J Wood Sci (2006) 52:2–8
DOI 10.1007/s10086-005-0790-z

REVIEW ARTICLE

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Look back over the studies of lignin biochemistry

Received: October 19, 2005 / Accepted: November 30, 2005 / Published online: February 1, 2006

Abstract The role of the cinnamate pathway in monolignol biosynthesis based on feeding experiments with lignifying plant stems and characterization of the enzymes in the pathway, *O*-methyltransferase (OMT), cinnamyl alcohol dehydrogenase (CAD), etc. is discussed. Monolignol biosynthesis via metabolic grids according to newly characterized enzymes in the pathway is also reviewed and discussed. The cleavage mechanisms of side chains and aromatic rings by lignin peroxidase and laccase elucidated by using ^{18}O, ^{2}H, and ^{13}C labeled lignin substructure dimers and DHP with $^{18}O_2$ and $H_2{}^{18}O$ are reviewed. Finally, the prospects of lignin biochemistry in the wood and paper industries are discussed according to the recent progress on gene technology on wood formation and microbial degradation of lignin.

Key words Lignin biochemistry · Monolignol biosynthesis · Microbial degradation of lignin

Introduction

Lignin is generally distributed with hemicelluloses in the spaces of intercellulose microfibrils in primary and secondary walls, and in middle lamellae as an adhesive component to connect cells and reinforce the cell walls of xylem tissues. The evolution of woody vascular plants on the earth could be ascribed to acquisition of lignin by the plants; lignin plays a key role to support the growth of large and tall vascular plants.

Lignins are generally classified into three major groups, guaiacyl lignin in softwoods (gymnosperms), guaiacyl–syringyl lignin in hardwoods (angiosperms), and guaiacyl–syringyl–*p*-hydroxyphenyl lignin in grasses (gramineae), based on their monomeric units. Softwood lignin is a three-dimensional heterogeneous polymer in which the monomeric guaiacylpropane units (>90%) are connected by both ether and carbon–carbon linkages: several substructures are involved in the lignin macromolecules, of which guaiacylglycerol-β-aryl ether is the most abundant interphenylpropane linkage (40%–60%), followed by the substructures, phenylcoumaran (10%), dibenzodioxin (10%), diarylpropane (<5%), pinoresinol (<5%), biphenyl (5%–10%), diphenyl ether (5%), etc.

The lignin of hardwoods is composed of guaiacylpropane and syringylpropane units connected by linkages similar to those found in conifer lignin; the ratio of the syringyl unit to the guaiacyl unit (1~3) is different among species. Grass lignin is composed of guaiacylpropane, syringylpropane, and *p*-hydroxyphenylpropane units also connected by similar linkages to those found in softwood lignin. *p*-Coumaric acid (5%–10% of lignin) is mostly esterified at the γ-position of the propyl side chains of the lignin. The lignin content of the woody stems of softwoods, hardwoods, and grasses (bamboo, wheat, etc) ranges from 15% to 36%.

As shown in Fig. 1, lignin is a three-dimentional polymer connected by several acid-resistant C-C linkages, is only partly degraded to monomeric compounds by hydrolysis, and is mostly degraded by oxidation. This is in contrast to other native polymers such as protein, cellulose, hemicelluloses, and starch, which are generally hydrolyzed to monomeric units in 100% yield.

These results indicate that the wood is a unique biological material in which cellulose fibers are chemically connected and covered with lignin to give a material that is strongly resistant against pathological and natural damage.[1–4]

Overview of lignin biosynthesis

From 1955 to 1963 Brown,[5] Brown and Neish,[6,7] Higuchi,[8,9] and Higuchi and Brown[10,11] started biosynthetic studies to

