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The Behavior of Deuterium-Labeled Monolignol and Monolignol Glucosides in Lignin Biosynthesis in Angiosperms

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To examine the behavior of monolignol and monolignol glucosides in lignin biosynthesis, pentadeutero-[9-D_2, 3-OCD_3]coniferyl alcohol and pentadeutero[9-D_2, 3-OCD_3]coniferin were synthesized and fed to growing *Eucalyptus camaldulensis* and *Magnolia kobus*. The differences in the incorporation patterns of these labeled precursors were studied using gas chromatography−mass spectrometry (GC-MS). Both precursors were incorporated into lignin, but the labeled coniferyl alcohol was incorporated more directly, resulting in a high proportion of pentadeutero-labeled guaiacyl and syringyl units in newly formed xylem, while labeled coniferin tended to be incorporated in lignin as tetradeutero units, especially in syringyl lignin in both trees. However, the incorporation efficiencies of the precursors into syringyl lignin were higher in *Magnolia* than in *Eucalyptus*, and the ratios of tetradeutero to pentadeutero in guaiacyl lignin were lower in *Magnolia* than in *Eucalyptus* when the trees were fed coniferin.

INTRODUCTION

Woody angiosperm lignin consists mainly of guaiacyl (G) and syringyl (S) units. The conversion of a guaiacyl unit to a syringyl unit in an angiosperm has been believed to occur at the level of cinnamic acid (*1*). Alternatively, CoA esters may be key intermediates for substitution on the lignin aromatic ring (*2*). Recently, novel pathways for synthesis of syringyl lignin have been suggested, which hold that the conversion occurs later in the pathway (*3*). Tracer experiments (*4*, *5*) demonstrated a pathway via cinnamyl alcohol. Another novel pathway involving the cinnamaldehyde stage has been shown in enzyme studies (*6*−*8*). We reported that pentadeutero[9-D_2, 3-OCD_3]coniferyl alcohol was incorporated into syringyl lignin, while two deuterium atoms were retained at C9, suggesting that the change to S from G occurred at the cinnamyl alcohol stage (*5*). In that study, the incorporation of coniferyl alcohol into syringyl lignin was less efficient than into guaiacyl lignin. Cinnamyl alcohols are considered direct precursors of lignin, but high levels are not stored in living plant cells. Conversely, monolignol glucosides are thought to be the storage and transport forms of monolignols (*9*). In feeding experiments using radiolabeled coniferin, coniferin was efficiently incorporated into lignin (*10*, *11*). In this study, we examined the behavior of the lignin precursors coniferin and coniferyl alcohol in differentiating xylem. Angiosperms can be categorized into two groups based on those with and without a coniferin pool (*12*). We studied *Magnolia kobus* DC as a representative angiosperm with a coniferin pool and *Eucalyptus camaldulensis* as one lacking a pool. To obtain more detailed information at the molecular level, stable isotope-labeled coniferin was used to elucidate the behavior of monolignol glucoside in different angiosperms.

MATERIALS AND METHODS

Chemical Synthesis of Stable Isotope-Labeled Precursors. Pentadeutero[9-D_2, 3-OCD_3]coniferyl alcohol, **1** (**Figure 1**), was synthesized from 3,4-dihydroxybenzaldehyde using the method of Umezawa et al. (*13*) Pentadeutero[9-D_2, 3-OCD_3]coniferin, **2** (**Figure 1**), was synthesized from pentadeutero[9-D_2, 3-OCD_3]coniferyl alcohol using the method of Matsui et al. (*14*).

Plant Material and Administration of Precursors. The precursors were administered to *E. camaldulensis* and *M. kobus* DC trees growing on the campus of Nagoya University. The upper parts of 1 year old shoots of a 5 year old eucalyptus were cut off, and small depressions were made at the top of the remaining stem. The depressions were filled with 30 mL of an aqueous 2 mM solution of the precursors in a mixture of 0.067 M KH_2PO_4 and 0.067 M Na_2HPO_4 (4:6, v/v; pH 7.1). The same technique was applied using 2 year old shoots of a 10 year old magnolia. The precursors were administered to lignifying shoots of eucalyptus and magnolia in November and July, respectively. After the solution had been absorbed, the cut ends were sealed with Parafilm. After 6 weeks, each shoot was harvested and 100 μm thick tangential sections were cut from the surface of bark-free xylem with a sliding microtome. Two sections from each sample were subjected to lignin analysis.

