

Variations in Lignin: What do Recent Studies on Lignin-Biosynthetic-Pathway Mutants and Transgenics Reveal about Lignification?

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Introduction

Lignification produces the complex lignin polymer in the plant cell wall that is vital for structural integrity of land-based plants, for defense against pathogens, and to facilitate various functions such as water transport. Lignin is unusual compared to other abundant natural polymers due to the low degree of order and the high degree of heterogeneity in its structure. We have recently been interested in understanding how plants respond to deficiencies in their ability to produce the normal precursors for lignification. Some such deficient plants occur as natural mutants; others can be developed using genetic engineering approaches. The potential to improve plant utilization by ruminants and in various other natural and industrial processes by engineering the amount, composition, and structure of lignin is currently attracting considerable interest.

Unexpected variation in lignin subunit composition has been found recently, particularly in an unusual mutation affecting the wood of loblolly pine (Ralph et al. 1997, MacKay et al. 1997, U.S. Dairy Forage Research Center 1997) and also in mutants and genetically engineered variants of higher plants. These results have significant implications regarding the traditional definition of lignin, and highlight the need for a better understanding of the lignin precursor biosynthetic pathway. We believe that the observed variation in composition and structure of lignin is still best explained by variation of the monolignol precursors and their abundance in the lignifying zone [see p.33]. The plasticity in lignin composition reveals new potential that extends beyond the traditional monolignol pathway for modification of the polymer by genetic engineering.

Our arguments for a greater level of plasticity in lignin, through variation in precursor composition, have recently been challenged (Gang et al. 1998, Lewis and Davin 1998). Here, we review and extend our results to support our structural findings and present our conclusions that these structures represent normal lignification with unusual precursors, consistent with a

traditional paradigm for lignification. Our view is in sharp contrast with a recent model of lignin biosynthesis requiring template dependent stereospecific control of lignin polymerization (Lewis and Davin 1998, Davin et al. 1997) [see p. 33]. Structural information has long been used to guide the search for underlying mechanisms for important biological processes, and the biosynthesis of lignin is no exception. The combination of current methods of structural chemistry, biochemistry, cell biology and genetics should continue to elucidate the nature and origin of the lignin polymer.

Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors. These alcohols give rise to *p*-hydroxyphenyl, guaiacyl and syringyl subunits in lignin (Fig. 1). The precursors are themselves derived from phenylalanine by deamination, followed by hydroxylation of the aromatic ring, methylation, and the reduction of the terminal acidic group to an alcohol. These alcohols have long been thought to be the direct precursors for lignin (monolignols). The lignin precursors can radically couple at several sites with each other, or, more frequently, with the growing lignin oligomer, to produce a complex polymer with a variety of intermolecular linkages. Here, we focus on a mutation in the last step of the precursor pathway: the formation of the monolignol coniferyl alcohol from coniferaldehyde. This step is catalyzed by the enzyme cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) encoded by a single gene in loblolly pine.

Discovery of a CAD-deficient pine mutant. The discovery of a recessive mutant allele of the *cad* gene in loblolly pine, *cad-n1*, has permitted the study of pines with severe deficiencies of CAD enzyme (MacKay et al. 1997, Ralph et al. 1997). The secondary xylem (wood) in *cad-n1* homozygous seedlings acquires a brown color, distinct from the nearly white color of wild-type pine wood. CAD deficiency causes dramatic changes in the

accumulation and nature of soluble phenolics; it also alters the structure of the lignin polymer that is deposited in the cell wall. The color and many of the changes in wood chemistry are similar to those observed in transgenic plants and in brown midrib mutants with suppressed CAD activity. However, the incorporation of one compound, dihydroconiferyl alcohol (DHCA), a major lignin precursor in pines with the *cad-n1* mutation, has not yet been noted in other plants with decreased CAD activity. These plants need to be re-examined using newer analytical methods. Detailed biochemical and molecular characterization of the CAD enzyme in loblolly pine and genetic analysis of the *cad-n1* allele have allowed us to establish a strong link between severe CAD deficiency and the incorporation of DHCA. Characterization of the gene and the CAD-deficient plants has been described in detail elsewhere (O'Malley et al. 1992, MacKay et al. 1995, MacKay et al. 1997).