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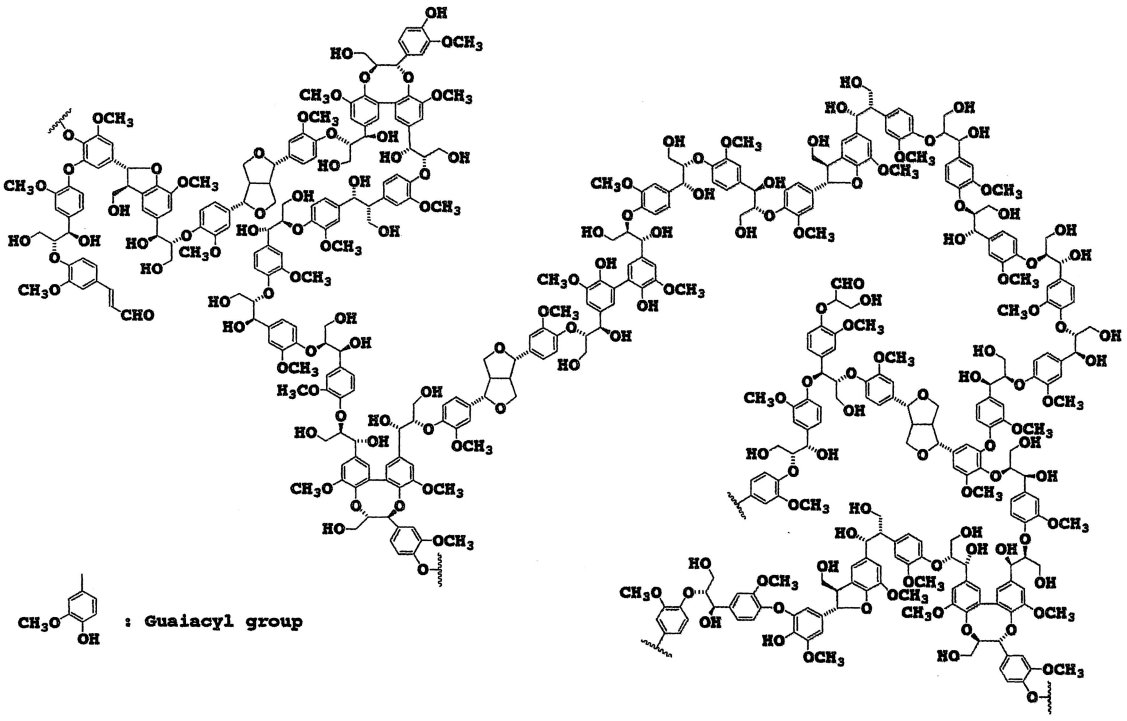


Fig. 1. A structural model of softwood lignin (guaiacyl lignin)

elucidate the biochemical nature of lignin, and established the pathway of lignin biosynthesis from glucose, shikimic acid, L-phenylalanine, and cinnamic acids (the cinnamate pathway). This work was based on feeding experiments with ^{14}C-labeled precursors using heading wheat plants, young twigs, and tissue cultures of softwoods and hardwoods. Since then, lignin biosynthetic study has been followed by the isolation and characterization of the respective enzymes involved in each reaction step in the cinnamate pathway.

Methylation of hydroxycinnamic acids

Two methylation steps to yield ferulate and sinapate are involved in the cinnamate pathway. In these reactions the *m*-phenolic hydroxyl group of the substrate is converted to a methoxyl group by the mediation of *O*-methyltransferase (OMT) with *S*-adenosylmethionine acting as the methyl group donor. To elucidate the formation of methoxyl groups of lignins, Higuchi[12,13] studied the characterization of OMTs of various woody plants such as bamboo shoots, young stems of poplar trees, and pine seedlings.

The purified enzyme of bamboo shoots efficiently catalyzed the methylation of caffeate, 5-hydroxyferulate, and 3,4,5-trihydroxycinnamate to ferulate, sinapate, and 5-hydroxyferulate and sinapate, respectively, with *S*-adenosylmethionine. On the other hand, the enzyme isolated from pine seedlings preferentially catalyzed ferulate formation from caffeate but not the formation of sinapate from 5-hydroxyferulate.

