J Wood Sci (1999) 45:487–491
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ORIGINAL ARTICLE

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Structural conversion of the lignin subunit at the cinnamyl alcohol stage in *Eucalyptus camaldulensis*

Received: November 17, 1998 / Accepted: March 16, 1999

Abstract The lignin biosynthetic pathway in *Eucalyptus camaldulensis* was investigated by feeding stems with deuterium-labeled precursor. Pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol was synthesized and supplied to shoots of *E. camaldulensis*, and incorporation of the labeled precursor into lignin was traced by gas chromatography-mass spectrometry. In addition to the direct incorporation of labeled precursor into the guaiacyl unit, a pentadeuterium-labeled syringyl unit was detected. This finding indicates that the γ-deuterium atoms in the hydroxymethyl group of labeled coniferyl alcohol remain intact during modification of the aromatic ring. The relative level of trideuterium-labeled syringyl monomer (the result of conversion via the cinnamic acid pathway) was negligible, suggesting that the pathway at the monolignol stage is used for conversion of exogenously supplied precursor. Our results provide conclusive evidence of a novel alternative pathway for generation of lignin subunits at the monolignol stage even in plants that do not accumulate coniferin in lignifying tissues.

Key words Biosynthetic pathway · Coniferyl alcohol · *Eucalyptus camaldulensis* · Lignin · Sinapyl alcohol

Introduction

Woody angiosperm lignins are mainly a mixture of guaiacyl (G) and syringyl (S) units. Conversion of guaiacyl units to syringyl units is believed to occur at the level of cinnamic acids or the corresponding coenzyme A (CoA) esters.[1–3] In our previous studies, we demonstrated that when γ-^{3}H-labeled lignin precursors were fed to growing trees a considerable amount of radioactivity was found in lignin units other than those that corresponded to the administered precursors; hence, a new pathway for the biosynthesis of monolignol has been proposed.[4–6] To clarify this pathway, pentadeuterium-labeled precursor was administered to *Magnolia kobus*, and incorporation of the labeled precursor into lignin was traced by gas chromatography-mass spectrometry (GC-MS).[7] It was shown that when coniferyl alcohol was exogenously fed to magnolia conversion of coniferyl alcohol to sinapyl alcohol could occur at the monolignol level. A large amount of coniferin accumulated before the lignification season and the enzymes capable of cleaving glucosides exist in lignifying cells, suggesting that the monolignol synthetic pathway via cinnamyl alcohol might be important for regulating the lignin composition in *Magnolia*.

Cinnamyl alcohol is considered to be the direct precursor of lignin, but it is relatively toxic and does not accumulate to high levels within living plant cells.[8] The monolignol glucosides are considered to be the storage and transport form of monolignols.[8] The storage of monolignol glucosides in gymnosperm species and some angiosperm species might be used to regulate lignin biosynthesis.[8,9] The accumulation patterns and levels of glucosides may vary greatly among angiosperm species. The physiological functions of monolignol glucosides and their effect on lignin biosynthesis in these angiosperm species are not clear, and it has been suggested that only part of the lignin synthesis occurs via coniferin.[10] It is important to investigate whether the cinnamyl alcohol pathway exists in those angiosperm species that do not accumulate coniferin in high levels. To further investigate the lignin structural conversion at the monolignol stage in higher plants, *Eucalyptus camaldulensis* was used in this study and the incorporation pattern of the labeled precursor into lignin was traced by GC-MS.

Materials and methods

Synthesis of the precursor

Pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol was synthesized from 3,4-dihydroxybenzaldehyde by the method of

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Umezawa et al.[11] by reacting it with benzylbromide and then with CD_3I (min. 99.5 atom % D; Aldrich Chemical, Milwaukee, WI, USA). The 3,4-dihydroxybenzaldehyde was converted to 4-benzyloxy-3-[OCD_3] methoxybenzaldehyde, which was reacted with a 30% solution of HBr to give *O*-trideuteromethyl[OCD_3] vanillin. Then the *O*-trideuteromethyl[OCD_3] vanillin was condensed with monoethyl malonate. The ethyl *O*-trideuteromethyl[OCD_3] ferulate obtained was reduced with $LiAlD_4$ (min. 98 atom % D; Isotec, Miamisburg, Oh, USA) to give coniferyl alcohol. After purification by passage through a silica gel column (eluted with a mixture of equal volumes of ethyl acetate and hexane) and recrystallization from CH_2Cl_2: hexane, colorless crystals of the pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol were obtained. The ^{1}H-NMR spectrum of the labeled precursor was recorded (EX-270; JEOL, Tokyo, Japan) in dimethyl-d_6 sulfoxide with tetramethylsilane as the internal standard to confirm the identity of the final product. ^{1}H-NMR: 4.77 (1H, s, CγOH-H), 6.20 (1H, d, CβH), 6.45 (1H, d, CαH), 6.70–7.06 (3H, m, Ar-H), 9.00 (1H, s, Ar-OH). The proportion of pentadeuterium-labeled coniferyl alcohol in the precursor was more than 99%.

