Lignin Biosynthesis

Ross Whetten[a,1] and Ron Sederoff[a,b]

[a] Forest Biotechnology Group, Department of Forestry, North Carolina State University, Raleigh, North Carolina 27695-8008
[b] Department of Genetics and Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695-8008

INTRODUCTION

Lignin is a polymer of aromatic subunits usually derived from phenylalanine. It serves as a matrix around the polysaccharide components of some plant cell walls, providing additional rigidity and compressive strength as well as rendering the walls hydrophobic and water impermeable. Terrestrial vascular plants may therefore have appeared only after the evolution of lignin biosynthesis, because structural support and water transport functions are central to the biology of higher land plants.

Trees have extreme needs for both structural support and water transport. Accordingly, they synthesize high levels of lignin in wood: 15 to 36% of the dry weight of wood is lignin (Sarkanen and Hergert, 1971). Lignin is therefore one of the world's most abundant natural polymers, along with cellulose and chitin. Despite the importance of lignin to land plant growth, relatively few plant cell types accumulate substantial amounts of lignin during normal development. Cells that accumulate lignin, such as xylem elements and sclerenchyma, frequently also have other cell wall modifications, such as secondary cell wall thickenings, that also contribute strength to the cell. Lignin deposition can be induced in many cell types by disease or wounding, indicating that the genes involved in lignin biosynthesis respond to both developmental and environmental signals.

Although much progress has been made through many years of research, there is still some uncertainty regarding the enzymes that participate in lignin formation and the order in which reactions take place. In 1968, Neish reviewed the formation of cinnamic acids and the reduction of the acids to alcohols as the precursors of lignin. Many of the uncertainties in the order and mechanisms of the pathway discussed by Neish (1968) still exist today. In this review, we provide a brief overview of the current state of knowledge on the biosynthesis of lignin. We focus on the biosynthetic pathway that produces lignin monomers (monolignols) and the mechanisms by which these monomers are polymerized to form lignin within the context of normal development, although it is worth noting that lignification can also be induced by stress.

The integration of new technologies in the molecular genetics and biochemistry of higher plants promises a continuation of the rapid progress that has been made in the study of lignin biosynthesis in recent years and should answer many long-standing questions about this fundamental biochemical pathway in higher plants. Extensive reviews on the properties, biosynthesis, and degradation of lignin have been published in recent years, including ones by Higuchi (1990), Lewis and Yamamoto (1990), Chen (1991), Sederoff et al. (1994), and Boudet et al. (1995).

OVERVIEW OF THE LIGNIN BIOSYNTHETIC PATHWAY

The concept that lignin is derived from polymerization of coniferyl alcohol dates from the late 19th and early 20th centuries (reviewed by Freudenberg, 1965). The general outline of the pathway that gives rise to coniferyl alcohol and other monolignols was proposed ∼30 years ago (Higuchi and Brown, 1963; Freudenberg, 1965; Freudenberg and Neish, 1968). Doubts still remain about the degree to which the pathway follows the same sequence in different plant species or even different cell types within a species. Three monolignols, differing only in the substitution pattern on the aromatic ring, can be polymerized into lignin (Figure 1). The relative abundance of the different monolignol residues in lignin varies between species and within species, as does the total lignin content. Mechanisms that control this variation are not well understood. The mechanisms and enzymology of polymerization of monolignols into lignin also have not yet been clearly defined (O'Malley et al., 1993; Dean and Eriksson, 1994; Savidge et al., 1994).

Coniferyl alcohol and other monolignols are derived from phenylalanine in a multistep process (Figure 2). Several other major classes of plant products in addition to lignin are derived from phenylalanine, including flavonoids, coumarins, stilbenes, and benzoic acid derivatives (see Dixon and Paiva, 1995, this issue; Holton and Cornish, 1995, this issue). The initial steps in the biosynthesis of all these compounds are shared through the general phenylpropanoid pathway. Phenylpropanoid compounds are so named because of the basic structure of a three-carbon side chain on an aromatic ring, which is derived from L-phenylalanine (Figure 2).

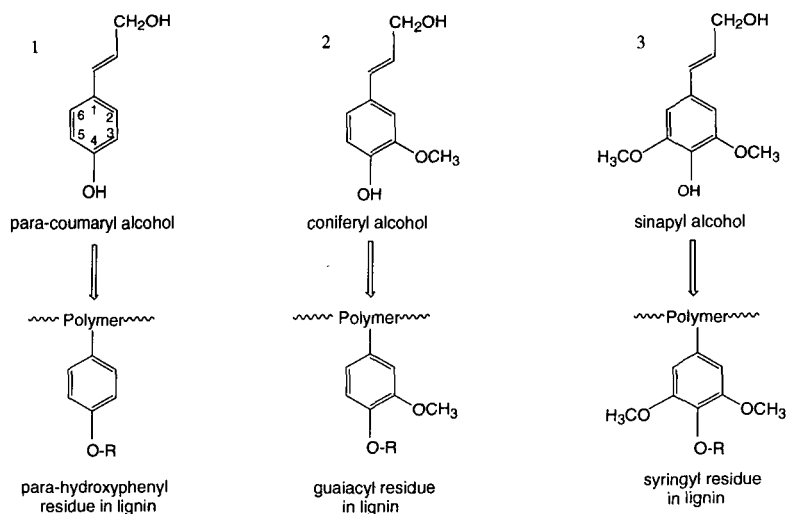[1] To whom correspondence should be addressed.

Figure 1. Structures of the Three Monolignols and the Resulting Lignin Residues.

The increasing degree of methoxylation of the aromatic ring from p-coumaryl alcohol (1) to coniferyl alcohol (2) to sinapyl alcohol (3) corresponds to a decrease in reactive sites on the aromatic ring. Ring positions 2 and 6 are nonreactive; only carbons 3 and 5, the 4-hydroxyl group, and the side chain carbons are potential sites for cross-linking. Methoxylation of carbon 3 in coniferyl alcohol and of carbons 3 and 5 in sinapyl alcohol blocks those sites and reduces the number of potential cross-links between monomers during polymerization. The R group on the 4-O position of the lignin residues can be either H (that is, creating a free hydroxyl group) or another cell wall polymer linked through ester or ether bonds.

The monolignols themselves are relatively toxic, unstable compounds that do not accumulate to high levels within living plant cells. Glycosylation of the phenolic hydroxyl group to produce monolignol glucosides stabilizes the compounds and renders them nontoxic. The glucosides probably serve as both storage and transport forms of the monolignols (Figure 3). Enzymes capable of cleaving the glycosidic bond exist in lignifying cells of many species of plants. The free monolignols are believed to be polymerized into lignin by a free radical mechanism that is initiated through oxidation of monolignols by cell wall–bound oxidases.

ENZYMES AND REACTIONS

Phenylalanine Ammonia-Lyase

Deamination of phenylalanine to cinnamate is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5). This enzyme is one of the most intensively studied in plant secondary metabolism because of the key role it plays in phenylpropanoid biosynthesis. PAL is found as a tetramer in vascular plants (Hanson and Havir, 1981; Jones, 1984). An analogous enzyme activity that deaminates tyrosine (tyrosine ammonia-lyase or TAL) to form p-coumarate has been detected, mainly in grasses (Neish, 1961). This activity frequently copurifies with PAL activity, and the question remains whether they are two different proteins or simply two activities of a single

polypeptide. Functional PAL enzyme has been expressed from a parsley cDNA in Escherichia coli (Schulz et al., 1989); the same experiment could be performed with a maize cDNA to test for PAL and TAL activities in the same polypeptide.

PAL was long thought to display negative cooperativity, attributed to allosteric effects of substrate on the tetrameric enzyme (Hanson and Havir, 1981). Extensive purification of PAL from cell suspension cultures of bean and alfalfa showed that these species express multiple forms of PAL with different kinetic properties. The individual PAL isozymes display Michaelis-Menten kinetics, but the mixture of isozymes shows the negative cooperativity previously considered characteristic of PAL (Bolwell et al., 1985; Jorrín and Dixon, 1990).

PAL subunits are typically encoded by multigene families in angiosperms, with two to 40 different members depending on the species (reviewed by Wanner et al., 1995). Genes encoding different PAL subunits show tissue-specific patterns of expression in several angiosperms (Bevan et al., 1989; Liang et al., 1989; Lois and Hahlbrock, 1992; Shufflebottom et al., 1993). There has been much speculation about the possibility that different PAL isoforms play different roles in the many aspects of phenylpropanoid metabolism, but little experimental evidence is available to test this hypothesis. PAL has been less intensively studied in gymnosperms, but there is no sign of multiple forms of the enzyme in elicited suspension cultures of Pinus banksiana (Campbell and Ellis, 1992b) nor do multiple genes appear to encode the PAL subunit in Pinus taeda (Whetten and Sederoff, 1992). Recent characterization of pal genes in P. banksiana indicates that four different pal genes

may be present in this species, although the relative levels of activity of the four genes have not been determined (S. Butland, M. Lam, and B.E. Ellis, unpublished results).

PAL catalyzes the committed step in phenylpropanoid metabolism and as such is well suited to play a regulatory role in controlling biosynthesis of all phenylpropanoid compounds, including lignin (Northcote, 1985). Transgenic plants with modified levels of PAL activity have provided opportunities to test hypotheses about the role of PAL in plant metabolism and development (Elkind et al., 1990). Bate et al. (1994) analyzed phenylpropanoid metabolites in transgenic tobacco plants with

decreasing amounts of PAL activity and found that lignin content is not greatly affected until PAL activity is reduced to 20 to 25% of wild-type levels. Levels of chlorogenic acid (a caffeic acid ester) and rutin (a flavonoid glycoside), in contrast, are affected by small changes in PAL activity.