Subsequent comparative studies on the substrate specificities of OMTs from gymnosperms and angiosperms showed that both caffeate and 5-hydroxyferulate are very good substrates, and 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuic aldehyde, and chlorogenate are also good substrates for angiosperm OMTs. The substrate specificity of the gymnosperm OMTs, on the other hand, was completely different, and caffeate was the most favorable substrate, followed by protocatechuic aldehyde and 3,4-dihydroxyphenyl acetate. However, 5-hydroxyferulate was a poor substrate for gymnosperm OMTs.

The relationship between the ratio of sinapate to ferulate (SA/FA) formed by OMTs, the ratio of syringaladehyde to vanillin (S/V) on alkaline nitrobenzene oxidation of the lignins, and the Mäule reaction showed that the plants with higher SA/FA ratio, such as angiosperms, give the greater S/V ratio and a positive Mäule reaction. On the other hand, plants with lower SA/FA ratio, such as conifers and ferns, give a lower S/V ratio and negative Mäule reaction. The result indicated an intimate correlation between the evolution of lignin biosynthesis and the two types of OMTs. The methylation of caffeate to ferulate by angiosperm OMTs was competitively inhibited by 5-hydroxyferulate, indicating a preferential formation of syringyl lignin in angiosperms. The purified OMT from Japanese black pine seedlings, on the other hand, mainly catalyzed the formation of ferulate. K_m values for caffeate and 5-hydroxyferulate were 0.05 mM and 2.8 mM, respectively, and sinapate formation was competitively inhibited by caffeate, indicating a preferential formation of guaiacyl lignin in conifers.

It is remarkable in relation to plant phylogeny that the angiosperm OMT is a difunctional enzyme that catalyzes the formation of both sinapate and ferulate from caffeate and 5-hydroxyferulate, and favors the formation of syringyl lignin, whereas gymnosperm and fern OMTs are essentially monofunctional and preferentially catalyze the formation of ferulate to give guaiacyl lignin.

Reduction of *p*-hydroxycinnamates to *p*-hydroxycinnamyl alcohols (monolignols)

With the use of tracer experiments, Higuchi and Brown[11] first found that ferulate was converted to coniferyl aldehyde and coniferyl alcohol. The following enzyme studies by Mansell et al.[14] showed that *p*-hydroxycinnamates are converted to the corresponding alcohols via *p*-hydroxycinnamaldehydes. Ferulate and sinapate were reduced to the corresponding cinnamyl alcohols by successive mediation of three enzymes, hydroxycinnamate : CoA ligase (4CL), hydroxycinnamoyl-CoA reductase (CCR), and hydroxycinnamyl alcohol oxidoreductase (CAD). However, the 4CL isolated from *Forsythia* and *Brassica* was not effective for sinapate. On the other hand, 4CL from soy bean cell cultures was separated into two isozymes by Ebel and Grisebach.[15] Isozyme 1 was found to have relatively low K_m and high V/K_m values for the three typical lignin precursors, *p*-coumarate, ferulate, and sinapate.

Kutsuki et al.[16] and Kutsuki[17] also indicated that most angiosperm and gymnosperm 4CLs are active with ferulate but not with sinapate. However, interestingly 4CLs of bamboo shoots, *Robinia pseudoacacia*, and *Erythrina crista-galli* were considerably active with sinapate.

Hydroxycinnamoyl-CoAs are reduced to the corresponding aldehydes by *p*-hydroxycinnamoyl-CoA reductase (CCR). The enzyme is specific for cinnamoyl-CoAs.

The last step in the formation of *p*-hydroxycinnamyl alcohols is the reduction of *p*-hydroxycinnamyl aldehydes to the corresponding alcohols mediated by CAD. Kutsuki et al.[18] showed that the angiosperm enzymes reduced both coniferyl and sinapyl aldehydes to the corresponding alcohols almost equally, but the gymnosperm enzymes were remarkably specific for the reduction of coniferyl aldehyde. The purified enzyme of Japanese black pine showed that the K_m to NADPH and coniferyl aldehyde were 6.8 μM and 9.1 μM respectively. The V_{max} to sinapyl aldehyde was only 2.2% of that for coniferyl aldehyde. We, therefore, presumed that CAD is one of the regulating enzymes like OMT, which controls the formation of guaiacyl and syringyl lignins. The enzyme is specific for cinnamyl aldehyde derivatives and is distributed in several organs of vascular plants, and is especially active in the cambial zone. Thus, all enzymes involved in the formation of monolignols of guaiacyl, syringyl, and *p*-hydroxyphenyl lignins have been characterized during the past few years after the feeding experiments.