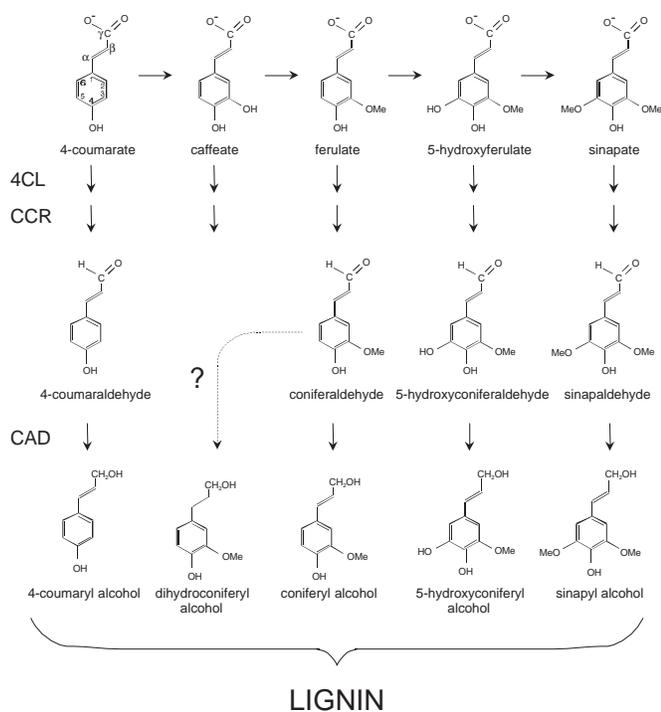


Figure 1. Lignin precursor biosynthesis. The general monolignol precursor biosynthetic pathway is outlined from 4-coumarate to five lignin precursors. Only three enzymes are shown: 4CL (hydroxycinnamate:CoA ligase), CCR (hydroxycinnamoylCoA:NADPH oxidoreductase) and CAD (cinnamyl alcohol dehydrogenase).

Accumulation of the substrate of CAD and the mutant. Chemical analyses of the wood from *cad-n1* homozygotes all indicate a dramatic increase in the levels of coniferaldehyde, the predominant substrate of CAD enzyme in pine, and in structures derived from coniferaldehyde. Simple extraction of ground wood with alcohol or other solvents revealed a several fold increase in soluble phenolics and a large increase in coniferaldehyde and vanillin. The resultant lignin polymer contains coniferaldehyde and vanillin at levels significantly higher than the wild type. This inference is based on results from isolated lignin samples analyzed by NMR, FTIR and UV spectroscopy, and from extractive free wood samples by microphoto-spectrometry, FTIR, PyrMS and thioacidolysis. The development of a brown or red-brown color in lignified tissues of plants that have suppressed CAD activity is well documented in both transgenics and mutants. In addition, *in vitro* synthesis of lignin oligomers (DHPs) starting with a mixture of coniferaldehyde and coniferyl alcohol generates dark red product, as opposed to a nearly colorless product when the aldehyde is absent or at low levels.

Discovery of dihydroconiferyl alcohol (DHCA) subunits in *cad-n1* homozygotes. The lignin from *cad-n1* homozygotes shows a large increase of unexpected subunits derived from DHCA. The identity and abundance of these subunits were first determined in an isolated lignin preparation by diagnostic NMR experiments. These findings were confirmed by direct chemical analysis of the isolated lignin using the DFRC method and analysis of extractive free wood samples by DFRC, Pyrolysis-MS, and thioacidolysis. Greatly elevated levels of DHCA have been observed in wood of several *cad-n1* homozygous seedlings and trees obtained from several crosses as outlined above; DHCA accumulation was not detected in any of the wild-type seedlings. The association between this phenotype and the *cad-n1* homozygous genotype is strong and was not dependent on environmental or biotic stress. DHCA is a *known* minor component of lignins. It is therefore not a new product in the mutant, but one found at highly elevated levels.