Analysis of Lignin. The derivatization followed by reductive cleavage (DFRC) method of Lu and Ralph (*15*) was used to degrade

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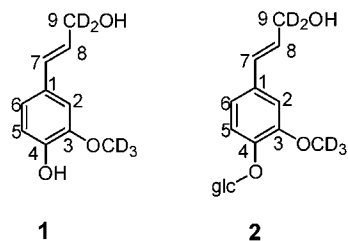† Nagoya University.
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Figure 1. Stable isotope labeled precursors; pentadeutero[9-D$_2$, 3-OCD$_3$]-coniferyl alcohol, **1**, and pentadeutero[9-D$_2$, 3-OCD$_3$]coniferin, **2**. The Arabic numerals in the chemical structures correspond to the order of carbon atoms.

the lignin. Acetylated derivatives were dissolved in CH$_2$Cl$_2$ and subjected to gas chromatography (GC) and gas chromatography−mass spectrometry (GC-MS) analysis.

GC and GC-MS Analysis of Monomeric Products. GC was performed with a GC353 system, equipped with a flame ionization detector (300 °C), and a 60 m × 0.25 mm i.d., TC1 fused silica capillary column (GL Sciences, Japan). Mass spectra were recorded at 70 eV with an MStation JMS 700 mass spectrometer (JEOL, Japan) combined with a model HP 6890 gas chromatograph with a 30 m × 0.32 mm i.d., DB1 fused silica capillary column (Hewlett-Packard, U.S.A.). The relative ratios of the 4D and 5D monomers of the DFRC products were determined using selected ion monitoring chromatograms at *m/z* 226 and 227 for G units and *m/z* 256 and 257 for S units.

RESULTS AND DISCUSSION

In this study, pentadeutero[9-D$_2$, 3-OCD$_3$]coniferyl alcohol, **1**, and pentadeutero[9-D$_2$, 3-OCD$_3$]coniferin, **2** (**Figure 1**), were administered to *E. camaldulensis* and *M. kobus* DC, and the incorporation patterns of labeled precursors were traced as DFRC lignin derivatives using GC-MS. Of potential lignin chemical analysis methods, the DFRC method is better suited for our strategy because it provides degradative products that give C$_6$−C$_3$ base ion peaks retaining C9-deuteriums on GC-MS. There were no differences in the DFRC product yields or the GC profiles of samples fed labeled precursors and the control (data not shown). The exogenously administered precursors were incorporated into lignin. **Figure 2** shows the mass spectra of G monomers derived from the samples fed labeled precursors. Predictably, pentadeuterium labeling (*m/z* 227) was conspicuous in each sample, indicating the direct incorporation of the labeled precursors into G lignin. Interestingly, considerable amounts of tetradeuterated monomers (*m/z* 226) were found in G derivatives from the eucalyptus stem fed labeled coniferin (**Figure 2b**). The S derivatives (**Figure 3**) also included tetradeuterated monomers (*m/z* 256), especially in the eucalyptus and magnolia fed coniferin (**Figure 3b,d**), in addition to pentadeuterated monomers (*m/z* 257). These results show that the pentadeuterated precursors administered to trees were partially oxidized to a cinnamaldehyde derivative, releasing one C9-deuterium atom, and then, they were converted back into cinnamyl alcohol before being incorporated into lignin (**Figure 4**). To estimate the molar ratios of tetradeutero (4D)- and pentadeutero (5D)-labeled units in both G and S derivatives in each sample, selected ion monitoring chromatogram (SIM) analysis was used (**Table 1**). When trees were fed the penta-deuterated coniferin, half of the labeled syringyl moieties were tetradeuterated moieties in both eucalyptus and magnolia, while only 30% of the syringyl moieties were tetradeuterated in plants fed the pentadeuterated coniferyl alcohol. These results show that when coniferyl alcohol is supplied to plants exogenously, it is incorporated into lignin more directly than coniferin. This confirms that coniferyl alcohol is a direct precursor of lignin.
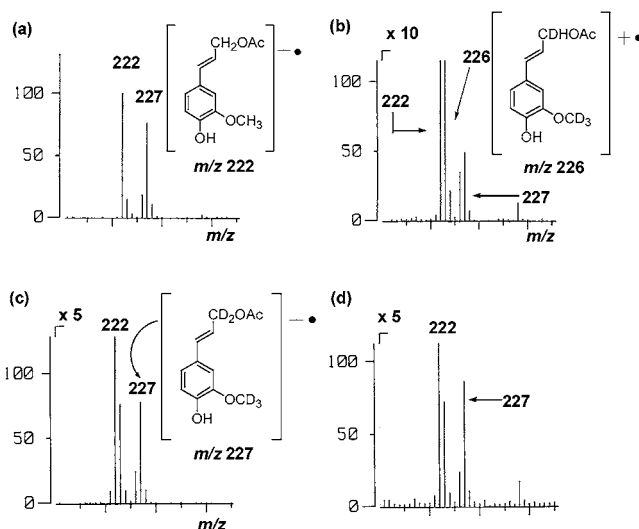