Higuchi and Ito[19] also found that both peroxidase and laccase are involved in the dehydrogenative polymerization of monolignols into the corresponding polymers, and proposed that peroxidase, which is widely distributed in higher plants, is involved in the dehydrogenative polymerization of monolignols into lignins.

Pathways for monolignol biosynthesis via metabolic grids

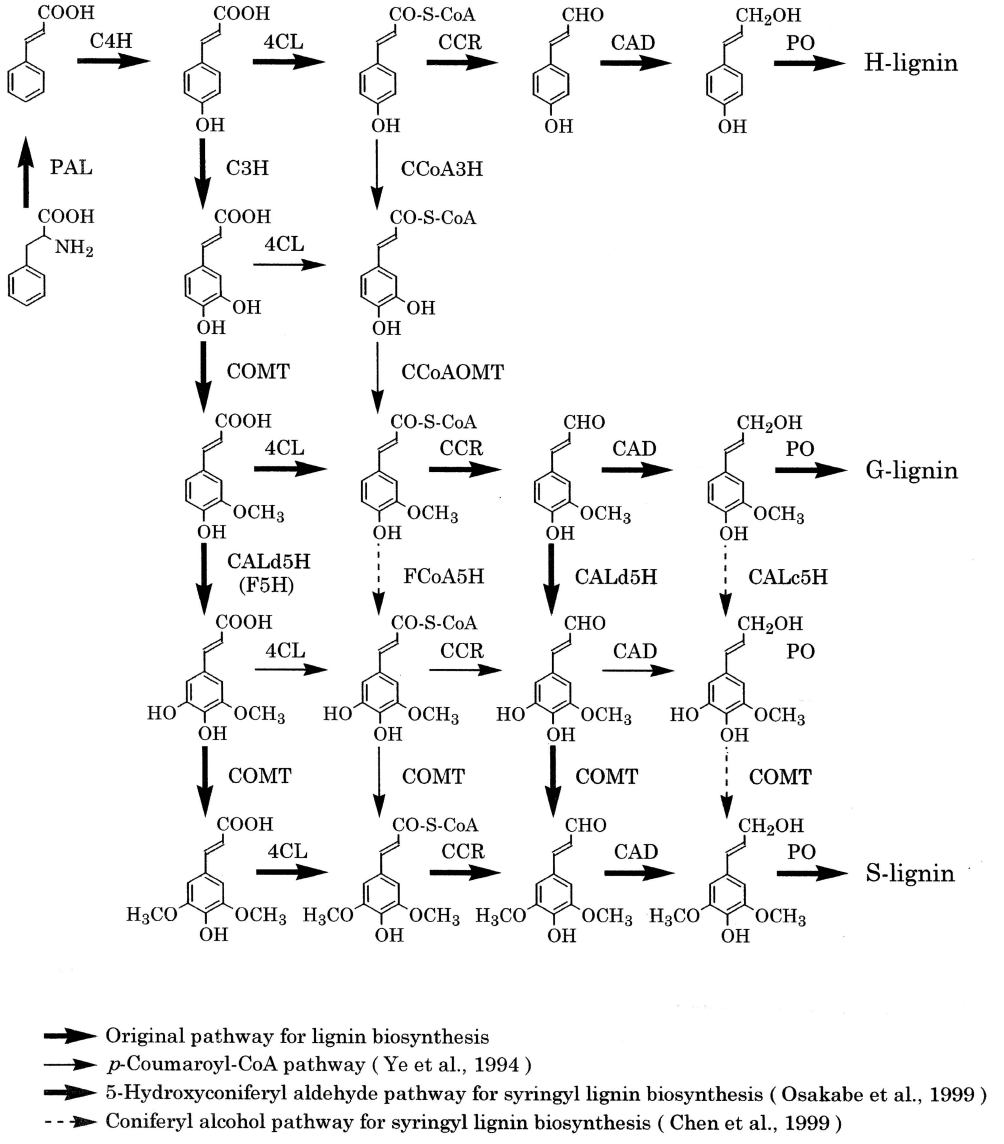
The original pathway proposed, based on the results of the feeding experiments with heading wheat, indicated that cinnamic acid formed from L-phenylalanine by the mediation of phenylalanine ammonia-lyase (PAL) is converted to *p*-coumarate, caffeate, ferulate, 5-hydroxyferulate, and sinapate, successively. *p*-Coumarate, ferulate, and sinapate are converted to the corresponding aldehyde and then monolignols, respectively. In grasses, *p*-coumarate is formed directly from L-tyrosine by the mediation of tyrosine ammonia-lyase (bifunctional phenylalanine ammonia-lyase)[20] and by hydroxylation of cinnamate.

