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Update on Genetic Improvement

Variation in Lignin Content and Composition[1]

Mechanisms of Control and Implications for the Genetic Improvement of Plants

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Lignin, a complex phenolic polymer, is important for mechanical support, water transport, and defense in vascular plants. Compressive strength and hydrophobicity of xylem cell walls are imparted by the lignin polymer, which is deposited during the terminal differentiation of tracheids and other cell types. The resistance of xylem to compressive stresses imposed by water transport and by the mass of the plants is important to growth and development. In addition, the insolubility and complexity of the lignin polymer makes it resistant to degradation by most microorganisms. Therefore, lignin serves an important function in plant defense. Variation in lignin content, composition, and location is likely to affect these essential processes. The constraints on the amount, composition, and localization of lignin for normal xylem function and plant defense are not known.

Lignin composition, quantity, and distribution also affect the agroindustrial uses of plant material. Digestibility and dietary conversion of herbaceous crops are affected by differences in lignin content and composition (Akin et al., 1986, 1991). Lignin is an undesirable component in the conversion of wood into pulp and paper; removal of lignin is a major step in the paper making process. Furthermore, the resistance of lignin to microbial degradation enhances its persistence in soils. Lignin is, therefore, a significant component in the global carbon cycle.

The mechanisms of control of lignin composition and quantity have wide implications regarding the adaptation and evolution of land plants and provide a basis for improved genetic manipulation of lignin for agroindustrial end uses. In this *Update*, we will focus on the levels of control of lignin variation, including (a) metabolic control, (b) regulation of individual enzymes in the biosynthetic pathway, and (c) regulation of gene expression. These levels of regulation affect variation in lignin content, quality, and distribution. Finally, the implications of these regulatory mechanisms for the genetic improvement of lignin for agroindustrial products will be described. Lignin structure, biosynthesis, degradation, and the regulation of lignification have been extensively reviewed (Higuchi, 1985, 1990; Lewis and Yamamoto, 1990; Chen, 1991; Sederoff et al., 1994).

LIGNIN IS A COMPLEX AND HIGHLY VARIABLE BIOPOLYMER

Lignin is a complex hydrophobic network of phenylpropanoid units that is thought to result from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors (Higuchi, 1985). These alcohols, 4-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol, give rise to *p*-hydroxyphenyl, guaiacyl, and syringyl lignins, respectively (Fig. 1). The three monolignol precursors differ in the extent of methoxylation. This variety of subunit substitution patterns means that a variety of intermolecular linkages can be formed during polymerization (Freudenberg and Neish, 1968; Lewis and Yamamoto, 1990). Lignin, therefore, varies in its subunit composition and intermolecular linkages.

In addition to being highly heterogeneous as a polymer, lignins can vary within a given cell wall (Agarwal and Atalla, 1986). Lignin heterogeneity is regulated during secondary cell-wall deposition, giving rise to layers of lignin that can differ in average monomer composition. Thus within a given cell wall, lignin subunit composition and overall quantity may vary depending on location in the wall, developmental state of the cell and tissue, and the influence of environmental stress. Lignin also varies in its composition and quantity between different cell types and between tissues within the same plant. For example, compression-wood lignin of gymnosperms is predominantly

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Abbreviations: *bm, brown midrib;* CAD, hydroxycinnamyl alcohol dehydrogenase; CCoAOMT, *S*-adenosyl-methionine:caffeoyl-CoA/5-hydroxyferuoyl-CoA *O*-methyltransferase; CCR, hydroxycinnamoyl-CoA:NADPH oxidoreductase; 4CL, hydroxycinnamate:CoA ligase; CoAOMT, *S*-adenosyl-methionine: caffeoyl-CoA *O*-methyltransferase; F5H, ferulate 5-hydroxylase; OMT, *S*-adenosyl-methionine:caffeate/5-hydroxyferulate *O*-methyltransferase; PAL, Phe ammonia-lyase; S:G ratio, syringyl:guaiocyl ratio.