The amino group cleaved from phenylalanine by PAL is released as ammonia. No data are available regarding the fate of this released ammonia, but the potential magnitude of nitrogen loss through this reaction indicates that the ammonia is probably recaptured within the plant. One possible route for recapture of the ammonia is through the action of glutamine
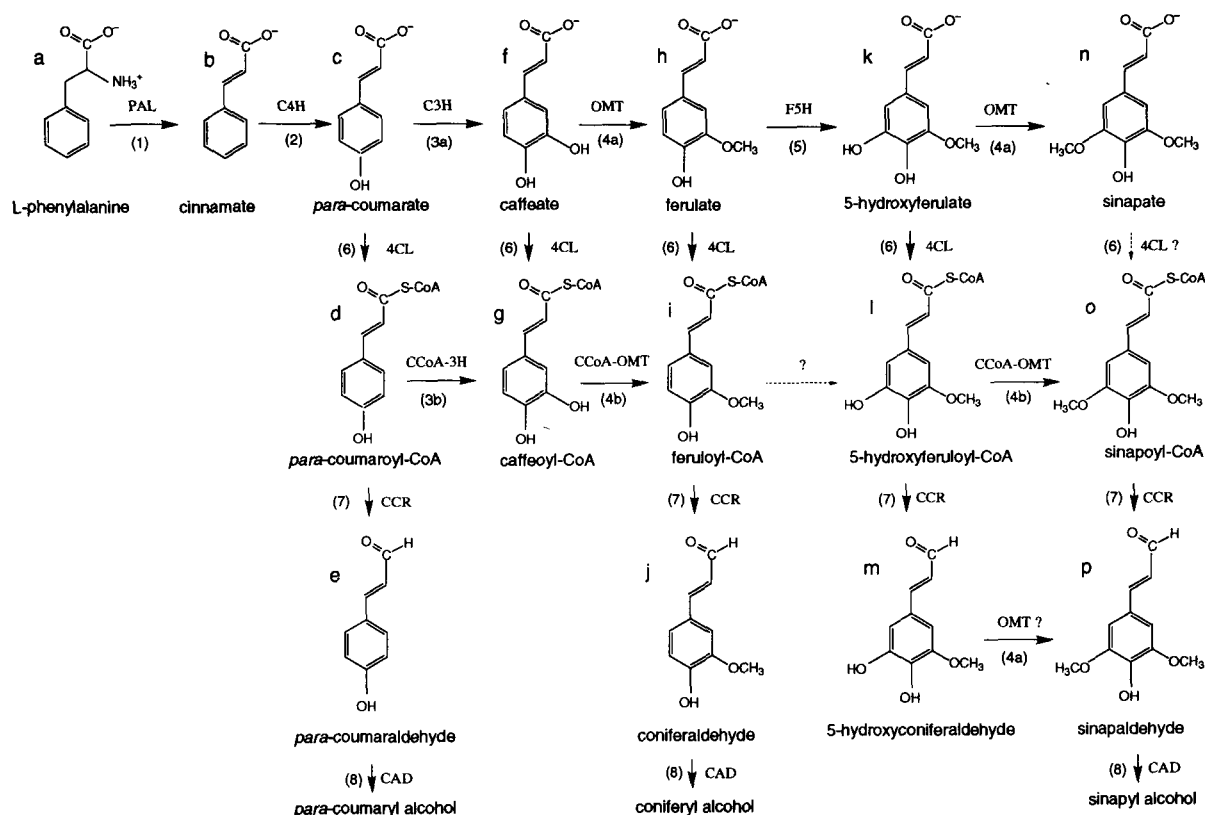

Figure 2. An Overview of the Monolignol Biosynthetic Pathway.

Phenylalanine (a) is deaminated by phenylalanine ammonia-lyase (PAL) (1) to yield cinnamate (b), and cinnamate is hydroxylated by cinnamate 4-hydroxylase (C4H) (2) to form p-coumarate (c). p-Coumarate can be conjugated by 4-coumarate:CoA ligase (4CL) (6) to coenzyme A to form an activated thioester precursor, p-coumaroyl-CoA (d). This compound is the precursor for synthesis of flavonoids, stilbenes, and other phenylpropanoids as well as the monolignol p-coumaryl alcohol (e). p-Coumarate and p-coumaroyl-CoA can both be hydroxylated at the 3 position to yield caffeate (f) and caffeoyl-CoA (g), respectively, although the enzymes (3a and 3b) that perform these reactions are not well studied. The newly added hydroxyl group can be methylated by an O-methyltransferase (OMT) (4a or 4b) to produce ferulate (h) or feruloyl-CoA (i). Caffeate and ferulate can be activated to the corresponding CoA thioesters by 4CL (6); feruloyl-CoA serves as a precursor for synthesis of the monolignol coniferyl alcohol (j). Ferulate can be hydroxylated by ferulate 5-hydroxylase (F5H) (5) to form 5-hydroxyferulate (k); the corresponding hydroxylation of feruloyl-CoA to form 5-hydroxyferuloyl-CoA (l) has not been demonstrated. 5-Hydroxyferulate is a substrate for 4CL, as shown. The 5-hydroxy group of 5-hydroxyferulate can be methylated by OMT (4a) to produce sinapate (m); the corresponding methylation of 5-hydroxyferuloyl-CoA to sinapoyl-CoA (n) has also been described and is probably catalyzed by caffeoyl-CoA 3-O-methyltransferase (CCoA-OMT) (4b). Activation of sinapate to sinapoyl-CoA is possible but occurs inefficiently in most plant extracts that have been tested. Reduction of the CoA thioesters to the corresponding aldehydes is catalyzed by cinnamoyl-CoA reductase (CCR) (7); caffeoyl aldehyde has no known physiological role and is not shown but may be produced in vivo. The aldehydes are further reduced by the action of cinnamyl alcohol dehydrogenase (CAD) (8) to give rise to the monolignols depicted in Figure 1. All acids are shown in the ionized form.
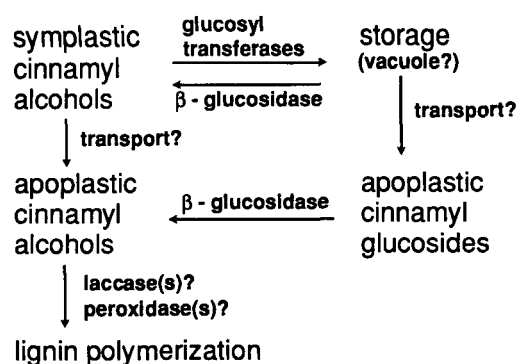
symplastic cinnamyl alcohols →(glucosyl transferases) / (β - glucosidase)← storage (vacuole?)

| transport?

| transport?

apoplastic cinnamyl alcohols ←(β - glucosidase)— apoplastic cinnamyl glucosides

| laccase(s)?
| peroxidase(s)?

lignin polymerization

Figure 3. Alternative Pathways for Storage, Transportation, and Deglycosylation of Monolignol Glucosides.

The more direct pathway—from symplastic cinnamyl alcohols to apoplastic cinnamyl alcohols to polymerization—may occur in angiosperms in which glucosides do not accumulate. Data from studies on conifers support the pathway on the right through storage of glucosides in the vacuole, transport of glucosides to the cell wall, and hydrolysis to the free cinnamyl alcohols in the apoplast.

synthetase (see Lam et al., 1995, this issue). Reductant for incorporation of ammonia into amino acids can be provided by the oxidative pentose phosphate pathway, even in non-photosynthetic plastids (Bowsher et al., 1992). This pathway is thought to be active in vascular tissue to provide reductant for monolignol biosynthesis (Pryke and ap Rees, 1977). Cytosolic glutamine synthetase has been immunolocalized to vascular bundles of rice leaves by tissue printing (Kamachi et al., 1992), and the promoter of the gene encoding pea cytosolic glutamine synthetase is active in vascular tissue of transgenic tobacco (Edwards et al., 1990).

Cinnamate 4-Hydroxylase

Hydroxylation of cinnamic acid to p-coumaric acid is catalyzed by cinnamate 4-hydroxylase (C4H; EC 1.14.13.11), a cytochrome P-450-linked monooxygenase. Molecular oxygen is cleaved during this reaction, with one oxygen atom added to the aromatic ring and the other reduced to water. C4H has been purified and characterized to different degrees from several plant species. Analysis of cDNA clones shows that C4H represents a class of cytochrome P-450s distinct from previously characterized P-450 enzymes (Fahrendorf and Dixon, 1993; Mizutani et al., 1993; Teutsch et al., 1993). C4H cDNAs have been expressed in yeast, and active enzyme has been recovered (Fahrendorf and Dixon, 1993; Pierrel et al., 1994). The plant enzyme is able to couple effectively with yeast NADPH–cytochrome P-450 reductase and catalyze the hydroxylation of cinnamate in microsomes from transformed yeast with high efficiency and specificity. Biochemical data are consistent with C4H catalysis of the first hydroxylation of cinnamic acid in the lignin biosynthetic pathway. No genetic data are available

regarding the role of this enzyme in modulating flux through the phenylpropanoid pathway.