Figure 2. Partial EI mass spectra of DFRC products (G monomer) from (**a**) eucalyptus fed pentadeuterated coniferyl alcohol[9-D$_2$, 3-OCD$_3$], (**b**) eucalyptus fed pentadeuterated coniferin[9-D$_2$, 3-OCD$_3$], (**c**) magnolia fed pentadeuterated coniferyl alcohol[9-D$_2$, 3-OCD$_3$], and (**d**) magnolia fed pentadeuterated coniferin[9-D$_2$, 3-OCD$_3$]. *m/z* 222, nonlabeled G monomer; *m/z* 226, tetradeuterated (4D) G monomer; and *m/z* 227, pentadeuterated (5D) G monomer. The intensities of the vertical axis in **b–d** are magnified by 5- or 10-fold.
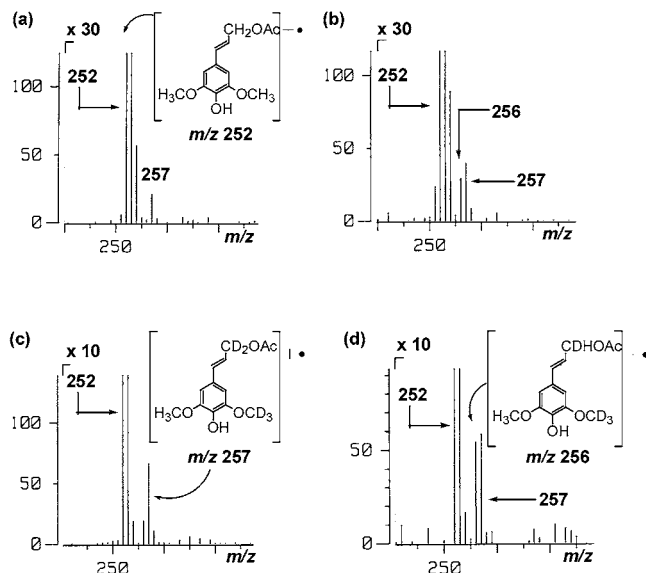

Figure 3. Partial EI mass spectra of DFRC products (S monomer) from (**a**) eucalyptus fed pentadeuterated coniferyl alcohol[9-D$_2$, 3-OCD$_3$], (**b**) eucalyptus fed pentadeuterated coniferin[9-D$_2$, 3-OCD$_3$], (**c**) magnolia fed pentadeuterated coniferyl alcohol[9-D$_2$, 3-OCD$_3$], and (**d**) magnolia fed pentadeuterated coniferin[9-D$_2$, 3-OCD$_3$]. *m/z* 252, nonlabeled S monomer; *m/z* 256, tetradeuterated (4D) S monomer; and *m/z* 257, pentadeuterated (5D) S monomer. The intensities of the vertical axis are magnified by 10- or 30-fold.