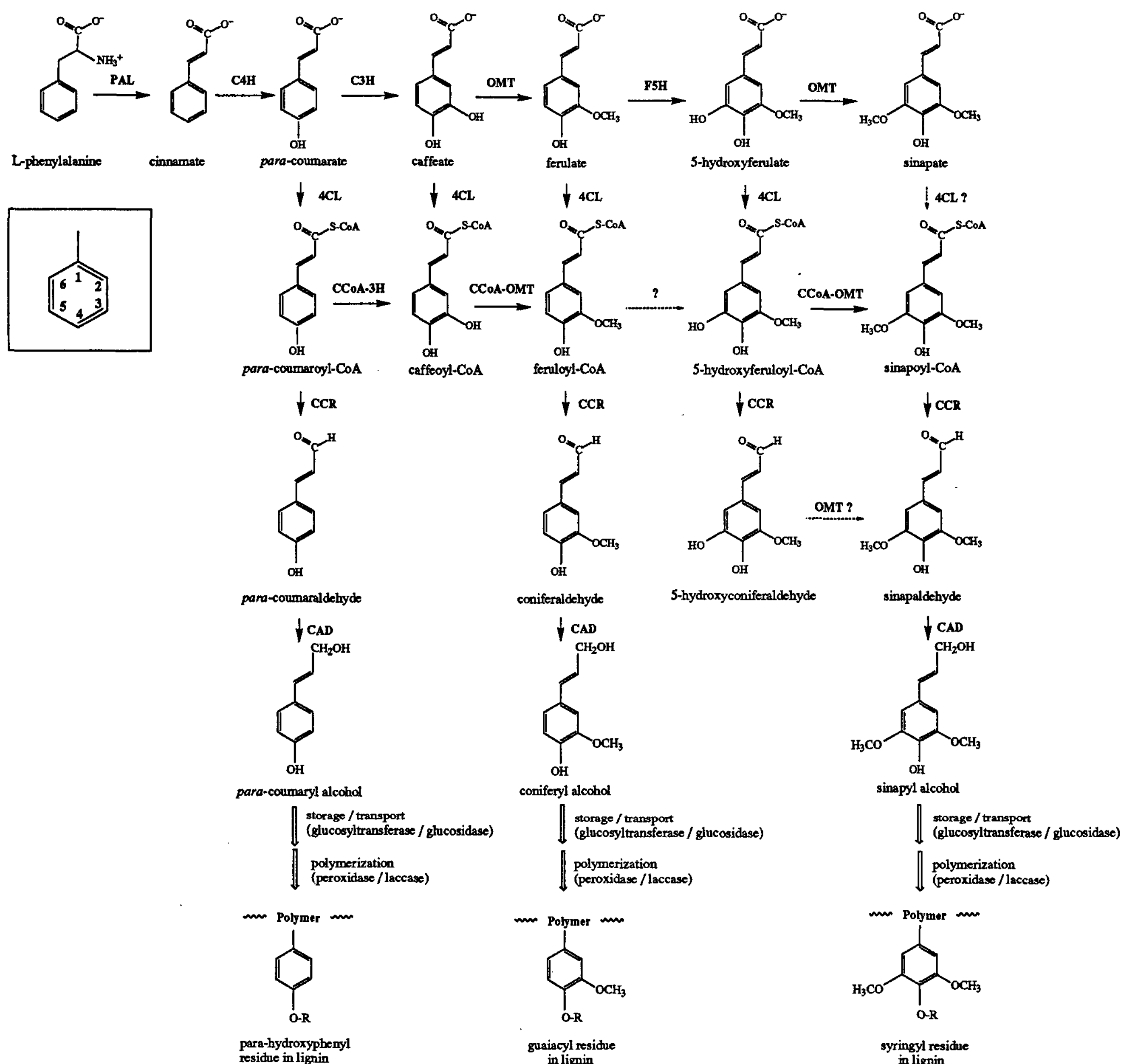

Figure 1. The lignin biosynthetic pathway. This general pathway for lignin biosynthesis has been inferred from studies of specific steps in several diverse species. To reduce the complexity of the diagram, co-factors for the enzymatic reactions have not been shown. The lignin biosynthetic enzymes and their respective EC numbers are: PAL, Phe ammonia-lyase (EC 4.3.1.5); C4H, cinnamate 4-hydroxylase (EC 1.14.13.11); C3H, 4-hydroxycinnamate 3-hydroxylase; OMT, *S*-adenosyl-methione:caffeate/5-hydroxyferulate-*O*-methyltransferase (EC 2.1.1.6); F5H, ferulate 5-hydroxylase; 4CL, hydroxycinnamate:CoA-ligase (EC 6.2.1.12); CCoA-3H, 4-hydroxycinnamoyl-CoA 3-hydroxylase; CCoA-OMT, *S*-adenosyl-methionine: caffeoyl-CoA/5-hydroxyferuloyl-CoA-*O*-methyltransferase (EC 2.1.1.104); CCR, hydroxycinnamoyl-CoA:NADPH oxidoreductase (EC 1.2.1.44); CAD, hydroxycinnamyl alcohol dehydrogenase (EC 1.1.1.195); glucosyltransferase, UDP-Glc:coniferyl alcohol 4-*O*-glucosyltransferase (EC 2.4.1.1); glucosidase, coniferin-specific 4-*O*-glucosidase (EC 3.2.1.21). The R-function in the monomeric units of lignin indicates that cross-linking via ester or ether bonds can occur at these positions. Reactions that have been demonstrated in vitro are shown with solid arrows. Reactions that have been inferred by feeding studies are shown with dashed arrows. The inset indicates the numbering convention for the carbons in the phenyl ring. See the text for further discussion of the validity of the pathway.

guaiacyl lignin, but it is augmented in *p*-hydroxyphenyl units (Kutsuki and Higuchi, 1981). Similarly, wood formed at the top of a mature conifer typically has a higher lignin content than wood from the stem (Zobel and van Buijtenen, 1989).

Lignin content is also variable within populations of plants of the same species. Within the genus *Pinus*, for example, the average content of lignin ranges from 25% (*Pinus monticola*) to 30% (*Pinus palustris*). Within a species of pine, the lignin content may range from 26 to 30% (Zobel and van Buijtenen, 1989). Lignin content, composition, and distribution vary among higher taxa as well. In different species of woody plants, lignin content can range between 15 and 36% of the dry weight of wood (Zobel and van Buijtenen, 1989). In gymnosperms (softwoods), lignins are typically composed predominantly of guaiacyl units with a minor proportion of unmethoxylated *p*-hydroxyphenyl units. The typical lignin of angiosperms (hardwoods) is guaiacyl-syringyl lignin, formed from co-polymerization of coniferyl and sinapyl alcohols (Higuchi, 1985). The presence of methoxylated syringyl units makes hardwood lignin more easily hydrolyzed during pulping (Chiang and Funaoka, 1990). Compression-wood lignin is more difficult to hydrolyze because it contains a higher proportion of condensed *p*-hydroxyphenyl units.