PAL and C4H have been shown to reside in the microsomal compartment by fractionation studies, and early workers reported that microsomes of oak convert phenylalanine first to p-coumarate then to caffeate (Alibert et al., 1972). Feeding experiments using phenylalanine and cinnamic acid with different radiolabels have shown that C4H utilizes cinnamic acid produced in situ by PAL more readily than exogenously supplied cinnamic acid (Czichi and Kindl, 1977; Hrazdina and Wagner, 1985). These findings suggest that cinnamate is preferentially transferred from PAL to C4H, rather than coming to equilibrium with the cytosol and diffusing to C4H. This preferential transfer of intermediates between enzymes of a pathway is an example of metabolic channeling (Srere, 1987; Hrazdina and Jensen, 1992). Channeling offers a mechanism by which the cell can maintain very low concentrations of toxic compounds while still allowing high levels of metabolic flux through the pathway. The degree to which channeling occurs in the remainder of the monolignol biosynthetic pathway is as yet unknown, but there have long been hypotheses that channeling might be important in regulation of monolignol biosynthesis (Stafford, 1974, 1981; Hrazdina and Jensen, 1992). Formation of multienzyme complexes is one mechanism by which channeling may be achieved, and colocalization of all the enzymes of the pathway to the same subcellular compartment is another.

Coumarate 3-Hydroxylase

Little is known about coumarate 3-hydroxylase, the enzyme that catalyzes the hydroxylation of p-coumarate to form caffeate. Several plant oxidases can carry out hydroxylation of phenolic molecules, and identifying the enzyme that catalyzes this reaction in the synthesis of monolignols has been difficult. Early workers suggested that the reaction is catalyzed by phenolase (EC 1.10.3.1), a copper-containing oxidase capable of oxidizing a wide variety of substrates (Vaughan and Butt, 1969). Inhibitor studies with tentoxin, a fungal compound that inhibits phenolase activity, showed that loss of phenolase activity has no detectable effect on caffeic acid synthesis in mung bean seedlings (Duke and Vaughn, 1982). This finding suggests that nonspecific phenolases are not responsible for the synthesis of caffeic acid. Kojima and Takeuchi (1989) characterized p-coumarate hydroxylase activity in tentoxin-treated seedlings and suggested that the enzyme is a phenolase with high specificity for p-coumarate. No genetic data are available to support this conclusion, and the enzyme has not been purified.

Coumaroyl-Coenzyme A 3-Hydroxylase

An alternative to hydroxylation of free p-coumarate has been described, first as part of the anthocyanin pigment biosynthetic pathway in Silene dioica (Kamsteeg et al., 1981) and later as

part of the defense response in elicitor-treated parsley cell cultures (Kneusel et al., 1989). A mutation that blocks anthocyanin pigment formation in *Silene* has been shown to block the activity of an FAD-dependent enzyme that hydroxylates p-coumaroyl-coenzyme A (CoA) to caffeoyl-CoA (Kamsteeg et al., 1981). No effect on lignin synthesis in mutant plants was reported, but the possibility of a similar enzyme involved in monolignol biosynthesis must be considered. Boniwell and Butt (1986), working with particulate fractions from potato, described an FAD- and NADPH-dependent enzyme active on both p-coumarate and p-coumaroyl-CoA. Kneusel et al. (1989) identified a zinc-dependent enzyme from elicitor-treated parsley cell cultures that catalyzes the hydroxylation of p-coumarate and p-coumaroyl-CoA, and they suggested that it may function in the synthesis of defensive compounds in plants attacked by fungal pathogens. The potential involvement of these enzyme activities in monolignol biosynthesis has not yet been adequately tested. Some of the hydroxylases active on p-coumarate are also active on p-coumaroyl-CoA, suggesting that a single enzyme could function in vivo to catalyze hydroxylation of either substrate.

The monolignol biosynthetic pathway may divide at p-coumarate, with one branch leading through p-coumaroyl-CoA to caffeoyl-CoA and feruloyl-CoA and the other branch leading through caffeate and ferulate to feruloyl-CoA (Figure 2). The question of whether formation of CoA thioesters precedes or follows hydroxylation/methylation reactions during monolignol biosynthesis is still unresolved, although data exist to suggest that both pathways are probably active to some extent, perhaps to different degrees in different plant tissues or cell types.

Caffeate O-Methyltransferase

Caffeic acid is methylated to form ferulic acid by caffeic acid 3-O-methyltransferase (C-OMT; EC 2.1.1.68), using S-adenosyl methionine as the methyl group donor. This methylation reaction limits the reactivity of the 3-hydroxy group, thus reducing the number of sites on the aromatic ring that can form bonds to other monolignol molecules during polymerization. The same enzyme is also believed to catalyze the methylation of 5-hydroxyferulate to sinapate. C-OMT is clearly implicated in synthesis of monolignols based on genetic evidence from both monocots and dicots. The brown-midrib phenotypes in maize, sorghum, and pearl millet are known to involve changes in lignin content and composition (Kuc et al., 1968; Porter et al., 1978; Cherney et al., 1988). Specific brown-midrib genes have been associated with changes in OMT activity in sorghum (Cherney et al., 1988) and maize (Grand et al., 1985). The maize *brown-midrib3* mutation has recently been shown to correspond to the gene encoding C-OMT (Vignols et al., 1995). Transgenic dicots with reduced OMT activity show changes in lignin content and monomer composition (Dwivedi et al., 1994; Ni et al., 1994). High-level antisense expression of aspen OMT in tobacco is associated with depressed C-OMT activity and a reduced ratio of syringyl to guaiacyl lignin subunits (Dwivedi et al., 1994).

The C-OMTs from different species show preference for different substrates. Comparison of C-OMT activity (assayed in crude extracts) from gymnosperms and angiosperms led to the conclusion that gymnosperm C-OMT typically shows preferential activity with caffeate over 5-hydroxyferulate, whereas angiosperm C-OMT shows greater activity with 5-hydroxyferulate as substrate (Shimada et al., 1973; Kuroda et al., 1981). Conifers generally contain primarily guaiacyl lignin, whereas angiosperms can produce guaiacyl-syringyl lignin; the difference in substrate specificity of C-OMT has been proposed to play a role in determining the monomer composition of lignin (Gross, 1985; Higuchi, 1985). Some gymnosperms produce guaiacyl-syringyl lignin, and some angiosperms produce only guaiacyl lignin (Lewis and Yamamoto, 1990), challenging generalizations about the distribution of guaiacyl-syringyl lignin across taxa. C-OMT purified from *Thuja orientalis* shows activity with 5-hydroxyferulate, although syringyl residues are not abundant in the lignin of this species (Kutsuki et al., 1981). The further characterization of C-OMT enzymes purified from different gymnosperms would contribute to our understanding of the role this enzyme may play in determining lignin monomer composition.

Caffeoyl-Coenzyme A O-Methyltransferase

An enzyme distinct from C-OMT, caffeoyl-CoA 3-O-methyltransferase (CCoA-OMT; EC 2.1.1.104), has been identified in connection with the defense response in several dicot plant species (Kühnl et al., 1989; Pakusch et al., 1989), and a cDNA clone has been isolated from parsley cell cultures (Schmitt et al., 1991). A cDNA of CCoA-OMT has recently been isolated from *Zinnia* mesophyll cultures in which differentiation of tracheary elements was induced (Ye et al., 1994). Based on the kinetics of induction of enzyme activity in cell cultures and the correlation of CCoA-OMT activity with lignification, Ye et al. (1994) proposed that CCoA-OMT plays a role in methylation of both caffeoyl-CoA and 5-hydroxyferuloyl-CoA during monolignol biosynthesis. Genetic evidence to support this hypothesis has not yet been obtained but should be available from transgenic plant experiments.

Ferulate 5-Hydroxylase

Hydroxylation of ferulate to 5-hydroxyferulate is catalyzed by another cytochrome P-450-linked monooxygenase, ferulate 5-hydroxylase (F5H; no EC number assigned). This enzyme has proven extremely difficult to work with; little has been published since the first reported assay of F5H activity in poplar microsomes (Grand, 1984). A genetic approach to the study of F5H function has yielded new tools for the study of this enzyme. A mutation in the Arabidopsis gene encoding F5H (*fah-1*) has been identified, and mutant plants were shown to lack

sinapate-derived residues in lignin (Chapple et al., 1992). As a result, the lignin in *fah-1* mutant Arabidopsis resembles a gymnosperm type of lignin composed of guaiacyl units derived from coniferyl alcohol. Isolation of the gene encoding F5H and expression of the protein product in a heterologous system will provide material for more intensive characterization of this little-studied enzyme.

F5H has also been implicated in the differences in lignin composition between angiosperms and gymnosperms. F5H activity is necessary for the hydroxylation of ferulate, an essential step for the formation of sinapyl alcohol. It is possible that F5H is present in all plants at varying levels and that low levels of F5H activity are insufficient to allow synthesis of significant levels of sinapyl alcohol and syringyl lignin.

4-Coumarate:Coenzyme A Ligase

4-Coumarate:CoA ligase (4CL; EC 6.2.1.12) catalyzes the formation of CoA thioesters of cinnamic acids in the biosynthesis of a wide variety of phenolic derivatives, including benzoic acid, condensed tannins, flavonoids, and the cinnamyl alcohols (Gross, 1985). 4CL depends strictly on ATP, and the reaction resembles the activation of fatty acids, proceeding through an intermediate acyl adenylate, which reacts with CoA to form the thioester. Early studies on 4CL suggested that multiple isoforms might control formation of different phenylpropanoid products. Grand et al. (1983) identified three isoforms of 4CL with different substrate specificities in stems of poplar and hypothesized that expression of different 4CL enzymes could regulate the relative abundance of the different precursors for lignin. Parsley contains two genes encoding virtually identical 4CL enzymes showing the same substrate specificity (Lozoya et al., 1988). These genes also show expression in xylem of transgenic tobacco (Hauffe et al., 1991, 1993). In loblolly pine xylem, however, Voo et al. (1995) found only one form of 4CL, encoded by a single gene. More genetic evidence is needed to determine whether 4CL has a significant role in regulating lignin microheterogeneity.