In other words, half of the pentadeuterated-labeled coniferin-[9-D$_2$, 3-OCD$_3$] incorporated into syringyl lignin is exchanged for tetradeuterated moieties [9-D, 3-OCD$_3$]. A considerable difference between eucalyptus and magnolia was the 4D:5D ratio of the guaiacyl moieties in the trees fed coniferin. In eucalyptus, the ratio was about 5:5, whereas in magnolia it was 2:3 to 3:7, which was similar to the ratio obtained with coniferyl alcohol feeding. To estimate the degree of uptake of the labeled precursors into lignin, the incorporation efficiencies were
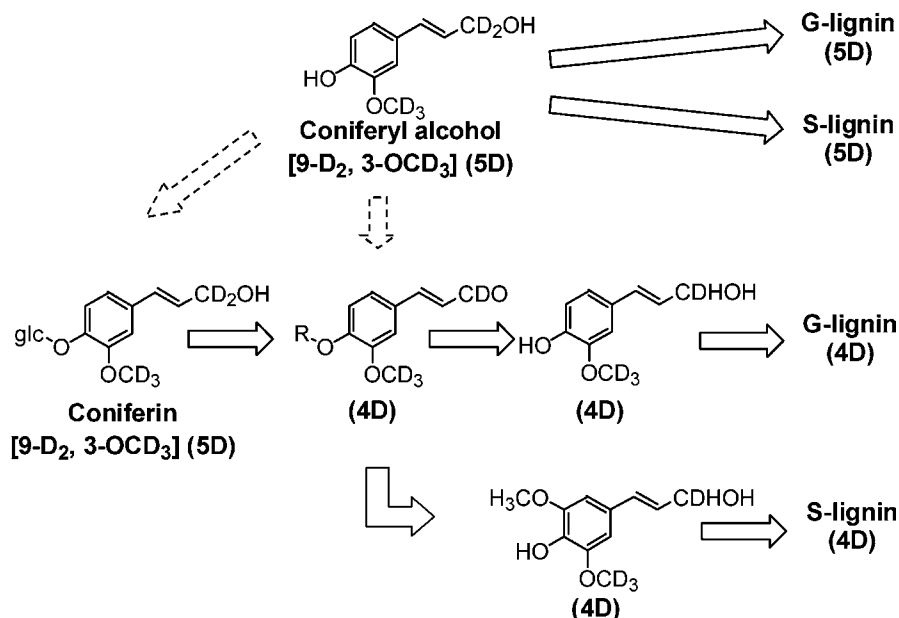
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Figure 4. Possible pathways for tetradeutero (4D)-labeled lignin biosynthesis from pentadeutero (5D)-labeled precursors. R = H or glc.

Table 1. Molar Ratio of 4D:5D-Labeled Lignin Subunits in DFRC Monomers and Incorporation Efficiency[a] of the Precursors into Lignin

	E. camaldulensis				M. kobus DC			
	G monomers (4D:5D)[f]	incorporation efficiency (%)	S monomers (4D:5D)[f]	incorporation efficiency (%)	G monomers (4D:5D)[f]	incorporation efficiency (%)	S monomers (4D:5D)[f]	incorporation efficiency (%)
CA-1[b,d]	22: 78	12.3	33: 67	0.69	26: 74	6.7	32:68	1.16
CA-2[b,e]	22: 78	7.5	32: 68	0.55	25: 75	8.3	32:68	1.76
CF-1[c,d]	45: 55	1.9	56: 44	0.58	17: 83	4.0	49: 51	1.49
CF-2[c,e]	43: 57	3.4	50: 50	0.41	28: 72	3.0	44: 56	0.95

[a] Each incorporation efficiency was calculated by the following formulas. The incorporation efficiency for G (%) = $(m/z\ 226 + m/z\ 227)/(m/z\ 222) \times 100$. The incorporation efficiency for S (%) = $(m/z\ 256 + m/z\ 257)/(m/z\ 252) \times 100$. [b] CA: the samples fed pentadeuterated [9-D2, 3-OCD3]coniferyl alcohol. [c] CF: the samples fed pentadeuterated [9-D$_2$, 3-OCD$_3$]coniferin. [d,e] 1,2: Two sections from each shoot fed the precursor were subjected to DFRC analysis. [f] 4D: the tetradeuterated derivatives from DFRC analysis; 5D: the pentadeuterated derivatives from DFRC analysis.

calculated using SIM data (**Table 1**). Within the same tree species, there was little difference in the incorporation efficiencies of the S monomer with coniferin and coniferyl alcohol feeding. However, the uptake into syringyl lignin was 1−1.8% in magnolia, whereas it was only 0.4−0.7% in eucalyptus, suggesting that magnolia metabolized more of the precursors into syringyl lignin than eucalyptus. By contrast, the uptake into guaiacyl lignins exceeded that into syringyl lignin in all of the samples, and there was no substantial difference between eucalyptus and magnolia. The incorporation of coniferyl alcohol into guaiacyl lignins was greater than that from coniferin. Because two sections were chosen for the DFRC analysis from newly formed xylem at a different stage in cell wall formation in each sample, each section had different S/G ratios (data not shown). Therefore, the differences observed between magnolia and eucalyptus shown in **Table 1** depend on the species, not on the degree of cell wall differentiation of the xylem.