THE LIGNIN BIOSYNTHETIC PATHWAY

A general pathway for lignin biosynthesis has been inferred from studies of specific steps in several diverse species (Fig. 1). It is not yet clear whether a single pathway can explain biosynthesis in all species, all tissues, and under varying conditions of environmental stress. There is no example for which the entire pathway of lignin biosynthesis has been characterized biochemically and genetically in a single tissue or a single species. Given the high degree of lignin heterogeneity among species and even within a plant, the regulation and nature of the pathway may differ among cell types and among species. In general, the necessity and sufficiency of the specific enzymatic steps in the biosynthetic pathway for lignification have not yet been demonstrated. The pathway may be subject to revision with new data; for example, methylation of at least some of the lignin precursors may not occur at the level of cinnamic acids but could occur later at the level of the activated CoA esters (Ye et al., 1994) or even at the level of the aldehydes (Matsui et al., 1994).

CONTROL OF LIGNIN CONTENT BY REGULATING FLUX INTO AND THROUGH THE BIOSYNTHETIC PATHWAY

Variation in lignin quantity, quality, and distribution must be based on the nature of the lignin biosynthetic pathway and the mechanisms of its regulation. Lignin variability may be due to the control of metabolite flux into and through the lignin biosynthetic pathway. Flux into the pathway is likely to be affected by entry-point enzymes, whereas flux through the pathway may be influenced by levels of enzyme activity and by metabolic channeling of substrates and products (Kacser and Burns, 1973).

Studies of inducible lignification in plant cell cultures have provided insights into the biochemical modulation of lignin quantity. Cell cultures can be induced by a variety of stimuli to proceed through temporally controlled metabolic and developmental programs. Tissue cultures have proven to be a very useful tool in the analysis of coordinate regulation of phenylpropanoid metabolism, particularly for isoflavonoid and flavonoid biosynthesis (Hahlbrock and Scheel, 1989). Cell culture systems are particularly useful when they reflect mechanisms of regulation that occur in the whole plant. However, cultured cells are highly stressed, and it is not possible to conclude that normal developmental processes are taking place in culture without parallel studies in intact plants.

The best-characterized cultured-cell system used to study inducible lignification is derived from excised *Zinnia* mesophyll cells. Isolated *Zinnia* mesophyll cells can be induced to differentiate into cells resembling tracheary elements. During the course of this differentiation, lignification occurs (Taylor et al., 1992). Coordinated activity of several lignin biosynthetic enzymes and the control of lignin patterning in the cell wall have been examined (Fukuda and Komamine, 1982; Church and Galston, 1988; Taylor et al., 1992). Activities of the enzymes involved in the synthesis of lignin precursors (PAL, 4CL, OMT, and CCoAOMT) increased concomitantly with lignification during tracheid differentiation. Ye et al. (1994) were able to correlate the presence of CCoAOMT with the differentiation of lignified cells in *Zinnia* plants. Peroxidase activity appeared long before the initiation of lignification, suggesting that peroxidase is not the limiting step in the biosynthesis of lignin in this system (Fukuda and Komamine, 1982).

Inducible lignification in jack pine (*Pinus banksiana*) using fungal-elicitor-treated cell cultures indicated that the metabolic mechanisms for the regulation of lignin quantity in jack pine are similar to those in *Zinnia* (Campbell and Ellis, 1992a, 1992b). Following elicitation, pine cells accumulate guaiacyl lignin, concomitantly with changes in monolignol biosynthetic enzyme activity. PAL activity increased rapidly but transiently after addition of elicitor, whereas 4CL and OMT activities were gradually induced parallel to lignin accumulation. CAD activity did not increase substantially; however, basal levels of this enzyme were quite high. In contrast, coniferin β-glucosidase, an enzyme related to the transport of monolignols to the cell wall, was induced in the elicited jack pine cultures parallel to lignification. Perhaps not all of the enzymes of lignin biosynthesis are coordinately controlled in *P. banksiana* cells under these conditions.

A rapid increase in PAL activity appears to be a hallmark of stress-induced lignification. In elicited jack pine suspension cultures, for example, PAL activity was rapidly induced to levels 10-fold greater than controls. This increase in enzyme activity decreased to control levels within 48 h (Campbell and Ellis, 1992a, 1992c). Eberhardt et al. (1993) also saw an increase in PAL activity in cells of loblolly pine (*Pinus taeda*) following treatment with plant growth regu-

lators. PAL activity increased from near undetectable levels to >200 pkat/mg protein. Elevated levels of PAL activity may be needed for lignification, to redirect Phe toward the biosynthesis of phenylpropanoids. The spatial and temporal regulation of PAL activity may be important in regulating the flux of metabolites into the lignin biosynthetic pathway in normal development as well.

Goffner at al. (1994) proposed that CCR may be an important regulatory enzyme that controls flux into monolignol biosynthesis. CCR catalyzes the conversion of hydroxycinnamoyl-CoA esters into the corresponding aldehydes and thus channels metabolites from general phenylpropanoid biosynthesis into the monolignol biosynthetic pathway. CCR can be viewed as the entry-point enzyme into monolignol biosynthesis. As is the case with other entry-point enzymes, CCR may regulate the flux into the monolignol-specific branch of phenolic metabolism. Entry-point enzymes may be good targets for the directed manipulation of lignin content.

TO WHAT EXTENT DOES ENZYMATIC SPECIFICITY INFLUENCE LIGNIN COMPOSITION AND DISTRIBUTION?

Lignin heterogeneity could be directly related to enzyme diversity and specificity. Substrate specificities of given enzymes in the lignin biosynthetic pathway affect metabolite flux into different branches of the biosynthetic pathway. Lignin heterogeneity within the plant could result from temporal and spatial distribution of isoforms for given steps in the biosynthetic pathway.