Plant material and administration of precursor

One-year-old shoots of a 5-year-old *Eucalyptus camaldulensis* tree that was growing on the campus of Nagoya University were used. The shoots were undergoing longitudinal and radial growth. The upper parts of the shoots were cut off in November 1997, and small pools were made at the top of the remaining stems. The pools were filled with 30 ml of an aqueous 10 mM solution of the precursor in a mixture of 1/15 M KH_2PO_4 and 1/15 M Na_2HPO_4 (4:6, v/v; pH 7.1). After the solution had been absorbed, the cut ends were sealed with Parafilm (American National Can, Greenwich, CN, USA). Each shoot was harvested after 6 weeks, and 100 μm thick tangential sections were cut from the surface of bark-free xylem with a sliding microtome, as described previously.[12] The sections were extracted exhaustively with benzene/ethanol (2:1, v/v) and hot water in a modified Soxhlet extraction unit before lignin analysis.

Analysis of lignin

The derivatization followed by reductive cleavage (DFRC) method of Lu and Ralph[13] was used to degrade the lignin: In a round-bottomed flask, 3 ml of a stock solution of acetyl bromide and acetic acid (20:80, v/v) was added to the sample (about 10 mg). The mixture was stirred gently at 50°C for 3 h, and the solvent was then completely removed by rotary evaporation below 50°C. The residue was dissolved in an acidic reduction solvent (dioxane/acetic acid/water 5:4:1, v/v), and zinc dust (50 mg) was added to the well-stirred solution. Stirring was continued for 30 min at room temperature. Then the mixture plus 10 ml of CH_2Cl_2 and 10 ml of a saturated solution of NH_4Cl that had been used to rinse the flask was transferred quantitatively into a separation funnel; about 0.1 mg of docosane dissolved in CH_2Cl_2 was added as an internal standard. The aqueous phase was adjusted to pH 2–3 with an aqueous 3% solution of HCl, and the whole mixture was extracted with CH_2Cl_2 (3×10 ml). The combined CH_2Cl_2 fraction was then dried over Na_2SO_4 and evaporated under a vacuum. The residue was acetylated in 1.5 ml CH_2Cl_2 (containing 0.2 ml acetic anhydride and 0.2 ml pyridine) for 40 min at room temperature. Excess acetylation reagent was removed completely by repeated azeotropic evaporation with ethanol under reduced pressure. Acetylated derivatives were dissolved in CH_2Cl_2 and subjected to gas chromatography (GC) and GC-MS analysis.

Analysis of monomeric products by GC and GC-MS

The acetylated derivatives were analyzed by GC and GC-MS. Gas chromatography was performed with a GC353 system (GL Sciences, Tokyo, Japan) equipped with a flame ionization detector (300°C) and a fused silica capillary column (60 m × 0.25 mm i.d.) (TC1; GL Sciences, Tokyo, Japan). The sample (3 μl) was injected with a moving-needle-type injector at 220°C. The carrier gas was nitrogen. The temperature was programmed to increase from 150° to 260°C at 3°C/min; after another 5-min interval the temperature was raised at 8°C/min to 300°C, where it was held for 30 min. Mass spectra were recorded at 70 eV with a Mstation JMS 700 mass spectrometer (JEOL, Tokyo, Japan) combined with a model HP 6890 gas chromatograph with a fused silica capillary column (30 m × 0.32 mm i.d.) (DB1; Hewlett Packard, Wilmington, DE, USA). The temperature was programmed to increase from 150° to 300°C at 3°C/min; the carrier gas was helium. The incorporation of labeled precursor was measured using the area ratios on each selected-ion-monitoring (SIM) chromatogram.