Dihydroconiferyl alcohol-derived subunits are components of lignin. Combined chemical degradation and NMR provide unambiguous evidence

that DHCA is a *bona fide* and abundant component of the lignin polymer. The non-extractable lignin fraction can be dissolved in propionyl bromide, where NMR confirms the presence of DHCA. Similar results in support of this claim are obtained whether the analyzed material is milled wood lignin, total wood, or fully solubilized residual lignin from the mutant pine. Comparison with synthetic lignins and model compounds indicates that approximately 50% of the subunits are in 5–5-coupled structures (Fig. 2). The presence of monomeric DHCA in solvent extracts and its predominance in 5–5-linked structures argues strongly against the suggestion that the DHCA components are the result of a modification of lignin following coupling of coniferyl alcohol. Detailed examination of dimers from degradation of normal pine lignin by the DFRC method provides evidence that the coniferyl alcohol monomer is essentially not involved in 5–5-coupling reactions. It has also been suggested that DHCA may be a dioxane:water extractable oligomerized lignan artifact (lignans are nonstructural dimeric phenolic metabolites). However, DHCA is present in all lignin fractions; the “careful recalculations” done by Lewis et al. are therefore flawed by their invalid assumption that DHCA is only an extractable artifact. In addition, the MWL of a CAD deficient tree had a weight-average molecular weight of ~17,000 and did not contain a low molecular weight fraction. Therefore, the abundance of DHCA is unlikely to be attributable to contaminating lignans. In fact, since lignans in *Pinus taeda* appear to be optically active, the inability to detect optical activity in the mutant lignin [see p. 35] may be a sufficient counter-argument.

The origin of DHCA in the mutant pine and in other normal softwoods is unknown. An NADPH-dependent enzyme activity reported to reduce the cinnamyl alcohol double bond in *b*-5 dilignol dehydroconiferyl alcohol to produce dihydrodehydroconiferyl alcohol will also convert coniferyl alcohol to dihydroconiferyl alcohol. This activity is an interesting candidate for involvement in the conversion of coniferaldehyde to dihydroconiferyl alcohol and should be carefully tested.

Composition and content of lignin in other mutants and transgenic plants. Mutant or

transgenic plants with genetic deficiencies affecting enzyme activity in the lignin biosynthetic pathway often have novel lignin structures or modified lignin composition, suggesting a high level of metabolic plasticity in the formulation of lignin precursors. A mutation in the enzyme ferulate-5-hydroxylase results in lignin without a syringyl component, whereas an overexpressing transgenic variant produces a lignin that is almost entirely composed of syringyl units. Minor components of normal lignins can become more significant when other key enzymes are depleted. Naturally occurring mutants (*e.g.*, the brown-midrib (bm3) mutants of maize and sorghum) and transgenic plants deficient in *O*-methyl transferase (OMT) contain significant amounts of units derived from 5-hydroxyconiferyl alcohol. Tobacco downregulated in CCR shows a striking increase in tyramine ferulate, a logical sink for the anticipated build-up of feruloyl-CoA. Coniferaldehyde becomes more significant in the CAD deficient pine mutant, radically coupling with aldehydes or with lignin monomers/oligomers. Similarly, sinapaldehyde becomes a major component of antisense-CAD tobacco transgenics.

Variability of lignin composition indicates a high level of metabolic plasticity based upon precursor supply. The ability of plants to adapt to diverse and