The most distinct variation in lignin composition occurs between gymnosperms and angiosperms. Hypotheses to account for differences in hardwood and softwood lignins are based on differences in the enzymes of the monolignol biosynthetic pathway (Shimada et al., 1973). The formation of sinapyl alcohol is presumed to require the hydroxylation of ferulic acid by F5H, the transmethylation of 5-hydroxyferulic acid to sinapic acid, and the subsequent reduction of sinapic acid to sinapyl alcohol through three steps common to the reduction of ferulic acid and 4-coumaric acid (Fig. 1). Two major biosynthetic factors may account for the difference in lignin monomer composition between gymnosperms and angiosperms, the enzyme F5H and the substrate specificity of the subsequent enzymatic steps catalyzed by OMT, 4CL, CCR, and CAD.

Erythrina cristagalli is an angiosperm that produces only guaiacyl lignin (gymnosperm type) but has typical angiosperm lignin biosynthetic enzymes. These include a 4CL, which uses sinapate; an OMT, which uses 5-hydroxyferulate; and a CAD, which uses sinapaldehyde (reviewed by Higuchi, 1981). Higuchi and co-workers hypothesized that the absence of F5H in *E. cristagalli* could account for this apparent paradox (Kutsuki and Higuchi, 1978; Higuchi, 1985). The absence of syringyl units in conifer lignin may be accounted for by the absence of F5H.

Alternative hypotheses could account for the difference in lignin composition between conifers and angiosperms. These hypotheses are based on the concepts of metabolic channeling and substrate specificities of lignin biosynthetic pathway enzymes other than F5H. For example, sinapate was a poor substrate for most of the conifer 4CL activities tested (Gross et al., 1975; Kutsuki et al., 1982b; Lüderitz et al., 1982; Voo et al., 1995). Sinapate 4CL activity is rare even in angiosperms, despite the fact that they produce syringyl lignins (Kutsuki et al., 1982b). Paradoxically, *E. cristagalli* catalyzes the conversion of sinapate to sinapoyl-CoA even though the vast majority of syringyl lignin-producing angiosperms do not.

Conifer CCR and CAD have higher affinity for substrates that are precursors to guaiacyl lignins (feruloyl-CoA and coniferaldehyde) than they do for the syringyl-precursor substrates (sinapoyl-CoA and sinapaldehyde) (Lüderitz and Grisebach, 1981; O'Malley et al., 1992; Galliano et al., 1993). In spruce, the maximal reaction rates for both CCR and CAD with the syringyl precursors were 10% of the rates with the guaiacyl precursors. Kutsuki et al. (1982a) proposed that the lower affinity of conifer CAD for sinapaldehyde accounted for the absence of syringyl lignins in conifers. The activity of CAD with sinapaldehyde versus coniferaldehyde ranged from 5 to 55% in different conifers surveyed in comparison to 65 to 169% in angiosperms. *E. cristagalli* still had a higher percentage of activity with sinapaldehye (65%) than did the conifers. Also, a gymnosperm had a high percentage of activity (55%) with sinapaldehyde despite the fact that it had only guaiacyl lignins. Furthermore, when conifers were fed radiolabeled sinapaldehyde, label was efficiently incorporated into sinapyl alcohol and lignin syringyl units (Terashima and Fukushima, 1988, 1989).

Purified angiosperm OMT appears to be bifunctional, i.e. both the conversion to ferulate and the conversion to sinapate are catalyzed by the same enzyme (Bugos et al., 1992). Crude enzyme extracts from conifers can catalyze both reactions as well (Higuchi, 1981; Gross, 1985). However, the relative affinity for the two substrates is significantly different for conifers in comparison to angiosperms. Gymnosperm OMT activities for the conversion of 5-hydroxyferulate to sinapate were 10 to 30% of that of the activities for the conversion of caffeate to ferulate, whereas angiosperm OMT activities with 5-hydroxyferulate were 90 to 320% of that of the activities with caffeate (Higuchi, 1981). In angiosperms, OMT could selectively channel hydroxycinnamic acids into sinapate. Nonetheless, some conifers clearly possess some sinapate-producing OMT activity. Similarly, *E. cristagalli* appeared to possess an "angiosperm-like" OMT activity (Kutsuki et al., 1982b). The absence of syringyl lignins in *Erythrina* must be achieved at some step other than OMT.

Metabolic channeling may be important in the regulation of lignin biosynthesis. It has been proposed that physical associations between enzymes may shunt precursors to subsequent steps in phenylpropanoid metabolism (Hrazdina and Jensen, 1992). For example, OMT, the enzyme that catalyzes the conversion of 5-hydroxyferulate to sinapate, might be necessary to channel sinapate to 4CL. Therefore, purified 4CL lacking OMT would not be active with sinapate.

Alternatively, some angiosperms may derive syringyl units from a pathway other than through sinapate. Conversion of

5-hydroxyferulate to its CoA ester occurs in extracts of both angiosperms and conifers (Kutsuki et al., 1982b; Lüderitz et al., 1982). In fact, 5-hydroxyferulate is the preferred substrate for some 4CL activities that use sinapate as a substrate (Grand et al., 1983). Methylation of 5-hydroxyferuloyl-CoA could be analogous to the conversion of caffeoyl-CoA to feruloyl-CoA in differentiating *Zinnia* mesophyll cells (Ye et al., 1994). Methylation may also occur after the CCR-catalyzed conversion of 5-hydroxyferuloyl-CoA to 5-hydroxyconiferaldehyde (Higuchi, 1985).