However, during the late 1980s and into the mid-1990s several new enzymes involved in the cinnamate pathway were isolated and characterized. Schoch et al.[21] found that 3-hydroxylation of *p*-coumarate mostly occurs via *p*-coumaroyl shikimate: *p*-coumaroyl shikimate is then converted to caffeoyl-CoA via caffeoyl shikimate. In addition, caffeoyl-CoA OMT specific for caffeoyl-CoA, and 5-hydroxyferuloyl-CoA were newly identified by Ye et al.[22] and Zhong et al.[23] The enzymes were found to be involved in the formation of guaiacyl and syringyl lignins by analyses of the down-regulated recombinant plant for the OMT.

Coniferyl aldehyde 5-hydroxylase (CALd5H) was isolated and characterized by Chiang's group[24] from sweet gum. The enzyme isolated from the recombinant sweet gum mediated the 5-hydroxylation of both ferulate and coniferyl aldehyde, but the hydroxylation of ferulate was inhibited by coniferyl aldehyde. They also found that the methylation of 5-hydroxyferulate and caffeate was inhibited by 5-hydroxyconiferyl aldehyde. Based on these results, they suggested that the main role of F5H originally found by Grand[25] as ferulate 5-hydroxylase could be the hydroxylation of conyferyl aldehyde to 5-hydroxyconiferyl aldehyde. They also suggested that the main role of COMT, which is known to catalyze the formation of ferulate and sinapate, could be the methylation of 5-hydroxyconiferyl aldehyde to sinapyl aldehyde.

The Chapple group[26,27] also isolated and characterized an enzyme from *Arabidopsis* that mediates the 5-hydroxylation of both coniferyl aldehyde and coniferyl alcohol. The Fukushima group found that deuterium-labeled coniferyl alcohol fed to robinia and oleander is converted to sinapyl alcohol in accordance with the 5-hydroxylase from *Arabidopsis*.[28]

Based on the characterization of newly found enzymes, the original cinnamate pathway has been moderately modified as grid pathways shown in Fig. 2.[29] The Fukushima group recently found that heptadeuterosinapate (8-D,3,5-OCD_3) fed to shoots of robinia and oleander trees was



PAL : phenylalanine ammonia-lyase
C4H : cinnamate 4-hydroxylase
4CL : 4-coumarate:CoA ligase
CCR : cinnamoyl-CoA reductase
CAD : cinnamyl alcohol dehydrogenase
PO : peroxidase
C3H : 4-coumarate 3-hydroxylase
COMT : caffeate *O*-metyltransferase
F5H : ferulate 5-hydroxylase
CCoA3H : cinnamoyl-CoA 3-hydroxylase
CCoAOMT : cinnamoyl-CoA *O*-metyltransferase
FCoA5H : feruloyl-CoA 5-hydroxylase (not identified yet)
CALd5H : coniferyl aldehyde-5-hydroxylase
CALc5H : coniferyl alcohol-5-hydroxylase (not identified yet)
H-lignin : *p*-hydroxyphenyl lignin
G-lignin : guaiacyl lignin
S-lignin : syringyl lignin