Determination of monolignol glucosides in *Eucalyptus camaldulensis*

Freshly cut shoot was separated into bark and wood fractions, which were placed in excessive amounts of hot 50% ethanol solution. The extracts were subjected to analysis by high-performance liquid chromatography (HPLC) on LC-6A (Shimadzu, Tokyo, Japan) equipped with a UNISIL PACK 5C-18 column (6 mm i.d. × 300 mm) (GL Sciences, Tokyo, Japan) and an ultraviolet (UV) detector (280 nm) according to the procedure described in the previous paper.[12]

Results and discussion

The three monolignols differ only in their patterns of substitution on the aromatic rings. A metabolic grid exists for modification of aromatic rings and reduction of side chains in the biosynthesis of monolignols; the potential routes (pathways) that might be involved in the regulation of these

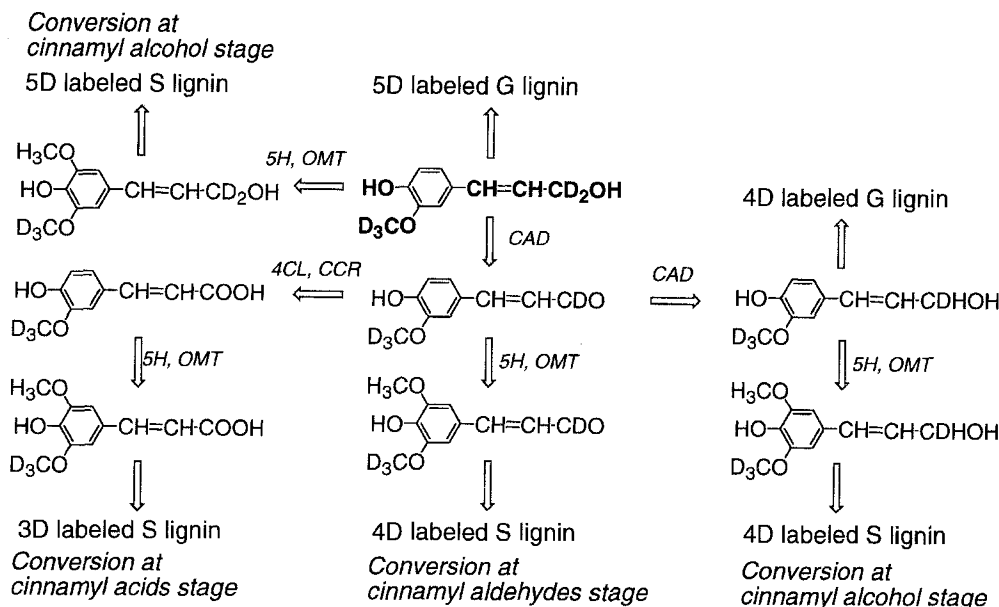

Fig. 1. Possible pathways for incorporation of a labeled precursor into guaiacyl and syringyl lignin. *CAD*: cinnamyl alcohol dehydrogenase; *5H*: 5-hydroxylase; *OMT*: *O*-methyltransferase; *CCR*: cinnamoyl-coenzyme A (CoA) reductase; *4CL*: 4-coumarate:CoA ligase

monolignols may be numerous. Studies on the behavior of γ-hydrogen atoms in lignin precursors during biosynthesis of monolignols should help us understand some aspects of this process. When pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol is fed to a growing plant, methoxylation of the aromatic ring may occur at various levels. Possible fates of the labeled coniferyl alcohol in newly formed xylem are shown in Fig. 1. Using the labeled precursor and tracing the distribution of isotope among lignin subunits, we tried to obtain information about the mechanisms of monolignol biosynthesis.

To further investigate whether the cinnamyl alcohol pathway exists in angiosperm species that do not accumulate high levels of coniferin, *Eucalyptus camaldulensis* was studied. As far as we know, there are no reports about the accumulation of coniferin during the lignification of eucalyptus. Our analysis results showed that no coniferin or syringin was detected in either the bark or wood fractions.

When labeled precursor is fed to a growing plant, it can be incorporated into newly formed xylem. To determine the most enriched region, a shoot that had been treated with pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol was freed of bark and continuously cut into tangential 100-μm sections on a sliding microtome. The sections were degraded using the DFRC method, and the relative labeling in G and S monomers in each section was analyzed by GC-MS. The incorporation pattern of labeled coniferyl alcohol in eucalyptus was similar to that found in magnolia.[7] Two peaks of incorporation, at 300 and 500 μm from the cambium, can be recognized (data not shown).

