, and found *CADA* as a candidate. The authors also analyzed the expression of all *CAD* paralogs in highly lignified tissues (stems) of *med5a/5b ref8–1* using RT-qPCR, identifying *CADG* as another candidate. However, when these genes were individually knocked out in the *med5a/5b ref8–1 cadc cadd* background using the CRISPR/Cas9 system, both resulting hextuple mutants showed no statistical difference in CAD activity, lignin content, and lignin composition when compared with the *med5a/5b ref8–1 cadc cadd* quintuple mutant. These results suggest that neither CADA nor CADG alone are involved in the reduction of *p*-coumaraldehyde to *p*-coumaryl alcohol in Arabidopsis lignifying tissues.

Given that the loss-of-function of four *CAD* genes was not sufficient to block the biosynthesis of *p*-coumaryl alcohol, the authors considered the involvement of CINNAMOYL-CoA REDUCTASE (CCR), a reductase that catalyzes the conversion of cinnamoyl-CoA esters to their corresponding cinnamaldehydes in the lignin biosynthetic pathway (Figure 1). Based on the observation that mevalonate can be produced via the two-step reduction of 3-hydroxy-3-methyl-glutaryl-coenzyme A through a mevaldehyde intermediate (Haines et al., 2013), they tested whether CCR can catalyze an analogous reaction: the two-step reduction of *p*-coumaroyl-CoA directly to *p*-coumaryl alcohol. However, incubation of *p*-coumaroyl-CoA with both recombinant CCR1 and CCR2 yielded only *p*-coumaraldehyde, whereas *p*-coumaryl alcohol was not detected even when *p*-coumaraldehyde was directly provided as substrate.

The *cadd* mutant used in this study is a T-DNA line with an insertion in the *CADD* promoter, which reduces transcript accumulation to very low levels without disrupting the coding sequence. Interestingly, the authors observed that *CADD* transcripts accumulate to nearly 20% of wild-type levels in *med5a/5b ref8–1 cadc cadd* plants. To test whether this leaky expression of *CADD* is sufficient to ensure the continued deposition of H-lignin, *CADD* was knocked out in the *med5a/5b ref8–1 cadc cadd* background using CRISPR/Cas9. The resulting plants showed compromised growth and decreased fertility, but their lignin composition was the same as that observed for *med5a/5b ref8–1 cadc cadd* plants. Altogether, these results suggest that none of the CAD enzymes previously established as monolignol-related alcohol dehydrogenases are effectively involved in the biosynthesis of *p*-coumaryl alcohol in Arabidopsis, suggesting a gap in our understanding of this process.

The lignin biosynthetic pathway has been extensively characterized and is considered well-established in Arabidopsis. However, this metabolic pathway still yields surprises, with additional enzymes identified in recent years (Vanholme et al., 2013; Barros et al., 2019). The work of Muro-Villanueva et al. throws in another unexpected twist, showing that the biosynthetic pathway for H-lignin biosynthesis is not completely understood. This branch of the monolignol pathway has been overlooked because H units account for only a minor portion of the lignin polymer. Additionally, plants engineered to synthesize H-enriched lignins (or other H-related compositions) often display drastic growth defects, which further complicates the characterization of *p*-coumaryl alcohol biosynthesis. For example, the overexpression of *p-COUMAROYL-CoA MONOLIGNOL TRANSFERASE* (*PMT*) in the *med5a/5b ref8–1* background to produce lignins built with *p*-coumaryl *p*-coumarate units resulted in seedling-lethal plants (Smith et al., 2022). These results suggest that the *p*-coumaryl *p*-coumarate units might be toxic to the plant, either because they cannot be properly integrated into the cell wall or because they cannot be stored in the vacuole (Smith et al., 2022). The conclusion made by Muro-Villanueva et al. that none of the monolignol-related CADs are involved in H-lignin biosynthesis in Arabidopsis raises the question: is there a reductase/alcohol dehydrogenase specifically involved in the reduction of *p*-coumaraldehyde to *p*-coumaryl alcohol? The identification of this mysterious dehydrogenase will not only contribute to our understanding of H-lignin biosynthesis but might also allow additional strategies to improve biomass digestibility.

Conflict of interest statement. None declared.

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