4CL from most plants shows low activity with sinapic acid (Kutsuki et al., 1982b). Two alternative pathways have been proposed to account for the production of sinapyl alcohol. Higuchi (1985) suggested a route to sinapaldehyde through hydroxylation of ferulate, activation of 5-hydroxyferulate to the CoA thioester, reduction to 5-hydroxyconiferaldehyde, and methylation to sinapaldehyde. 4CL preparations from several angiosperm and gymnosperm species have shown activity with 5-hydroxyferulate (Knobloch and Hahlbrock, 1977; Kutsuki et al., 1982b; Lüderitz et al., 1982; Grand et al., 1983). Whether reduction to the aldehyde occurs before or after methylation of the 5-hydroxy position is not known. Ye et al. (1994) suggested a pathway from caffeic acid through caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA, and sinapoyl-CoA, based on the ability of CCoA-OMT to methylate the CoA thioesters. This alternative pathway to sinapyl alcohol requires hydroxylation of feruloyl-CoA to 5-hydroxyferuloyl-CoA before methylation

by CCoA-OMT. Assays of F5H from xylem of sweetgum and aspen show no hydroxylation activity with feruloyl-CoA as substrate (V. Chiang, personal communication), supporting the hypothesis that hydroxylation occurs at the acid level, whereas methylation may occur at the CoA thioester level.

Cinnamoyl-Coenzyme A Reductase

Reduction of hydroxycinnamoyl-CoA thioesters to the corresponding aldehydes is catalyzed by cinnamoyl–CoA reductase (CCR; EC 1.2.1.44). CCR does not in general exhibit much specificity for one hydroxycinnamoyl-CoA substrate over another, although feruloyl-CoA is reported to be the best substrate for CCR from *Forsythia* (Gross and Kreiten, 1975), soybean (Wengenmayer et al., 1976), hybrid poplar (Sarni et al., 1984), and *Eucalyptus* (Goffner et al., 1994). Goffner et al. (1994) hypothesized that CCR plays a key regulatory role in lignin biosynthesis as the first committed step in the production of monolignols from phenylpropanoid metabolites.

Cinnamyl Alcohol Dehydrogenase

The reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols is catalyzed by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195). CAD has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthetic pathway (Walter et al., 1988). However, CAD is expressed in cells that do not make lignin (O'Malley et al., 1992; Grima-Pettenati et al., 1994). CAD is also expressed in response to stress (Galliano et al., 1993), pathogen elicitors (Campbell and Ellis, 1992a), and wounding. CAD is therefore regulated by both developmental and environmental pathways, much like other well-studied enzymes of phenylpropanoid metabolism.

Differences in substrate affinities of CAD enzymes from angiosperms and gymnosperms may play a role in controlling the formation of different types of lignin (Kutsuki et al., 1982a). Isoforms of CAD with markedly different substrate affinities are detected in such species as soybean (Wyrambik and Grisebach, 1975), wheat (Pillonel et al., 1992), *Eucalyptus* (Goffner et al., 1992), and *Salix* (Mansell et al., 1976). Many species, however, are believed to contain a single form of the enzyme. For example, there is a single CAD enzyme encoded by a single gene in loblolly pine (O'Malley et al., 1992; MacKay et al., 1995). CAD preparations from gymnosperms are generally much more active on coniferaldehyde, whereas angiosperm CAD preparations show more equal activities with coniferaldehyde and sinapaldehyde (reviewed in Gross, 1985).

A related enzyme, coniferyl alcohol dehydrogenase (EC 1.1.1.194), has been described in *Salix* (Mansell et al., 1976) and soybean (Wyrambik and Grisebach, 1975). This enzyme is reported to catalyze the reduction of coniferyl alcohol only. The physiological role of coniferyl alcohol dehydrogenase is not known.

The gene encoding CAD has been a target for modification of lignin content in plants through genetic engineering. Tobacco plants transformed with an antisense CAD construct show varying degrees of reduction in CAD activity and modification of phenolic products (Halpin et al., 1994). Two lines of transgenic plants were analyzed in detail. Plants with 7% of wild-type CAD activity had changes in monolignol composition, whereas plants with 20% of wild-type CAD activity did not. The changes in composition included increases in the ratio of aldehyde- to alcohol-derived products, with a preferential effect on syringyl subunits. An increase in the aldehyde content of lignin was expected in plants with reduced CAD activity, but the preferential effect on syringyl subunits was not. Both lines also showed an increase in the content of alkali-extractable phenolics and thioglycolic acid–extractable lignin. Extraction with alkali removes phenolic acid residues bound by ester linkages to cell wall polymers, and subsequent thioglycolic acid treatment breaks some but not all bonds between monolignol residues in lignin. The increased extractability of lignin in plants with lowered CAD activity suggests that the types of bonds formed between phenolic acids, monolignols, and other cell wall polymers depend on both the concentrations and types of different subunits available.

Glycosylation, Storage, and Transport

The monolignols p-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol are relatively toxic and unstable compounds. Glycosylation on the phenolic hydroxyl group, a reaction performed by UDP-glucose:coniferyl alcohol glucosyltransferase (EC 2.4.1.111), forms the monolignol glucosides p-hydroxycinnamyl alcohol glucoside, coniferin, and syringin, respectively. These glucosides accumulate in some species of plants, most notably in conifers. Freudenberg and Harkin (1963) reported the purification of all three monolignol glucosides from "cambial sap" (that is, dry matter from an aqueous cell-free extract of differentiating xylem) of Norway spruce, with yields of 80 g of coniferin, 1.8 g of syringin, and trace amounts of p-hydroxycinnamyl alcohol glucoside from 1.27 kg of extract. These yields reflect the stability and ease of purification as well as the abundance of the different glucosides and only approximate the actual amount of the various monolignols in spruce lignin (Freudenberg and Harkin, 1963).

Terazawa et al. (1984) analyzed monolignol glucoside content in 19 dicot tree species in 15 genera and six gymnosperm species in six genera. The only dicots in which monolignol glucosides were detected were five species of the Magnoliaceae and Oleaceae families, whereas coniferin was detected in all gymnosperm species analyzed. The authors hypothesized that lignin biosynthesis may have been regulated by storage of monolignol glucosides in ancestral plants and that, whereas gymnosperms and some less-derived angiosperm species have retained the property of accumulating monolignol glucosides, most species of angiosperms have evolved alternatives to forming monolignol glucosides.

Although some angiosperms accumulate monolignol glucosides, further investigation of variation in monolignol glucoside content in differentiating xylem of Syringa vulgaris (Oleaceae) and Larix leptolepis (Pinaceae) during the annual growth cycle offers support for the hypothesis that monolignol glucosides now serve different physiological functions in angiosperms than in gymnosperms (Terazawa and Miyake, 1984). Glucosides show different tissue distribution and different patterns of accumulation during the growing season and accumulate to very different levels in the two species. The conifer Larix accumulates coniferin to levels of 15% of the wet tissue weight, but only in differentiating xylem. Savidge (1989) also found that coniferin content is highest in developing xylem of several conifer species and is associated with tracheid differentiation. Leinhos and Savidge (1993) have shown that coniferin is also found at high levels within protoplasts made from developing xylem of Pinus banksiana and Pinus strobus. The coniferin content of the protoplasts can account for most if not all of the coniferin present in the tissue. Both large and small protoplasts contain coniferin, suggesting that both ray parenchyma and fusiform cells (differentiating tracheids) contain coniferin. These results support the view that coniferin is stored in the vacuole. Terazawa and Miyake (1984) found that in Syringa, by contrast, syringin accumulates in both differentiating xylem and inner phloem to levels ∼1% of wet tissue weight. Monolignol glucosides have the potential to play physiological roles in plants other than as lignin precursors, but specific precursor–product relationships have not been defined.

Coniferyl alcohol glucosyltransferase is found in many species of plants, as is coniferin β-glucosidase (EC 3.2.1.126) (Ibrahim and Grisebach, 1976; Hösel et al., 1982). Coniferin glucosidases have recently been identified and characterized from differentiating xylem of two species of pines. Leinhos et al. (1994) purified two proteins from jack pine (Pinus banksiana), whereas Dharmawardhana et al. (1995) purified one protein from lodgepole pine (P. contorta). The proteins from these closely related species are quite different. The lodgepole pine protein has a mass of 28 kD, and N-terminal protein sequence analysis shows high similarity with known plant β-glucosidases (Dharmawardhana et al., 1995). The jack pine proteins, by contrast, have molecular masses of over 100 kD, and their N-terminal amino acid sequences show no similarity with other plant β-glucosidases (Leinhos et al., 1994). Histochemical localization of lodgepole pine coniferin β-glucosidase activity, using a chromogenic substrate specific for the coniferin-hydrolyzing enzyme, shows activity located in differentiating xylem (Dharmawardhana et al., 1995). Although other β-glucosidases have been suggested to be involved in lignification, these pine enzymes are clearly associated in both time and place with lignification, and they have the substrate specificity expected of enzymes involved in hydrolysis of monolignol glucosides.

The inferred presence of monolignol glucosides in the vacuoles of differentiating conifer xylem cells (Leinhos and Savidge, 1993) and the presence of specific glucosidases in pine xylem cell walls (Leinhos et al., 1994; Dharmawardhana et al.,

1995) imply that in pines, the monolignol glucosides are transported from the vacuole to the cell wall at the appropriate time during xylem differentiation. The mechanism of this transport is unknown, as are the factors that regulate it. Ultrastructural studies on angiosperms have detected vesicle traffic between the cytosol and the plasmalemma in differentiating tracheids and have shown labeling of those vesicles in tissues fed labeled phenylalanine and cinnamic acid (Pickett-Heaps, 1968). Angiosperms that do not accumulate monolignol glucosides may have different transport mechanisms than gymnosperms, which do accumulate glucosides. The regulatory significance of transport of monolignols or monolignol glucosides to the cell wall is unknown, but a regulatory role has been suggested by studies showing differences in patterns of incorporation of different labeled monolignol glucosides in pine (Terashima and Fukushima, 1989).