When the γ-position of the side chain of lignin precursor is labeled with deuterium atoms, the deuterium atoms are not released during dehydrogenative polymerization. Hence, such γ-labeled precursor can be used for tracing the details of lignin biosynthesis.[6] To obtain structural information about γ-labeled lignin, the DFRC method was used to degrade the lignins. The ether cleavage of lignins by the

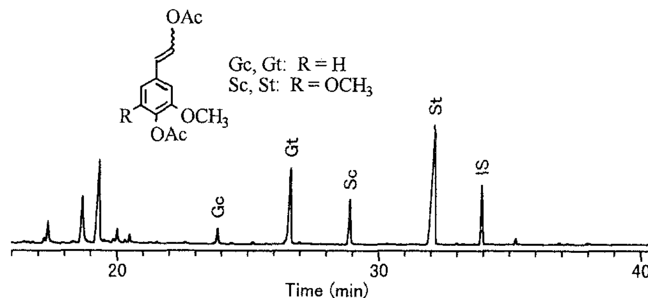

Fig. 2. Gas chromatogram of derivatization followed by reductive cleavage (DFRC)-derived monomers derived from the differentiating xylem of *Eucalyptus camaldulensis*. *Gc*, *cis*-4-acetoxyconiferyl acetate; *Gt*, *trans*-4-acetoxyconiferyl acetate; *Sc*, *cis*-4-acetoxysinapyl acetate; *St*, *trans*-4-acetoxysinapyl acetate; *IS*, internal standard (docosane)

DFRC method produces 4-acetoxycinnamyl acetate monomers (*trans* and *cis* isomers), which can be quantified by GC and GC-MS. The gas chromatogram of the products after DFRC treatment of a labeled cut section of eucalyptus sample is shown in Fig. 2. The major peaks are due to guaiacyl and syringyl units that are involved only in ether linkage. Figure 3 shows the mass spectra of G monomers derived from DFRC treatment of a control sample and a sample that had been fed with labeled coniferyl alcohol. The molecular ion peak for G monomer is at m/z 264 (4-acetoxyconiferyl acetate). The base peak (m/z 222) is due to the aromatic acetate losing a ketene (M-42). There is no interference at 226, 227. Because deuterium atoms on methoxyl groups are not prone to exchange during lignin biosynthesis, the relative ratios of 225/222, 226/222, and 227/222 can be viewed as the relative proportions of deuterated materials in the G unit. An examination of isotopic distributions allows us to characterize the deuterium labeling of lignin G unit. As we had anticipated, pentadeuterium-labeling (m/z 227) was conspicuous in the labeled sample,

Table 1. Relative proportions of deuterated materials in guaiacyl and syringyl units

Sample	222	223	224	225	226	227	252	253	254	255	256	257
Control	100	14.15	1.72	0.14	0	0	100	20.63	2.70	0.28	0.04	0
Ca[a]	100	12.37	1.45	0.17	1.68	5.39	100	20.77	2.75	0.32	0.11	0.21

Results are expressed as the percentage of labeled materials to natural guaiacyl or syringyl units measured at m/z 222 and 252, respectively
[a] CA: sample fed with pentadeuterium-labeled coniferyl alcohol

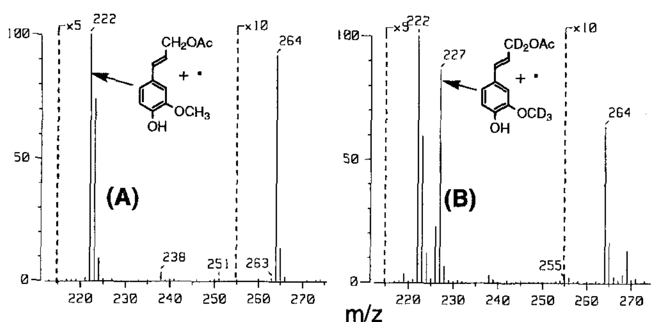

Fig. 3. Incorporation of deuterium-labeled lignin precursor into guaiacyl lignin in differentiating xylem of *Eucalyptus camaldulensis*. **A** Control, without feeding. **B** fed with pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol

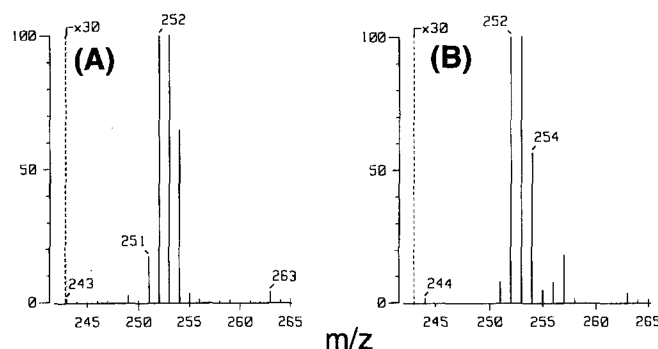

Fig. 4. Incorporation of deuterium-labeled lignin precursor into syringyl lignin in differentiating xylem of *Eucalyptus camaldulensis*. **A** Control, without feeding. **B** Fed with pentadeutero[γ,γ-D_2, OCD_3] coniferyl alcohol