Fig. 2. Multiple pathways for monolignol biosynthesis via metabolic grids

incorporated into syringyl lignin supporting the original cinnamate pathway in that sinapate is converted to sinapyl alcohol via sinapoyl-CoA.[30] They further found that crude extracts of differentiating xylem or stems from these plants clearly showed 4CL activity for sinapate in agreement with the original cinnamate pathway. In contrast, magnolia and *Arabidopsis* 4CL activity toward sinapate could not be detected, and the labeled sinapate was not incorporated into their lignin. These results show that syringyl lignin biosynthesis in angiosperms operates via multiple pathways that depend on the plant species. Further investigations are necessary to determine whether syringyl lignin is synthesized via the original cinnamate pathway or grid pathways by checking phylogenetically different angiosperm plants.

Henriksson and coworkers[31] recently proposed that in polymerization of monolignols to lignin, manganese oxalate works as a diffusible redox shuttle; first Mn^{II} is oxidized to

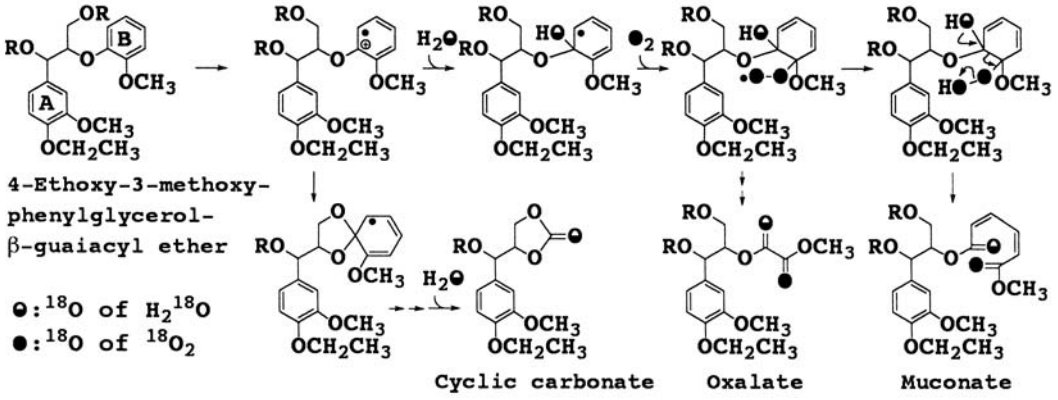


Fig. 3. Mechanism of aromatic ring cleavage of *β-O*-4 lignin substructure models by LiP of *Phanerochaete chrysosporium*

Mn^{III} by a peroxidase and is then reduced to Mn^{II} by a simultaneous oxidation of monolignols to the radicals that formed covalent linkages of the lignin.

Microbial degradation of lignin

As mentioned above, lignin is a unique heterogeneous polymer that is only slightly degraded by hydrolysis but is well degraded by oxidation. The main cleavage mechanisms of side chains and aromatic rings of lignin model compounds and synthetic lignin (DHP) by white-rot fungi and their enzyme lignin peroxydase (LiP) and laccase (LA) have been elucidated by Higuchi and coworkers[32–37] using ^{2}H, ^{13}C, and ^{18}O-labeled lignin substructure dimers with $^{18}O_2$ and $H_2{}^{18}O$ (Fig. 3).

It was shown that the side chain and aromatic rings of these substrates were oxidatively cleaved via aryl cation radical and phenoxy radical intermediates in reactions mediated by LiP/H_2O_2, and LA/O_2/mediator. Hydrogen peroxide is only required for the conversion of native LiP and manganese peroxydase (MnP) into two electron-deficient reactive species (compound I). Compound I of LiP abstracts two electrons stepwise to yield aryl cation radicals or aryl cations, which are attacked by O_2 or nucleophiles such as H_2O and R-OH, respectively.