MULTIENZYME FAMILIES AND LIGNIN HETEROGENEITY

At several steps in the lignin biosynthetic pathway, multiple genes and enzymes have been identified that could carry out similar reactions during development or the defense response (reviewed by Sederoff et al., 1994). Multiple enzymes may also indicate diversity of function or alternative pathways for biosynthesis. Multiple forms of PAL have been extensively studied in bean, parsley, and alfalfa. Similarly, isoforms of 4CL and CAD have also been described. These isoforms are encoded by multigene families, whose members are differentially expressed in development, and in response to environmental stress. Lignin heterogeneity could be accounted for, at least in part, by different isoforms of monolignol biosynthetic enzymes that prefer different substrates. The timing and location of expression of given family members could also generate heterogeneity in lignin.

An exception to the multigene/enzyme isoform families is found in at least some conifers. For example, Campbell and Ellis (1992c) found that a single isoform of PAL was elicited in pine cell cultures during the inducible lignification response. This is in contrast to angiosperms in which several PAL isoforms are induced with elicitor. PAL appears to be encoded by a single gene in loblolly pine (Whetten and Sederoff, 1992), suggesting that a single *PAL* gene responds to both environmental and developmental cues in this species.

Multiple isoforms of 4CL that differ in substrate specificity and/or tissue specificity have been identified in several species (reviewed by Voo et al., 1995). 4CL is a branch-point enzyme for several end-product-specific pathways in addition to lignin biosynthesis. Different isoforms of 4CL with different substrate specificities and developmental regulation could determine the microheterogeneity of lignin (Grand et al., 1983). In conifers, the composition of lignin changes during the formation of the xylem cell wall. Terashima and Fukushima (1988, 1989) examined the types of monomers that are deposited during the formation of xylem cell walls. They proposed that the first lignin deposited during the differentiation of xylem is enriched in *p*-hydroxyphenyl lignin in the middle lamella and the cell corners. Guaiacyl lignin, which predominates, is subsequently deposited in the middle lamella and the secondary wall. A small amount of syringyl lignin may be deposited late in the formation of the secondary wall. Hypothetically, different forms of 4CL with different substrate specificities could be active at different times during cell-wall biosynthesis, giving rise to lignin microheterogeneity.

Compression wood is formed in gymnosperms in response to mechanical stress and is characterized by higher wood density, increased lignin content and an increased proportion of *p*-hydroxyphenyl lignin (Kutsuki and Higuchi, 1981). 4CL could be involved in the modulation of lignin quantity and composition during the formation of compression wood by regulating the proportion of *p*-coumarate reduced to *p*-coumaryl alcohol, compared to the proportion of ferulate converted to coniferyl alcohol. In pine, the lignin content increases from 26 to 34% and the methoxyl content is reduced from 15 to 12.6%, reflecting the increased proportion of *p*-hydroxyphenyl lignin (Kutsuki and Higuchi, 1981). A specific isoform of 4CL with increased affinity for *p*-coumarate might be adequate to modify the composition of lignin. However, a recent study of loblolly pine indicates that differentiating xylem of normal wood and compression wood have the same single form of 4CL and that there is only a single functional gene for 4CL active in xylem (Voo et al., 1995). In this system, therefore, the microheterogeneity of lignin cannot be explained by different isoforms of 4CL and must be due to a different mechanism of regulation of monolignol biosynthesis.

CAD isoforms have also been demonstrated in a number of species, including wheat, eucalyptus, soybean, and bean (reviewed by MacKay et al., 1995). Differential expression of isoforms with different substrate specificities has been put forth as a potential mechanism to account for lignin heterogeneity (Mansell et al., 1976). In contrast, loblolly pine appears to have only a single form of CAD encoded by a single functional *CAD* gene (MacKay et al., 1995). Microheterogeneity in lignin monomer composition must be accounted for at a step other than CAD. Loblolly pine CAD must be responsive to both environmental and developmental cues.

The existence of multienzyme/multigene families for PAL, 4CL, and CAD in some species offers an opportunity to genetically modify lignin by targeting only those members of the family that affect lignin deposition under conditions of interest. If a decrease in lignin content is desirable, one could down-regulate only the family member that affects lignification during stem development while leaving the member that responds to pathogen attack intact. In species in which there appears to be only one enzyme/gene involved in the lignin biosynthetic pathway, modification of those targets is likely to produce pleiotropic effects if one enzyme is responsive to all cues. In this instance it may be more useful to modify the regulation of these targets.

OTHER BIOSYNTHETIC MECHANISMS FOR THE CONTROL OF LIGNIN HETEROGENEITY: MONOMER TRANSPORT AND POLYMERIZATION

The biosynthesis of lignin occurs at the cell wall; therefore, lignin precursors must somehow be translocated from the cytoplasm, where they are synthesized, to the cell wall for polymerization. The steps following monolignol bio-

synthesis may also modulate the composition of lignin. Regulation of transport or polymerization could affect the quantity of lignin produced through specific nonenzymatic chemical interactions or through enzymatic effects.

Observations of the turnover of coniferin (coniferyl alcohol-β-D-glucoside) during the development of *Picea abies* seedlings suggested that this compound might be an intermediate of lignin biosynthesis (reviewed by Grisebach, 1981). During the process of lignification there was a concomitant increase in the activities of UDP-Glc:coniferyl alcohol 4-*O*-glucosyltransferase and a coniferin-specific 4-*O*-glucosidase. In developing spruce seedlings, the glucosyltransferase was immunohistochemically detected in the parietal cytoplasmic layer, whereas the β-glucosidase was found in the cell wall. Consequently, it was hypothesized that coniferyl alcohol was converted to coniferin in the cytoplasm, transported as the glucoside to the cell wall, deglucosylated to liberate the aglycone, and incorporated into lignin.