indicating direct incorporation of the labeled precursor into G lignin. A considerable amount of tetradeuterated monomers (m/z 226) was found in G derivatives, whereas trideuterated monomers (m/z 225) remained at a low level (Table 1). Thus, part of the labeled precursor was reversed to cinnamyl aldehyde, releasing one γ-deuterium atom, and then returned to cinnamyl alcohol before being incorporated into lignin. Reversion of the labeled coniferyl alcohol to the corresponding acid or CoA ester seems unlikely. This result is in agreement with our previous results.[7] Figure 4 shows the mass spectra of S monomers in control and labeled samples. The base peak for the S monomer is at m/z 252 (M-42). In the labeled sample the peak at m/z 257 is relatively high, and the ratio of 257/252 is much higher than that of the control sample (Table 1). This is the unambiguous evidence of pentadeuterium labeling in the syringyl unit. The labeled coniferyl alcohol must have been converted to S lignin while retaining two γ-deuterium atoms.

There is the pool of coniferin in lignifying tissues of magnolia[14] but no pool of syringin; however, magnolia xylem has a considerable amount of syringyl lignin. We have reported that the administered coniferin could be converted to guaiacyl and syringyl lignin in magnolia at the monolignol stage.[7,8] These results and the fact that no syringin accumulates in magnolia xylem strongly support the idea that the pooled coniferin in the lignifying xylem tissues can be used as a precursor of syringyl lignin and guaiacyl lignin. It is also suggested that regulation of the G/S ratio in lignin can be attained even in the final stage – cinnamyl alcohols – of monolignol biosynthesis. This regulation system may be convenient for the plant that has a large pool of coniferin.

There was no information about this pathway for plants such as *Eucalyptus*, which has no pool of coniferin. In this study we showed that the cinnamyl alcohol pathway indeed exists in *Eucalyptus*. However, incorporation of the labeled precursor to S lignin was much lower (ca 0.2%, based on natural abundant moieties) than that in *Magnolia*[7] (ca. 1%), whereas incorporation to G lignin was nearly the same in both trees (ca. 5%). This finding suggests that involvement of the cinnamyl alcohol pathway in lignin biosynthesis of eucalyptus is relatively low.

Caffeic acid is methylated by caffeic 3-*O*-methytransferase (C-OMT) to yield ferulic acid, and the same enzyme is believed to catalyze the methylation of 5-hydroxyferulate to sinapate.[8] It has also been proposed that an enzyme distinct from C-OMT, caffeoyl-CoA 3-*O*-methyltransferase (CCoA-OMT), plays a role in the methylation of both caffeoyl-CoA and 5-hydroxyferuloyl-CoA.[2] Recently, a novel multifunctional *O*-methyltransferase, catalyzing the methylation of caffeic acid, 5-hydroxyferulic acid, caffeoyl CoA, and 5-hydroxyferuloyl CoA with similar specific activities, was identified in loblolly pine.[3] The enzyme involved in the cinnamyl alcohol pathway is not known. If methoxylation of coniferyl alcohol to sinapyl alcohol requires two steps (hydroxylation and methylation), as in the cinnamic acid pathway,[15] both coniferyl alcohol 5-hydroxylase and 5-hydroxylconiferyl alcohol *O*-methyltransferase might be needed to convert coniferyl alcohol to sinapyl alcohol. Characterization of this enzyme would contribute to our understanding of the role of this pathway in determining lignin monomer composition.

Our results suggest that the pathway at the monolignol stage is dominant in the conversion of exogenously supplied coniferyl alcohol to S lignin in eucalyptus. However, we cannot estimate the extent of involvement of this pathway in endogenous biosynthesis of lignin precursors at this time. A better understanding of the biosynthetic pathway of lignin will have important consequences with respect to the efforts to modify the lignification of plants.

Acknowledgments The authors thank Prof. J. Ralph (U.S. Dairy Forage Research Center, USDA, Madison, WI, USA) for the standard sample for GC analyses and Dr. M. Shibata (Kameyama Research Center, Forestry Research Institute, Oji Paper, Japan) for supplements of *Eucalyptus camaldulensis* seedlings. This study is partly supported by a Grant-in-Aid for Scientific Research (No. 90096280) from the Ministry of Education of Japan.

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