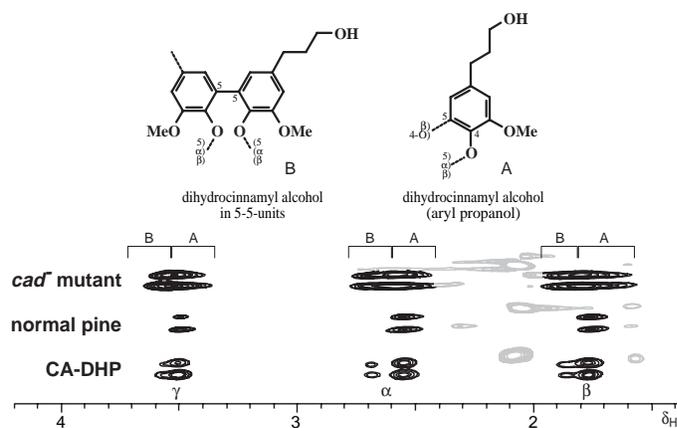


Figure 2. Comparison of DHCA units in the CAD deficient mutant, normal pine, and a coniferyl alcohol synthetic lignin (DHP). The normal pine, with only low levels of DHCA has units which couple only with traditional monolignols. In the CAD deficient mutant, the level is so high that DHCA units are coupling with each other, resulting in ~50% of the units being 5-linked. In the DHP, the level of DHCA is about 3% of the coniferyl alcohol; a small amount of homocoupling occurs. The spectra are partial 2D HMQC-TOCSY NMR plots through a and b on DHCA units showing corrections to the a, b, and g sidechain protons.

large changes in the precursor supply indicates that there is considerable metabolic plasticity in the assembly of the lignin polymer. Lignin must encompass a wider array of phenolic structures, with its composition and structure primarily guided by the precursor supply in the lignifying zone within plant tissues. The precursor supply varies among plant taxa; it also varies among cell types and within the cell wall, thus resulting in macro and micro heterogeneity of lignin itself. The increase in *p*-hydroxyphenyl subunits in lignin from compression wood is well-established. The precursor supply is also affected by genetic lesions or variants that create additional variation in lignin structure as discussed here.

Other nontraditional subunits are found in lignins.

It is important to recognize that lignins, even in 'normal' plants, are extremely variable in their composition. The definition of lignin as a polymer resulting from the three monolignols has long been recognized as too narrow. Many plants have lignins containing significant levels of other unusual components (Fig. 3), and it is likely that no plant contains lignins that are solely derived from the three "primary" precursors. For example, all lignins contain aldehyde groups – it is this feature that provides the diagnostic lignin staining reaction with acid phloroglucinol. Evidence from mutants and genetic variants where aldehydes accumulate strongly supports the view that aldehydes are incorporated as

precursors, because, in these variants, more aldehydes are found in the lignin.

Many lignins are biosynthesized by incorporating esterified monolignols into the lignification scheme. Thus, grasses utilize *p*-coumarates, hardwoods and some dicots such as kenaf utilize acetates, and some plants, notably bamboo, aspen and willow, use *p*-hydroxybenzoates as 'monomers' for lignification. Ferulates and diferulates are found intimately incorporated into all grass and some dicot lignins, where they are equal partners in the free-radical polymerization process and may even be nucleation sites for lignification. Amides may also be incorporated; although it is a wounding response product, tyramine ferulate is found in various lignins, *e.g.*, in tobacco. A general definition of lignin must include more than the traditional three hydroxycinnamyl alcohols, or the phenolic polymers in many plants serving the structure and function of lignin, *e.g.*, in grasses, might not be considered lignin. Lignin components do appear to be derived from phenylpropanoids as a general class; this classification has been used frequently.

Conclusions

Recent genetic studies have shown that manipulating specific lignin-biosynthetic-pathway genes produces profound alterations in the phenolic components of