Enzymatic Oxidative Polymerization

Erdtman (1933) proposed that phenolic precursors are polymerized into lignin by an oxidative mechanism involving free radical intermediates. Two different classes of enzyme, peroxidases and laccases, have been proposed to perform the polymerization of monolignols into lignin. The evidence in support of the involvement of both types of enzyme is extensive but circumstantial. Laccase (EC 1.10.3.2), an oxygen-dependent oxidase containing four copper atoms, and peroxidase (EC 1.11.1.7), an H_2O_2-dependent hemoprotein, are both capable of oxidizing monolignols to free radicals in vitro. Freudenberg and Richtzenhain (1943) first used crude enzyme preparations for polymerization of monolignols in vitro into a dehydrogenation polymer. Early experiments used extracts of mushroom and plants, whereas later experimenters used purified proteins (reviewed by Freudenberg, 1965).

Despite 50 years of investigation, the exact mechanisms and enzymology of lignin polymerization remain in doubt. The evidence for and against involvement of laccase and peroxidase in lignification has recently been reviewed (O'Malley et al., 1993; Dean and Eriksson, 1994), and new evidence continues to accumulate (for example, see Liu et al., 1994; McDougall et al., 1994). A convincing answer to this question will require demonstration of changes in lignin content or composition upon experimental manipulation of enzyme activity, either in transgenic plants or in mutants defective in specific enzymes. Such experiments are under way in several laboratories.

New approaches, such as nuclear magnetic resonance and Raman microprobe studies for analysis of lignin structure in situ, have provided support for the concept that lignin polymerization is not random but organized (Atalla and Agarwal, 1985; Agarwal and Atalla, 1986; Lewis et al., 1987). For example, the phenyl rings of lignin appear to be aligned preferentially in the plane of the cell walls (Atalla and Agarwal, 1985; Agarwal and Atalla, 1986). Hemicelluloses, it has been suggested, play a role in organizing cellulose structure (Atalla et al., 1993), and the possibility exists that hemicelluloses also participate in organizing lignin precursors before polymerization (Houtman and Atalla, 1995). Detailed analyses of reaction products of monolignols with oxidative enzymes have shown that different enzymes produce different oligolignols, suggesting that free radical polymerization need not give rise to a random structure (Sterjiades et al., 1993; Okusa et al., 1995). Crude plant extracts have been shown to catalyze a stereoselective synthesis of dilignols, again suggesting that polymerization of monolignols need not be a random process (Davin et al., 1992).

KEY QUESTIONS

How Are the Proportions of Different Monomers Controlled?

The mechanisms controlling the relative abundance of the p-hydroxyphenyl, guaiacyl, and syringyl subunits in lignin are not well understood, although hypotheses regarding the roles of different enzymes have been formulated. Enzymatic specificity that could affect lignin composition has been demonstrated for OMT, 4CL, and CAD, and the differences between angiosperm and gymnosperm lignin monomer composition have been attributed to these differences in enzyme substrate specificity (Higuchi, 1985). The degree to which each of these enzymes affects lignin monomer composition has yet to be determined for any plant species and need not be the same for other species. Lignin monomer composition also varies among cell types within individual plants and between different regions of the wall of a single cell. The precise spatial control of monomer composition within the cell wall seems unlikely to be determined by differences in substrate specificity of enzymes acting during monolignol biosynthesis. Metabolic control theory suggests that assigning all of the control of a metabolic process to any single enzyme in a pathway is usually unjustified; control is more frequently distributed across several enzymes in the pathway (Kacser and Burns, 1973; also see reviews in Cornish-Bowden and Cárdenas, 1990).

There is considerable interest in how lignin monomer composition is controlled, because the monomer composition of lignin is a major factor in determining the difficulty of lignin degradation. The guaiacyl–syringyl lignin typical of angiosperms is more easily removed by the kraft pulping process than is the guaiacyl lignin typical of conifers (Chiang et al., 1988). Digestibility of forage and fodder crops is also affected by lignin content and composition (Cherney et al., 1988, 1991). The hydroxylase enzymes would seem to be key branch points for determining the relative abundance of different monomer types: C3H for regulating the ratio of p-hydroxyphenyl to guaiacyl and syringyl subunits, and F5H for regulating the ratio of guaiacyl to syringyl subunits. Neither C3H nor F5H is well studied, and as a result, no data are available to test the hypothesis of a role for these enzymes in controlling monomer composition.

Substrate channeling has long been suggested as playing a role in controlling the intracellular concentrations of toxic

intermediates while allowing high levels of carbon flux into lignin (Stafford, 1974, 1981; Hrazdina and Jensen, 1992). Such interactions between various enzymes in the pathway may also regulate the amount of each of the monolignols that is produced in response to either developmental or environmental cues. A recently developed tool in yeast genetics, the two-hybrid system, provides a means of testing for associations between polypeptides encoded by cloned cDNAs (Fields and Song, 1989). This method could be used to test for interactions between enzymes in the monolignol biosynthetic pathway, given the current availability of cDNA clones corresponding to many of the enzymes. The two-hybrid system is limited to detecting interactions that are a function of the proteins encoded by the cloned cDNAs, however, and cannot assay for potential tissue type– or cell type–specific interactions controlled by other factors not present in yeast.

How Are Genes Encoding Enzymes of Lignin Biosynthesis Regulated?

The genetic regulation of the enzymes of lignin biosynthesis must involve complex and interrelated systems of gene activation. All of the steps in the synthesis of monolignols are likely to be regulated by developmental signals and by environmental stimulation as well. In a few cases, it is apparent that multiple genes are involved that are differentially regulated, whereas in other cases, single genes must be regulated by different signal transduction pathways. The early genes in the phenylpropanoid pathway are expressed in the biosynthesis of many different phenylpropanoid products in addition to lignin. The regulatory control of lignin biosynthesis must therefore be part of a complex combinatorial network. It is likely that transcription factors will be identified that regulate phenylpropanoid genes in a tissue- and pathway-specific manner, whether due to development or environmental stress, and that the nature of the genetic regulatory network that determines coordinate or differential control will soon be defined.

Specific promoter elements common to PAL, 4CL, and CAD genes have been defined and may be involved in regulation related to lignin biosynthesis. Conserved sequences have been identified in the promoters of the genes encoding parsley 4CL (Hauffe et al., 1991), bean PAL2 (Leyva et al., 1992), Arabidopsis PAL1 (Ohl et al., 1990), and other lignin biosynthetic enzymes, including CAD (Feuillet et al., 1995). Proteins that can bind to such elements are being identified; in particular, the Myb class of transcriptional regulators has been implicated in regulation of phenylpropanoid-related genes (Sablowski et al., 1994).

How Is Deposition of Lignin in the Cell Wall Controlled?

Feeding experiments suggest that incorporation of different monomers into lignin occurs at different phases of wall synthesis and lignin formation in both conifers and woody angiosperms (Terashima and Fukushima, 1989). As mentioned previously, lignin content and composition in angiosperms can vary among cell types and within the wall of an individual cell (reviewed in Chen, 1991). It has been suggested that changes in lignin monomer composition are due to changes in the patterns of monolignol biosynthesis during cellular differentiation (Grand et al., 1983). Few data address the question of how the accumulation of monolignol glucosides in conifers affects the control of monolignol synthesis and differential deposition into lignin at varying stages of cellular differentiation. Terashima (1990) has argued convincingly that the lignin polymer formed in cell wall biosynthesis is a structurally ordered macromolecule formed by a regulated process.

A related question concerns the cellular origin of the lignin precursors and the time of lignin deposition. The work of Terashima and co-workers argues for regulated deposition during cell wall biosynthesis because of specific changes in composition that are found in the cell wall layers. Paradoxically, it appears that lignin deposition can occur in cells that are no longer alive (Pickett-Heaps, 1968), a process that might require transport from nearby cells still producing monolignols, perhaps ray parenchymal cells. Gene fusion experiments in transgenic plants have shown that promoters of several genes encoding monolignol biosynthetic enzymes are active in ray parenchyma, including parsley 4CL (Hauffe et al., 1993), bean PAL2 (Shufflebottom et al., 1993), and *Eucalyptus* CAD (Feuillet et al., 1995).

CONCLUSION

The conversion of L-phenylalanine into one of the three lignin precursors, or monolignols, takes place through a series of well-defined reactions. The sequence in which these reactions occur, however, is not yet clearly established. The monomer composition of lignin is known to differ between species, between cell types within a species, and between developmental stages of a single cell. The order in which hydroxylation, methylation, thioactivation, and reduction reactions occur during monolignol synthesis may vary at some or all of these levels as well, although the extent and physiological significance of this variation are not clear. The developmental mechanisms responsible for variation in lignin composition are unknown but will be the subject of intense interest as tools for the analysis of developmental regulation become available. Research into the mechanisms of lignin polymerization is also continuing.