As with enzymes of monolignol biosynthesis, the amount of activity as well as the substrate specificities of the enzymes associated with lignin transport could play a role in modulating lignin content and composition (reviewed by Grisebach, 1981). Spruce glucosyltransferase functions with sinapyl alcohol almost as effectively as with coniferyl alcohol. Spruce β-glucosidase activity is as effective with syringin as it is with coniferin. Dharmawardhana et al. (1995) recently demonstrated that a coniferin β-glucosidase, tightly associated with lignification in differentiating xylem of lodgepole pine (*Pinus contorta*), used both coniferin and syringin as efficiently as substrates. Conifers fed sinapaldehyde and sinapyl alcohol incorporate these compounds into lignins as efficiently as do angiosperms (Terashima and Fukushima, 1988, 1989). It is likely that these enzymes play a role in the timing and localization of lignin deposition rather than regulating the syringyl content of conifer lignin.

The polymerization of monolignols to make lignins is believed to be catalyzed by some combination of peroxidase and laccase (Higuchi, 1985; O'Malley et al., 1993; Dean and Eriksson, 1994). Several cell culture-based systems have pointed to the importance of changes in peroxidase isoform patterns during lignin induction. As in *Zinnia* (Church and Galston, 1988), peroxidase isoforms were induced during lignification in castor bean and pine cultures (Bruce and West, 1989; Campbell and Ellis, 1992a). A role for these enzymes in the lignification process has been suggested based on correlation with the lignification response. These cell culture results are substantiated by findings in the whole plant, in which peroxidase activity was correlated with regions of lignification (reviewed by O'Malley et al., 1993; Dean and Eriksson, 1994). Similar results were obtained for other enzymes implicated in lignin polymerization, including laccase and coniferyl alcohol oxidase (reviewed by O'Malley et al., 1993; Dean and Eriksson, 1994). Laccase and peroxidase appear to play a role in the timing and extent of lignification as well as affecting the localization of lignin. Genetic modification of these enzymes is therefore proposed to impact the quantity, composition, and localization of lignin.

GENETIC MECHANISMS UNDERLYING LIGNIN HETEROGENEITY: TRANSCRIPTIONAL REGULATION OF LIGNIFICATION

The transcription of genes that encode lignin biosynthetic enzymes may be important in the spatial and temporal control of lignification and in regulating the quantity and composition of lignin. The expression of genes encoding lignin biosynthetic enzymes in response to different developmental and environmental cues may further influence the timing and localization of lignification. The extent to which lignin can be genetically modified in a directed fashion will depend on our knowledge of the organization and expression of the genes encoding the biosynthetic enzymes. It has been shown by standard approaches that many of these genes are transcriptionally regulated. For example, bean *PAL2* (Shufflebottom et al., 1993), poplar *OMT* (Bugos et al., 1991), zinnia *CCoAOMT* (Ye at al., 1994), parsley *4CL* (Hauffe et al., 1993), and eucalyptus *CAD* (Feuillet et al., 1995) all exhibit regulation at the transcriptional level. Promoters of some of these genes contain common sequence elements that may direct tissue-specific expression. The identification of these regulatory sequences and the transcription factors that act upon them point to mechanisms for the directed modification of genes encoding lignin biosynthetic enzymes. Members of the Myb class of transcriptional regulators have been implicated in regulating the expression of genes encoding monolignol biosynthetic enzymes (Hatton et al., 1995). It may be possible to engineer the response of lignin biosynthetic genes to specific developmental or environmental cues by modifying these regulatory motifs and/or transcription factors.

MUTANTS DEMONSTRATE THE ROLES OF SPECIFIC ENZYMES IN THE CONTROL OF LIGNIN HETEROGENEITY

Mutants affecting lignin content and composition have been characterized in maize, sorghum, and *Arabidopsis thaliana*. For example, *bm* mutations affect lignin composition and content in maize, sorghum, and pearl millet. The typical mutant phenotype is that of a red-brown midrib where normal midrib color is white-yellow. All mutants are recessive and show a reduced lignin content. In some cases, the mutation has affected the composition of lignin. Reduced lignin in *bm* mutants has been associated with increased digestibility of silage, increased lodging, and decreased yield (reviewed by Cherney et al., 1991).

The brown midrib phenotype results from mutational lesions in more than one step in the lignin biosynthetic pathway. In maize, four independent *bm* mutations have been identified and characterized (Kuc et al., 1968). The *bm1* mutation shows a lower overall lignin content in mature plants but a higher content in plants less than 6 weeks old (Kuc and Nelson, 1964). Gee et al. (1968) suggested that the *bm1* mutation affected the reduction of phenolic acids to alcohols. The *bm2* mutation reduces lignin as much as 30% (Kuc et al., 1968; Chabbert et al., 1994b), but the biochemical lesion is not known. With *bm2*, the syringyl

content of lignin is increased and *p*-coumaric acid is reduced (Chabbert et al., 1994b). The *bm3* mutation reduces lignin content by 12% and is associated with a decrease in OMT activity (Grand et al., 1985). With *bm3*, *p*-coumaric acid, ferulic acid, and 5-hydroxyferulic acid are increased and so is the proportion of *p*-hydroxyphenyl units in lignin (Chabbert et al., 1994a). Recently, Vignols et al. (1995) were able to demonstrate that *bm3* mutations correspond to insertions or deletions at the maize locus encoding *O*-methyltransferase. A mutation in *bm4* reduces the level of syringaldehyde and also reduces lignin content by 8% (Kuc et al., 1968).