The subsequent reactions of the cation radicals and cations are not controlled by the enzyme just as non-enzyme-directed couplings of phenoxy radicals of monolignol in lignin biosynthesis. Thus, the role of LiP, LA, and probably MnP in lignin biodegradation could be explained by the following unifying view.

Enzymatic reaction

1. $LiP/H_2O_2 \rightarrow$ Phenoxy radicals of phenolic units, and aryl cation radicals or cation radicals of nonphenolic units
2. $LA/O_2 \rightarrow$ Phenoxy radicals of phenolic units
3. LA/O_2 + Mediators $\rightarrow$ Phenoxy radicals of phenolic units, and aryl cation radicals or cation radicals of nonphenolic units
4. $MnP/H_2O_2 + Mn^{2+} \rightarrow$ Phenoxy radicals of phenolic units
5. $MnP/H_2O_2 + Mn^{2+}$ + Mediators $\rightarrow$ Phenoxy radicals of phenolic units and aryl cation radicals or cation radicals of nonphenolic units

Nonenzymatic reaction

1. Homolytic or heterolytic cleavage of side chains (Cα-Cβ, alkyl-phenyl), and aromatic rings
2. O_2 attack on carbon-centered radical intermediates
3. Nucleophilic attack on aryl cations and Cα cations by H_2O and R-OH $\rightarrow$ degradation products

Prospects of lignin biochemistry

Recently the large-scale generation of expressed sequence tags (ESTs) from xylem tissues has progressed for loblolly pine,[38] poplar,[39] *Arabidopsis*,[40] and *Zinnia*.[41] The lignin transcriptional profiling using microarray technology of poplar genes in the different developmental stages of wood-forming tissues has identified genes encoding the enzymes involved in lignin, hemicelluloses, and cellulose biosynthesis as well as other potent regulators of xylogenesis. Elucidation of the mechanism of lignin biosynthesis at the molecular level should provide fundamental information on xylem differentiation in woody plants.[42] It is considered that wood biotechnology greatly improves the quality and quantity of wood components for industrial use. Preparation of lignin-free plants by biotechnology has been conducted with the aim to obtain easily digestible cattle feeds. For example, 4-hydroxycinnamate:CoA ligase (4CL) down-regulated aspen by biotechnology exhibited a 45% reduction of lignin but 15% increase in cellulose.[43] The growth rate of hybrid aspen plants transformed with the horseradish peroxidase gene (HRP prxC1a) was substantially increased. The average stem length of transformed plants was 25% greater than that of control plants. The fast-growing hybrid aspen had elevated peroxidase activity. On the other hand, the transformed tobacco with transcription factor Ntlim1 showed considerably lower activities of PAL, 4CL,

and CAD, and the lignin content was 27% less than that of control plants.[44]

Lignin is nature's second-most abundant polymer behind cellulose, and its annual biosynthetic rate has been estimated to be approximately 2×10^{10}t.[45] Therefore, wood biotechnology for lignin biosynthesis leads to important economic benefits for utilization of wood materials in the pulp, paper, and fiberboard industries. The physical properties of wood could also be improved in near future by wood biotechnology for applications in house building and furniture.

Recent molecular investigations[46] on lignolytic enzymes have shown that *Phanerochaete chrysosporium* has two gene families including ten Lip-type and three MnP-type genes coding different isoenzymes expressed during secondary metabolism. Many ligninolytic peroxidase genes from other white-rot fungi, and two versatile peroxidase (VP) genes from *Pleurotus eryngii* have been cloned. Biochemical and biotechnological approaches to lignin biodegradation open up a new field in biomass conversion, such as biopulping, biobleaching, and treatment of Kraft bleaching effluents and related pollutants by lignin-degrading basidiomycetes and their enzymes.[47–50] The reader is referred to the review article by Boudet and Grima-Pettenati[51] for a discussion of molecular biology and engineering of lignin biosynthesis and biodegradation.

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