The complexity of lignin structure has made analysis of polymerization mechanisms a challenging topic. It has been difficult to demonstrate that lignin structure deviates significantly from a random polymer. Increasing evidence now suggests that monomer residues are organized within the lignin polymer, and research is focusing on the mechanisms that give rise to order. Methods capable of analyzing subtle differences in monomer organization will be essential to progress

in this area. The role of other cell wall polymers in organizing lignin deposition also deserves further evaluation. The possibility that polysaccharide components of the cell wall participate in organizing lignin precursors can be addressed using mutations that alter cell wall polysaccharides (Reiter et al., 1993; Potikha and Delmer, 1995) or using cell cultures treated with inhibitors (Taylor et al., 1992). The tools are now available for a much more detailed investigation of lignin biosynthesis than has been possible. We can expect to understand, within the next decade, facets of the structure and biosynthesis of this complex polymer that have eluded explanation for the past 100 years.

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REFERENCES

Agarwal, U.P., and Atalla, R.H. (1986). In-situ microprobe studies of plant cell walls: Macromolecular organization and compositional variability in the secondary wall of Picea mariana (Mill.) B.S.P. Planta 169, 325–332.

Alibert, G., Ranjeva, R., and Boudet, A.M. (1972). Recherches sur les enzymes catalysant la biosynthèse des acides phénoliques chez Quercus pedunculata. III. Formation séquentielle, à partir de la phénylalanine, des acides cinnamique, p-coumarique et caféique, par des organites isolés. Physiol. Plant. 27, 240–243.

Atalla, R.H., and Agarwal, U.P. (1985). Raman microprobe evidence for lignin orientation in the cell walls of native woody tissue. Science 227, 636–638.

Atalla, R.H., Hackney, J.M., Uhlin, I., and Thompson, N.S. (1993). Hemicelluloses as structure regulators in the aggregation of native cellulose. Int. J. Biol. Macromol. 15, 109–112.

Bate, N., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., and Elkind, Y. (1994). Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc. Natl. Acad. Sci. USA 91, 7608–7612.

Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R., and Schuch, W. (1989). Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J. 8, 1899–1906.

Bolwell, G.P., Bell, J.N., Cramer, C.L., Schuch, W., Lamb, C.J., and Dixon, R. A. (1985). L-Phenylalanine ammonia-lyase from Phaseolus vulgaris: Characterization and differential induction of multiple forms from elicitor-treated cell suspension cultures. Eur. J. Biochem. 149, 411–419.

Boniwell, J.M., and Butt, V.S. (1986). Flavin nucleotide-dependent 3-hydroxylation of 4-hydroxyphenylpropanoid carboxylic acids by particulate preparations from potato tubers. Z. Naturforsch. 41C, 56–60.

Boudet, A. M., Lapierre, C., and Grima-Pettenati, J. (1995). Biochemistry and molecular biology of lignification. New Phytol. 129, 203–236.

Bowsher, C.G., Boulton, E.L., Nayagam, S., and Emes, M.J. (1992). Reductant for glutamate synthase is generated by the oxidative pentose phosphate pathway in non-photosynthetic root plastids. Plant J. 2, 893–898.

Campbell, M.M., and Ellis, B.E. (1992a). Fungal elicitor-mediated responses in pine cell cultures. I. Induction of phenylpropanoid metabolism. Planta 186, 409–417.

Campbell, M.M., and Ellis, B.E. (1992b). Fungal elicitor-mediated responses in pine cell cultures. III. Purification and characterization of phenylalanine ammonia-lyase. Plant Physiol. 98, 62–70.

Chapple, C.C.S., Vogt, T., Ellis, B.E., and Somerville, C.R. (1992). An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 4, 1413–1424.

Chen, C.-L. (1991). Lignin: Occurrence in woody tissues, isolation, reactions, and structure. In Wood Structure and Composition, M. Lewin and I.S. Goldstein, eds (New York: Marcel Dekker), pp. 183–261.

Cherney, J.H., Axtell, J.D., Hassen, M.M., and Anliker, K.S. (1988). Forage quality characterization of a chemically-induced brown-midrib mutant in pearl millet. Crop Sci. 28, 783–787.

Cherney, J.H., Cherney, J.R., Akin, D.E., and Axtell, J.D. (1991). Potential of brown-midrib, low-lignin mutants for improving forage quality. Adv. Agron. 46, 157–198.

Chiang, V.L., Puumala, R.J., and Takeuchi, H. (1988). Comparison of softwood and hardwood kraft pulping. TAPPI J. 71, 173–176.

Cornish-Bowden, A., and Cárdenas, M.L. (1990). Control of Metabolic Processes, NATO ASI Series, Series A: Life Sciences, Vol. 190 (New York: Plenum Press).

Czichi, U., and Kindl, H. (1977). Phenylalanine ammonia-lyase and cinnamic acid hydroxylases as assembled consecutive enzymes on microsomal membranes of cucumber cotyledons: Cooperation and subcellular distribution. Planta 134, 133–143.

Davin, L., Bedgar, D.L., Katayama, T., and Lewis, N. (1992). On the stereoselective synthesis of (+)-pinoresinol in Forsythia suspensa from its achiral precursor, coniferyl alcohol. Phytochemistry 31, 3869–3874.

Dean, J.F.D., and Eriksson, K.-E.L. (1994). Laccase and the deposition of lignin in vascular plants. Holzforschung 48, 21–33.

Dharmawardhana, D.P., Ellis, B.E., and Carlson, J.E. (1995). A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. Plant Physiol. 107, 331–339.

Dixon, R.A., and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Duke, S.O., and Vaughn, K.C. (1982). Lack of involvement of polyphenol oxidase in ortho-hydroxylation of phenolic compounds in mung bean seedlings. Physiol. Plant. 54, 381–385.

Dwivedi, U.N., Campbell, W.H., Yu, J., Datla, R.S.S., Bugos, R.C., Chiang, V.L., and Podila, G.K. (1994). Modification of lignin biosynthesis in transgenic Nicotiana through expression of an antisense O-methyltransferase gene from Populus. Plant Mol. Biol. 26, 61–71.

Edwards, J.W., Walker, E.L., and Coruzzi, G. M. (1990). Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. Proc. Natl. Acad. Sci. USA 87, 3459–3463.

Elkind, Y., Edwards, R., Mavandad, M., Hedrick, S.A., Ribak, O., Dixon, R.A., and Lamb, C.J. (1990). Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic

tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc. Natl. Acad. Sci. USA **87**, 9057–9061.

Erdtman, H. (1933). Dehydrierung in der Coniferylreihe. 1. Dehydrodieugenol und dehydrodiisoeugenol. Z. Biochem. **258**, 172–180.

Fahrendorf, T., and Dixon, R.A. (1993). Stress responses in alfalfa (*Medicago sativa* L.). XVIII. Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P-450. Arch. Biochem. Biophys. **305**, 509–515.

Feuillet, C., Lauvergeat, V., Deswarte, C., Pilate, G., Boudet, A., and Grima-Pettenati, J. (1995). Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants. Plant Mol. Biol. **27**, 651–667.

Fields, S., and Song, O. (1989). A novel genetic system to detect protein–protein interactions. Nature **340**, 245–246.

Freudenberg, K. (1965). Lignin: Its constitution and formation from *p*-hydroxycinnamyl alcohols. Science **148**, 595–600.

Freudenberg, K., and Harkin, J.M. (1963). The glucosides of cambial sap of spruce. Phytochemistry **2**, 189–193.

Freudenberg, K., and Neish, A.C. (1968). Constitution and Biosynthesis of Lignin: Molecular Biology, Biochemistry and Biophysics, Vol. 2, A. Kleinzeller, G.F. Springer, and H.G. Wittman, eds (New York: Springer-Verlag).

Freudenberg, K., and Richtzenhain, H. (1943). Enzymatische versuche zur entstehung des lignins.Berichte der Deutsche Chemische Gesellschaft; currently known as Chemische Berichte **76**, 997–1006.

Galliano, H., Heller, W., and Sandermann, H. (1993). Ozone induction and purification of spruce cinnamyl alcohol dehydrogenase. Phytochemistry **32**, 557–563.

Goffner, D., Joffroy, I., Grima-Pettenati, J., Halpin, C., Knight, M.E., Schuch, W., and Boudet, A.M. (1992). Purification and characterization of isoforms of cinnamyl alcohol dehydrogenase from *Eucalyptus xylem*. Planta **188**, 48–53.

Goffner, D., Campbell, M.M., Campargue, C., Clastre, M., Borderies, G., Boudet, A., and Boudet, A.M. (1994). Purification and characterization of cinnamoyl–coenzyme A:NADP oxidoreductase in *Eucalyptus gunnii*. Plant Physiol. **106**, 625–632.

Grand, C. (1984). Ferulic acid 5-hydroxylase: A new cytochrome P-450–dependent enzyme from higher plant microsomes involved in lignin synthesis. FEBS Lett. **169**, 7–11.

Grand, C., Boudet, A., and Boudet, A.M. (1983). Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems—Properties and tissue distribution. Planta **158**, 225–229.

Grand, C., Parmentier, P., Boudet, A., and Boudet, A.M. (1985). Comparison of lignins and of enzymes involved in lignification in normal and brown midrib (*bm3*) mutant maize seedlings. Physiol. Veg. **23**, 905–911.

Grima-Pettenati, J., Campargue, C., Boudet, A., and Boudet, A.M. (1994). Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from *Phaseolus vulgaris*. Phytochemistry **37**, 941–947.

Gross, G. (1985). Biosynthesis and metabolism of phenolic acids and monolignols. In Biosynthesis and Biodegradation of Wood Components, T. Higuchi, ed (New York: Academic Press), pp. 229–271.

Gross, G., and Kreiten, W. (1975). Reduction of coenzyme A thioesters of cinnamic acids with an enzyme preparation from lignifying tissue of *Forsythia*. FEBS Lett. **54**, 259–262.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.M., Boon, J.J., Chabbert, B., Tollier, M.-T., and Schuch, W.