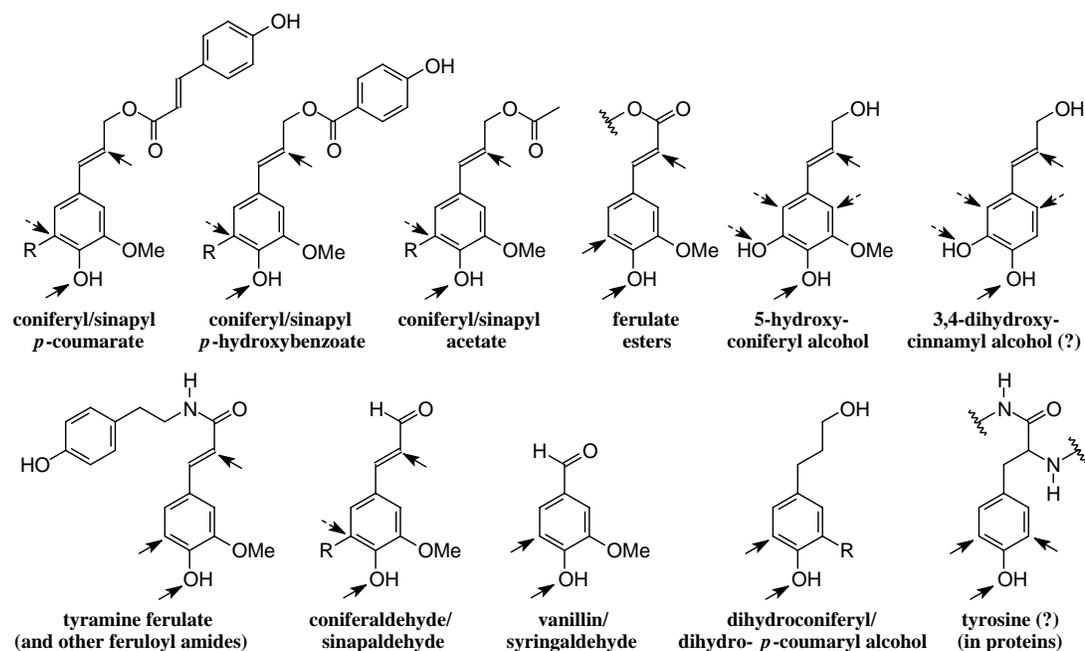


Figure 3. Non-monolignol "monomers" that are known or thought to be intimately incorporated into lignins. Sites for radical coupling are indicated with an arrow; a dashed arrow indicates other possible sites (coupling behavior not known).

plants. Whether the polyphenolic components produced by radical coupling reactions should be called lignin is little more than semantics. Although the 'lignins' in mutant and transgenic plants may appear to be strikingly different from 'normal lignins,' findings indicate that they represent merely broad compositional shifts. All of the novel units that have been found to date appear to be minor units in normal lignins. The recognition that such minor units can incorporate into lignin provides significantly expanded opportunities for engineering the composition and consequent properties of lignin.

References

- Davin, L. B.; Wang, H.-B.; Crowell, A. L.; Bedgar, D. L.; Martin, D. M.; Sarkanen, S.; Lewis, N. G. Stereoselective biomolecular phenoxy radical coupling by an auxiliary (Dirigent) protein without an active center. *Science* 1997, 275, 362-366.
- Gang, D. R.; Fujita, M.; Davin, L. D.; Lewis, N. G. The 'abnormal lignins': mapping heartwood formation through the lignan biosynthetic pathway. In *Lignin and Lignan Biosynthesis*; N. G. Lewis and S. Sarkanen, Eds.; Amer. Chem. Soc.: Washington, DC, 1998; pp 389-421.
- Lewis, N. G.; Davin, L. B. The biochemical control of monolignol coupling and structure during lignan and lignin biosynthesis. In *Lignin and Lignan Biosynthesis*; N. G. Lewis and S. Sarkanen, Eds.; Amer. Chem. Soc.: Washington, DC, 1998; pp 334-361.
- MacKay, J. J.; Liu, W.; Whetten, R. W.; Sederoff, R. R.; O'Malley, D. M. Genetic analysis of Cinnamyl alcohol dehydrogenase (CAD) in loblolly pine: single gene inheritance, molecular characterization and evolution. *Mol. and General Genetics* 1995, 247, 537-545.
- MacKay, J. J.; O'Malley, D. M.; Presnell, T.; Booker, F. L.; Campbell, M. M.; Whetten, R. W.; Sederoff, R. R. Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.* 1997, 94, 8255-8260.
- O'Malley, D. M.; Porter, S.; Sederoff, R. R. Purification, characterization and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). *Plant Physiol* 1992, 98, 1364-1371.
- Ralph, J.; MacKay, J. J.; Hatfield, R. D.; O'Malley, D. M.; Whetten, R. W.; Sederoff, R. R. Abnormal lignin in a loblolly pine mutant. *Science* 1997, 277, 235-239.
- U.S. Dairy Forage Research Center. 1997. U.S. Dairy Forage Research Center 1997 Research Summaries. U.S. Department of Agriculture, Agricultural Research Service, pp. 33-35.