In sorghum, *bmr* mutants resemble the *bm* mutants of maize. The sorghum *bmr6* mutation is the best characterized. In *bmr6* plants, reduction of CAD activity and an increase in cinnamaldehyde concentration relative to cinnamyl alcohols suggests an effect on CAD (Pillonel et al., 1991). An unexplained aspect of the *bmr6* mutation, however, is that the level of OMT activity is also reduced. In a brown midrib mutation found in pearl millet, lignin was reduced by 20% and decreased amounts of *p*-coumarate (38%) were detected (Hartley et al., 1992). The brown midrib mutations indicate that different steps in the lignin biosynthetic pathway can be altered to give rise to a similar mutant phenotype.

Chapple et al. (1992) identified and characterized a lignin mutation (*fah1*) affecting F5H in Arabidopsis. The *fah1* mutant had reduced levels of sinapic acid esters in the seed and modified lignin composition. Wild-type Arabidopsis produces the guaiacyl-syringyl lignins typical of angiosperms. In contrast, the *fah1* mutant produced only guaiacyl lignin. The lignin in the *fah1* mutant resembles gymnosperm lignin in histochemical tests and oxidation products. A T-DNA-tagged mutation allelic to *fah1* has been cloned. The sequence is related to known plant P450 monooxygenases (Chapple et al., 1994), in keeping with the results of Grand (1984), who showed that F5H was a microsomal, Cyt P450 mixed-function monooxygenase. The *fah1* mutant supports the hypothesis that the difference between angiosperm and conifer lignins is due to the absence of F5H in conifers. It is now possible to introduce F5H into a conifer and to examine the effect on the biosynthesis of syringyl lignin. The use of the F5H cDNA in transgenic studies will test the effect of F5H on the variation in lignin monomer composition.

TRANSGENIC PLANTS WITH ALTERED LIGNIN PROPERTIES

The directed genetic engineering of lignin content in crop plants as well as forest tree species has been considered for almost a decade. The feasibility of genetically engineering lignin properties has been tested using tobacco and poplar as a model species. Transgenic tobacco plants with modified expression of PAL, CAD, COMT, or an anionic peroxidase have been obtained.

The effectiveness of modification of lignin by *PAL* transgenes depends on the extent of inhibition of PAL activity, the relative activity of the enzymes in the downstream parts of the pathway, and the pleiotropic effects of the suppression of an early step in the pathway (Bate et al., 1994). Tobacco containing bean *PAL2* transgenes showing sense suppression have a range of activity from wild-type levels to 0.2% of wild type (Elkind et al., 1990; Bate et al., 1994). The level of PAL activity in stems becomes the rate-determining step regulating the deposition of lignin at levels 3- to 4-fold below wild-type levels (Bate et al., 1994). PAL appears to determine overall flux in the phenylpropanoid pathway, whereas downstream steps may control partitioning. In plants with PAL reduced 15-fold compared to wild type, histochemically detectable lignin was almost completely blocked. Therefore, the regulatory architecture of monolignol biosynthesis combines control at the first committed step with downstream regulation of flux.

Transgenic tobacco containing an antisense construct of either the aspen bifunctional OMT gene (Dwivedi et al., 1994) or the alfalfa OMT gene (Ni et al., 1994) has been analyzed for lignin content and composition. The plants containing the antisense alfalfa OMT gene had a significantly lower lignin content but did not exhibit a change in the ratio of syringyl to guaiacyl units.

Halpin et al. (1994) have examined the effects of antisense *CAD* gene expression in transgenic tobacco. CAD activity was reduced to 10% of normal levels without affecting the development of the plants or the quantity of lignin in the cell wall. It is interesting that suppression of PAL activity to 20% of wild-type activity affected the deposition of lignin in tobacco, whereas inhibition of CAD, a terminal step in monolignol biosynthesis, did not show effects on lignin content and composition at the same level of suppression. Higher levels of CAD activity may be needed in more stringent environments, to mount a defense response, or for some other important function of monolignols or their derivatives. In the most affected *CAD* antisense plants, however, the phenolic composition of the cell wall was altered, including higher incorporation of cinnamyl aldehydes versus alcohols into the lignin polymer. Additionally, the cell walls had a greater amount of esterified phenolics. The net result was that the lignin was more extractable. More extractable lignin would be an advantage in processing for pulp and paper or for digestibility as fodder.

An intriguing feature of some plants produced by antisense suppression of CAD is that they have red xylem (Halpin et al., 1994). The appearance of the xylem in these plants resembles that of *bm* mutants. Higuchi et al. (1994) showed that coniferyl aldehyde was converted to a wine-red dehydrogenation polymer by horseradish peroxidase and hydrogen peroxide. The red-brown color may be attributed to the polymerization products of coniferylaldehyde, which accumulates when CAD activity is reduced. Metabolic blocks at other steps in the lignin biosynthetic pathway also produce the *bm* phenotype, suggesting that other accumulated intermediates in the pathway, when polymerized, produce a red-brown phenotype also.

Transgenic tobacco have been produced that overexpress an anionic peroxidase believed to be involved in lignification (Lagrimini et al., 1990). Pith sections cut from transgenic plants overexpressing the anionic peroxidase

browned more rapidly than controls (Lagrimini, 1991). The anionic peroxidase isozyme is implicated by these results in cross-linking of cell-wall polymers, but evidence for a role in lignin formation is still inconclusive. It may be that redundancy in the peroxidase family will compensate for changes in "lignin-specific" peroxidase isoforms. Alternatively, it may be that a lignin-specific peroxidase has not yet been identified.