(1994). Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. Plant J. **6**, 339–350.

Hanson, K.R., and Havir, E.A. (1981). Phenylalanine ammonia-lyase. In The Biochemistry of Plants: A Comprehensive Treatise, Vol. 7, Secondary Plant Products, E.E. Conn, ed (New York: Academic Press), pp. 577–625.

Hauffe, K.D., Paszkowski, U., Schulze-Lefert, P., Hahlbrock, K., Dangl, J.L., and Douglas, C.J. (1991). A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell **3**, 435–443.

Hauffe, K.D., Lee, S.P., Subramaniam, R., and Douglas, C. J. (1993). Combinatorial interactions between positive and negative *cis*-acting elements control spatial patterns of *4CL-1* expression in transgenic tobacco. Plant J. **4**, 235–253.

Higuchi, T. (1985). Biosynthesis of lignin. In Biosynthesis and Biodegradation of Wood Components, T. Higuchi, ed (New York: Academic Press), pp. 141–160.

Higuchi, T. (1990). Lignin biochemistry: Biosynthesis and biodegradation. Wood Sci. Technol. **24**, 23–63.

Higuchi, T., and Brown, S.A. (1963). Studies of lignin biosynthesis using isotopic carbon. XIII. The phenylpropanoid system in lignification. Can. J. Biochem. Physiol. **41**, 621–628.

Holton, T.A., and Cornish, E.C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7**, 1071–1083.

Hösel, W., Fiedler-Preiss, A., and Borgmann, E. (1982). Relationship of coniferin β-glucosidase to lignification in various plant cell suspension cultures. Plant Cell Tissue Organ Cult. **1**, 137–148.

Houtman, C.J., and Atalla, R.H. (1995). Cellulose–lignin interactions: A computational study. Plant Physiol. **107**, 977–984.

Hrazdina, G., and Jensen, R.A. (1992). Spatial organization of enzymes in plant metabolic pathways. Annu. Rev. Plant. Physiol. Plant Mol. Biol. **43**, 241–267.

Hrazdina, G., and Wagner, G.J. (1985). Metabolic pathways as enzyme complexes: Evidence for the synthesis of phenylpropanoids and flavonoids on membrane-associated enzyme complexes. Arch. Biochem. Biophys. **237**, 88–100.

Ibrahim, R., and Grisebach, H. (1976). Purification and properties of UDP-glucose: Coniferyl alcohol glucosyltransferase from suspension cultures of Paul's Scarlet rose. Arch. Biochem. Biophys. **176**, 700–708.

Jones, D.H. (1984). Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. Phytochemistry **23**, 1349–1359.

Jorrín, J., and Dixon, R.A. (1990). Purification, characterization and induction of phenylalanine ammonia-lyase isoforms from elicitor-treated cell suspension cultures. Plant Physiol. **92**, 447–455.

Kacser, H., and Burns, J.A. (1973). The control of flux. Symp. Soc. Exp. Biol. **27**, 65–104.

Kamachi, K., Yamay, T., Hayakawa, T., Mae, T., and Ojima, K. (1992). Vascular bundle-specific localization of cytosolic glutamine synthetase in rice leaves. Plant Physiol. **99**, 1481–1486.

Kamsteeg, J., Van Brederode, J., Verschuren, P.M., and Van Nigtevech, G. (1981). Identification, properties and genetic control of p-coumaroyl–coenzyme A 3-hydroxylase isolated from petals of *Silene dioica*. Z. Pflanzenphysiol. **102**, 435–442.

Kneusel, R.E., Matern, U., and Nicolay, K. (1989). Formation of *trans*-caffeoyl–CoA from *trans*-4-coumaroyl–CoA by Zn²⁺ dependent

enzymes in cultured plant cells and its activation by an elicitor-induced pH shift. Arch. Biochem. Biophys. **269**, 455–462.

Knobloch, K.-H., and Hahlbrock, K. (1977). 4-Coumarate:CoA ligase from cell-suspension cultures of *Petroselinum crispum* Hoffm. Arch. Biochem. Biophys. **184**, 237–248.

Kojima, M., and Takeuchi, W. (1989). Detection and characterization of *p*-coumaric acid hydroxylase in mung bean, *Vigna mungo*, seedlings. J. Biochem. **105**, 265–270.

Kuc, J., Nelson, O.E., and Flanagan, P. (1968). Degradation of abnormal lignins in the brown-midrib mutants and double mutants of maize. Phytochemistry **7**, 1435–1436.

Kühnl, T., Koch, U., Heller, W., and Wellmann, E. (1989). Elicitor induced *S*-adenosyl-methionine: caffeoyl-CoA 3-*O*-methyltransferase from carrot cell suspension cultures. Plant Sci. **60**, 21–25.

Kuroda, H., Shimada, M., and Higuchi, T. (1981). Characterization of a lignin-specific *O*-methyltransferase in aspen wood. Phytochemistry **20**, 2635–2639.

Kutsuki, H., Shimada, M., and Higuchi, T. (1981). The role of *O*-methyltransferase in the formation of lignin and lignans in the genus *Thuja*. Mokuzai Gakkaishi **27**, 39–48.

Kutsuki, H., Shimada, M., and Higuchi, T. (1982a). Regulatory role of cinnamyl alcohol dehydrogenase in the formation of guaiacyl and syringyl lignins. Phytochemistry **21**, 19–23.

Kutsuki, H., Shimada, M., and Higuchi, T. (1982b). Distribution and roles of *p*-hydroxycinnamate:CoA ligase in lignin biosynthesis. Phytochemistry **21**, 267–271.

Lam, H.-M., Coschigano, K., Schultz, C., Melo-Oliveira, R., Tjaden, G., Oliveira, I., Ngai, N., Hsieh, M.-H., and Coruzzi, G. (1995). Use of Arabidopsis mutants and genes to study amide amino acid biosynthesis. Plant Cell **7**, 887–898.

Leinhos, V., and Savidge, R.A. (1993). Isolation of protoplasts from developing xylem of *Pinus banksiana* and *Pinus strobus*. Can J. For. Res. **23**, 343–348.

Leinhos, V., Udagama-Randeniya, P.V., and Savidge, R.A. (1994). Purification of an acidic coniferin-hydrolysing β-glucosidase from developing xylem of *Pinus banksiana*. Phytochemistry **37**, 311–315.

Lewis, N., and Yamamoto, E. (1990). Lignin: Occurrence, biogenesis and biodegradation. Annu. Rev. Plant. Physiol. Plant Mol. Biol. **41**, 455–496.

Lewis, N.G., Yamamoto, E., Wooten, J.B., Just, G., Ohashi, H., and Towers, G.H.N. (1987). Monitoring biosynthesis of wheat cell-wall phenylpropanoids in situ. Science **237**, 1344–1346.

Leyva, A., Liang, X., Pintor-Toro, J.A., Dixon, R.A., and Lamb, C.J. (1992). *cis*-Element combinations determine phenylalanine ammonia-lyase gene tissue-specific expression patterns. Plant Cell **4**, 263–271.

Liang, X., Dron, M., Cramer, C.L., Dixon, R.A., and Lamb, C.J. (1989). Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. J. Biol. Chem. **264**, 14486–14492.

Liu, L., Dean, J.F.D., Friedman, W.E., and Eriksson, K.-E. (1994). A laccase-like phenoloxidase is correlated with lignin biosynthesis in *Zinnia elegans* stem tissue. Plant J. **6**, 213–224.

Lois, R., and Hahlbrock, K. (1992). Differential wound activation of member of the phenylalanine ammonia-lyase and 4-coumarate:CoA ligase gene families in various organs of parsley plants. Z. Naturforsch. **47C**, 90–94.

Lozoya, E., Hoffmann, H., Douglas, C., Schulz, W., Scheel, D., and Hahlbrock, K. (1988). Primary structures and catalytic properties

of isoenzymes encoded by the two 4-coumarate:CoA ligase genes in parsley. Eur. J. Biochem. **176**, 661–667.

Lüderitz, T., Schatz, G., and Grisebach, H. (1982). Enzymic synthesis of lignin precursors—Purification and properties of 4-coumarate: CoA ligase from cambial sap of spruce (*Picea abies* L.). Eur. J. Biochem. **123**, 583–586.

MacKay, J.J., Liu, W., Whetten, R.W., Sederoff, R.R., and O'Malley, D.M. (1995). Genetic analysis of cinnamyl alcohol dehydrogenase (*Cad*) in loblolly pine: Single gene inheritance, molecular characterization and evolution. Mol. Gen. Genet., in press.

Mansell, R.B., Babbel, G.R., and Zenk, M.H. (1976). Multiple forms and specificity of coniferyl alcohol dehydrogenase from cambial regions of higher plants. Phytochemistry **15**, 1849–1853.

McDougall, G.J., Stewart, D., and Morrison, I.M. (1994). Oxidation of coniferyl alcohol to lignin-like products by tobacco xylem cell walls. Phytochemistry **37**, 683–688.

Mizutani, M., Ward, E., DiMaio, J., Ohta, D., Ryals, J., and Sato, R. (1993). Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 (P450C4H) possessing cinnamate 4-hydroxylase activity. Biochem. Biophys. Res. Comm. **190**, 875–880.

Neish, A.C. (1961). Formation of *m*- and *p*-coumaric acids by enzymatic desamination of the corresponding isomers of tyrosine. Phytochemistry **1**, 1–24.

Neish, A.C. (1968). Monomeric intermediates in the biosynthesis of lignin. In Constitution and Biosynthesis of Lignin, K. Freudenberg and A.C. Neish, eds (New York: Springer-Verlag), pp. 3–43.

Ni, W., Paiva, N.L., and Dixon, R.A. (1994). Reduced lignin in transgenic plants containing a caffeic acid *O*-methyltransferase antisense gene. Transgenic Res. **3**, 120–126.

Northcote, D. H. (1985). Cell organelles and their function in biosynthesis of cell wall components: Control of cell wall assembly during differentiation. In Biosynthesis and Biodegradation of Wood Components, T. Higuchi, ed (New York: Academic Press), pp. 87–108.

Ohl, S., Hedrick, S.A., Chory, J., and Lamb, C.J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell **2**, 837–848.

Okusa, K., Miyakoshi, T., and Chen, C.-L. (1995). Comparative studies on dehydrogenative polymerization of coniferyl alcohol by laccases and peroxidases. Holzforschung, in press.

O'Malley, D.M., Porter, S., and Sederoff, R.R. (1992). Purification, characterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (*Pinus taeda* L.). Plant Physiol. **98**, 1364–1371.

O'Malley, D.M., Whetten, R., Bao, W., Chen, C.-L., and Sederoff, R. (1993). The role of laccase in lignification. Plant J. **4**, 751–757.

Pakusch, A.-E., Kneusel, R.E., and Matern, U. (1989). *S*-Adenosyl-methionine:*trans*-caffeoyl–coenzyme A 3-*O*-methyltransferase from elicitor-treated parsley cell suspension cultures. Arch. Biochem. Biophys. **271**, 488–494.

Pickett-Heaps, J. D. (1968). Xylem wall deposition: Radioautographic investigations using lignin precursors. Protoplasma **65**, 181–205.

Pierrel, M.A., Batard, Y., Kazmaier, M., Mignotte-Vieux, C., Durst, F., and Werck-Reichhart, D. (1994). Catalytic properties of the plant cytochrome P450 CYP73 expressed in yeast: Substrate specificity of a cinnamate hydroxylase. Eur. J. Biochem. **224**, 835–844.

Pillonel, C., Huynziker, P., and Binder, A. (1992). Multiple forms of the constitutive wheat cinnamyl alcohol dehydrogenase. J. Exp. Bot. **43**, 299–305.

Porter, K.S., Axtell, J.S., Lechtenberg, V.L., and Colenbrander, V.F. (1978). Phenotype, fiber composition, and in vitro dry matter

disappearance of chemically induced brown-midrib (bmr) mutants of sorghum. Crop Sci. 18, 205–209.

Potikha, T., and Delmer, D.P. (1995). A mutant of Arabidopsis thaliana displaying altered patterns of cellulose deposition. Plant J. 7, 453–460.

Pryke, J.A., and ap Rees, T. (1977). The pentose phosphate pathway as a source of NADPH for lignin synthesis. Phytochemistry 16, 557–560.

Reiter, W.-D., Chapple, C.C.S., and Somerville, C.R. (1993). Altered growth and cell walls in a fucose-deficient mutant of Arabidopsis. Science 261, 1032–1036.

Sablowski, R.W.M., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C., and Bevan, M. (1994). A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J. 13, 128–137.

Sarkanen, K.V., and Hergert, H.L. (1971). Definition and nomenclature. In Lignins: Occurrence, Formation, Structure and Reactions, K.V. Sarkanen and C.H. Ludwig, eds (New York: Wiley Intersciences), pp. 43–94.

Sarni, F., Grand, C., and Boudet, A.M. (1984). Purification and properties of cinnamoyl–CoA reductase and cinnamyl alcohol dehydrogenase from poplar stems. Eur. J. Biochem. 139, 259–265.

Savidge, R.A. (1989). Coniferin, a biochemical indicator of commitment to tracheid differentiation in conifers. Can. J. Bot. 67, 2663–2668.

Savidge, R.A., Udagama-Randenaya, P.V., and Leinhos, V. (1994). Properties of two enzymes of lignification in conifers. In Proceedings of the Second International Symposium on the Applications of Biotechnology to Tree Culture, Protection and Utilization, C.H. Michler, M.R. Becwar, D. Cullen, W.L. Nance, R.R. Sederoff, and J.M. Slavicek, eds (St. Paul, MN: U.S. Department of Agriculture, Forest Service), p. 10.

Schmitt, D., Pakusch, A.-E., and Matern, U. (1991). Molecular cloning, induction, and taxonomic distribution of caffeoyl-CoA 3-O-methyltransferase, an enzyme involved in disease resistance. J. Biol. Chem. 266, 17416–17423.

Schulz, W., Eiben, H.-G., and Hahlbrock, K. (1989). Expression in Escherichia coli of catalytically active phenylalanine ammonia-lyase from parsley. FEBS Lett. 258, 335–338.

Sederoff, R., Campbell, M., O'Malley, D.M., and Whetten, R. (1994). Genetic regulation of lignin biosynthesis and the potential modification of wood by genetic engineering in loblolly pine. In Genetic Engineering of Plant Secondary Metabolism, B.E. Ellis, G.W. Kuroki, and H.A. Stafford, eds (New York: Plenum Press), pp. 313–355.

Shimada, M., Fushiki, H., and Higuchi, T. (1973). Mechanism of biochemical formation of the methoxyl groups in softwood and hardwood lignins. Mokuzai Gakkaishi 19, 13–21.

Shufflebottom, D., Edwards K., Schuch, W., and Bevan, M. (1993). Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. Plant J. 3, 835–845.

Srere, P.A. (1987). Complexes of sequential metabolic enzymes. Annu. Rev. Biochem. 56, 89–124.

Stafford, H.A. (1974). Possible multi-enzyme complexes regulating the formation of C6-C3 phenolic compounds and lignin higher plants. In Recent Advances in Phytochemistry, Vol. 8, H.A. Stafford, ed (New York: Plenum Press), pp. 53–79.

Stafford, H.A. (1981). Compartmentation in natural product biosynthesis by multienzyme complexes. In The Biochemistry of Plants: A Comprehensive Treatise, Vol. 7, Secondary Plant Products, E.E. Conn, ed (New York: Academic Press), pp. 118–137.

Sterjiades, R., Dean, J.F.D., Gamble, G., Himmelsbach, D., and Eriksson, K.-E.L. (1993). Extracellular laccases from sycamore maple (Acer pseudoplantanus) cell-suspension cultures: Reactions with monolignols and lignin model compounds. Planta 190, 75–87.

Taylor, J.G., Owen, T.P., Jr., Koonce, L.T., and Haigler, C.H. (1992). Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning. Plant J. 2, 959–970.

Terashima, N. (1990). A new mechanism for formation of a structurally ordered protolignin macromolecule in the cell wall of tree xylem. J. Pulp Paper Sci. 16, J150–155.

Terashima, N., and Fukushima, K. (1989). Biogenesis and structure of macromolecular lignin in the cell wall of tree xylem as studied by microautoradiography. In Plant Cell Wall Polymers: Biogenesis and Biodegradation, ACS Symposium Series 399, N.G. Lewis and M.G. Paice, eds (Washington, DC: American Chemical Society), pp. 160–168.

Terazawa, M., and Miyake, M. (1984). Phenolic compounds in living tissue of woods. II. Seasonal variations of phenolic glycosides in the cambial sap of woods. Mokuzai Gakkaishi 30, 329–334.

Terazawa, M., Okuyama, H., and Miyake, M. (1984). Phenolic compounds in living tissue of woods. I. Phenolic β-glucosides of 4-hydroxycinnamyl alcohol derivatives in the cambial sap of woods. Mokuzai Gakkaishi 30, 322–328.

Teutsch, H.G., Hasenfratz, M.P., Lesot, A., Stoltz, C., Garnier, J.-M., Jeltsch, J.-M., Durst, F., and Werck-Reichhart, D. (1993). Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc. Natl. Acad. Sci. USA 90, 4102–4106.

Vaughan, P.F.T., and Butt, V.S. (1969). The hydroxylation of p-coumaric acid by an enzyme from leaves of spinach beet (Beta vulgaris L.). Biochem. J. 113, 109–115.

Vignols, F., Rigau, J., Torres, M.A., Capellades, M., and Puigdomènech, P. (1995). The brown midrib3 (bm3) mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. Plant Cell 7, 407–416.

Voo, K.S., Whetten, R., O'Malley, D., and Sederoff, R.R. (1995). 4-Coumarate:CoA ligase in xylem of loblolly pine. Plant Physiol. 108, 85–97.

Walter, M.W., Grima-Pettenati, J., Grand, C., Boudet, A.M., and Lamb, C. (1988). Cinnamyl-alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor. Proc. Natl. Acad. Sci. USA 85, 5546–5550.

Wanner, L.A., Li, G., Ware, D., Somssich, I.E., and Davis, K.R. (1995). The phenylalanine ammonia-lyase gene family in Arabidopsis thaliana. Plant Mol. Biol. 27, 327–338.

Wengenmayer, H., Ebel, J., and Grisebach, H. (1976). Enzymic synthesis of lignin precursors: Purification and properties of a cinnamoyl-CoA:NADPH reductase from cell supension cultures of soybean (Glycine max). Eur. J. Biochem. 65, 529–536.

Whetten, R., and Sederoff, R.R. (1992). Phenylalanine ammonia-lyase from loblolly pine: Purification of the enyzme and isolation of complementary DNA clones. Plant Physiol. 98, 380–386.

Wyrambik, D., and Grisebach, H. (1975). Purification and properties of isoenzymes of cinnamyl alcohol dehydrogenase from soybean. Eur. J. Biochem. 59, 9–15.

Ye, Z.-H., Kneusel, R.E., Matern, U., and Varner, J.E. (1994). An alternative methylation pathway in lignin biosynthesis in Zinnia. Plant Cell 6, 1427–1439.