PROSPECTS FOR THE GENETIC MODIFICATION OF LIGNIN

To date, few transgenic plants have been produced with modified lignin. So far, the results obtained with genetically engineered plants with altered lignin have been largely unpredictable. For example, antisense *CAD* has no effect on morphology in transgenic tobacco. Only two of about 100 transformed lines have sufficiently reduced CAD activity to affect lignin extractability; however, lignin content was unchanged (Halpin et al., 1994). Paradoxically, antisense transformants with an *OMT* gene showed little effect on the ratio of syringyl/guaiacyl units in lignin but had modified lignin content (Ni et al., 1994). *PAL* transformants, on the other hand, showed suppression of lignin content and, as might be expected, had pleiotropic effects on the plants (Elkind et al., 1990). Plants expressing antisense peroxidase did not have lower lignin content but did exhibit some pleiotropic effects. Transgenic plants created by antisense or sense suppression may have pleiotropic effects on related genes in multigene families or on genes of different functions.

What is needed to advance the objective of directed modification of lignin? First, a combination of genetic and biochemical experiments is needed to define the steps that are necessary and sufficient for lignification. It will also be necessary to learn to what extent the pathway varies in different tissues, in different plants, and under different environmental or developmental circumstances. We also need to increase our understanding of the mechanisms of regulation and metabolic control of lignin biosynthesis. It is also necessary to define the extent to which lignin can be modified in content and quality without compromising important aspects of development, physiology, and stress compensation. Other phenolic compounds that are derived from the same biosynthetic route as lignins play important roles in plant development (Binns et al., 1987; Lynn et al., 1987). Therefore alterations in this pathway may have pleiotropic effects like those observed with PAL sense suppression in transgenic tobacco. It therefore becomes neces-

Table I. *Summary of mutations or conditions that modify lignin content or composition*

Mutation or Stimulus	Species or Tissue	Enzyme(s)	Lignin Content	Phenolic Composition	Reference
bm1	Maize		Minus 18%	Decreased *p*-coumarate	Kuc et al., 1968
bm2	Maize		Minus 30% Increased S:G ratio	Decreased *p*-coumarate	Chabbert et al., 1994b
bm3	Maize	OMT	Minus 12%	Increased cinnamic acids	Chabbert et al., 1994a; Vignols et al., 1995
bm4	Maize		Minus 8%	Decreased syringaldehyde	Kuc et al., 1968
bmr6	Sorghum	CAD	Reduced	Increased aldehydes Increased ferulate Decreased *p*-coumarate	Pillonel et al., 1991
bmr12/18	Sorghum		Reduced	Reduced syringyl moieties Decreased *p*-coumarate	Akin et al., 1986
bmr	Pearl millet		Minus 20%	Decreased *p*-coumarate Increased guaiacyl units	Cherney et al., 1991
fah-1	Arabidopsis	F5H		Reduced S:G ratio Reduced sinapate derivatives	Chapple et al., 1992
Antisense OMT	Tobacco	OMT	Minus 12%	S:G ratio unchanged	Ni et al., 1994
Antisense CAD	Tobacco		(none)	More aldehyde monomers	Halpin et al., 1994 Increased extractibility
Compression wood	Conifers		Increased	Increased *p*-hydroxyphenyl lignin	Timell, 1986
Juvenile wood	Conifers		Increased		Zobel and van Buijtenen, 1989
Elicitor	Pine cells	PAL, 4CL, OMT, CAD, β-glucosidase, peroxidase isoforms	Induced	Increased phenolics	Campbell and Ellis, 1992a, 1992b, 1992c
Plant growth regulators	Excised *Zinnia* mesophyll cells	PAL, 4CL, CCoAOMT, peroxidase isoforms	Induced	Lignification	Ye et al., 1994; Fukuda and Komamine, 1982; Church and Galston, 1988

sary to know more about the regulation of gene expression to identify tissue-specific promoters or tissue-specific regulatory factors that could suppress or enhance gene expression in tissues of interest without having an impact on other tissues or environmental responses. Additionally, it may be more important to modify the composition of lignin to improve its properties rather than simply to reduce content.

CONCLUSIONS

Modulation and variation in lignin content and composition has been studied in a wide variety of species, in natural mutations, and transgenic plants (Table I). These cases provide the basis for strategies of modification of lignin content and composition through genetic engineering. This directed modification of lignin is likely to proceed through a series of steps, continuing to provide new information and novel phenotypes related to lignin biosynthesis. An important barrier to application continues to be the inadequate gene-transfer systems for many of the most commercially important species, including many grasses and woody plants. The large scale of the industrial uses of many plant products will provide economic incentives even for small improvements in quality through modification in lignin. We are on a threshold of great advances in both understanding and application related to variation of lignin biosynthesis.

NOTE ADDED IN PROOF

Following the preparation of this manuscript, Atanassova et al. (1995) reported results on the characterization of transgenic tobacco plants expressing COMT sequences in sense and antisense orientations. Plants that had 2 to 12% of normal COMT activity had normal lignin content, but showed a marked decrease in syringyl units and the novel appearance of 5-hydroxy guaiacyl units. These results support the view that there is an alternative pathway for the synthesis of guaiacyl units in lignin, and provide more support for the feasibility of genetic engineering of the lignin biosynthetic pathway in commercially important species.

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