The detection of significant amounts of tetradeutero lignin derivatives in the DFRC products from the trees fed pentadeuterated precursors suggests that some of the labeled precursors were oxidized to cinnamyl aldehyde or its glucoside, releasing a C9-deuterium atom, and then converted back into cinnamyl alcohol before polymerization into lignin. Alternatively, the oxidation and reduction might have occurred after polymerization. The latter hypothesis can be eliminated, since the 4D: 5D ratios in lignin, especially in S lignin, differed in the samples fed coniferin and coniferyl alcohol. If the conversion into 4D-labeled lignin units from 5D precursors occurred after poly-

merization, the ratios in the samples fed coniferin and coniferyl alcohol should be almost the same. The oxidation and reduction of pentadeuterated precursors are catalyzed by cinnamyl alcohol dehydrogenase, which is localized in the cytosol (*16*). Coniferin must be deglucosylated before it is taken up in lignin, and this is catalyzed by coniferin β-glucosidase, which is located in the cell wall (*17−19*). However, coniferin specific β-glucosidase has not been reported in angiosperm trees. We consider that this is because little research has examined β-glucosidase in angiosperm trees rather than the absence of its activity. The autoradiographic study using tritium-labeled coniferin showed that the coniferyl alcohol derived from the coniferin was incorporated into angiosperm lignin in magnolia, lilac, beech, and poplar (*20*). This strongly suggests that a β-glucosidase acted on the coniferin administered to these angiosperms. On the basis of these reports and the behavior of coniferin that we observed, it would be interesting to determine the substrate transport mechanism between the cell wall and the cytoplasm.

When eucalyptus and magnolia were fed coniferin, the proportion of 4D moieties reached 50% of all of the labeled moieties (4D + 5D), especially in syringyl lignin. By contrast, the proportion was only 30% in plants fed coniferyl alcohol. This implies that the "5D to 4D pathway" is centered on coniferin, not coniferyl alcohol. Some of the unincorporated pentadeuterated coniferyl alcohol might be stored as coniferin after glucosylation. Subsequently, this might be incorporated into lignin, causing the shift from 5D to 4D that could explain the observation of some 4D-labeled moieties with coniferyl

alcohol feeding. Interestingly, the total proportion of labeled syringyl monomers consisting of 4D-labeled monomers reached 50% when magnolia was fed the labeled coniferin. The percentage of 4D in the labeled guaiacyl lignin in magnolia fed coniferin was only 20−30%. The pathway from 5D to 4D clearly involves syringyl lignin biosynthesis rather than guaiacyl lignin biosynthesis. A recent lignin study found that the conversion of G to S occurs at the cinnamaldehyde stage (*6−8*). Consequently, half of the stored coniferin might be incorporated into S lignin via sinapaldehyde derived from coniferaldehyde that was synthesized from coniferin. Because the 4D and 5D units occurred equally in S lignin, the conversion pathway acting on the cinnamyl alcohol stage should be no less active than the pathway involving the aldehyde stage (*4, 5*). The presence or absence of a native coniferin storage pool could explain the difference in the incorporation efficiency of the precursors into syringyl lignin in magnolia and eucalyptus. The efficiency of incorporation into S lignin was less in eucalyptus than in magnolia (**Table 1**). Because magnolia has a native pool of coniferin, the possibility of converting excess coniferyl alcohol into coniferin and storing it for reuse should be higher than that in eucalyptus, which does not have a coniferin pool. Coniferyl alcohol glucosyl transferase activity has been detected in some angiosperms in which coniferin was not found (*21*). It remains unclear why many angiosperms have this activity when no coniferin pool is detected. Terazawa et al. suggested that these angiosperms acquired the system not to store monolignols and did not originally synthesize coniferin (*22*). However, there is no evidence that these angiosperms do not produce coniferin. It is possible that the tetradeutero (4D) unit detected in lignin in the shoot administered pentadeutero (5D)coniferyl alcohol is oxidized with the shift of "5D to 4D" via coniferin, even in eucalyptus. Moreover, the deuterium labels detected in the lignin of eucalyptus fed labeled coniferin suggest that a deglucosylation step could operate in vivo at any point before the polymerization in angiosperms with undetectable coniferin.

This study demonstrated that tetradeuterated lignin was biosynthesized in trees fed pentadeuterated coniferyl alcohol or coniferin and that this was more pronounced in the syringyl lignin from the trees fed coniferin rather than coniferyl alcohol. This suggests that there is greater control over the uptake of coniferin into lignin, as compared with coniferyl alcohol, especially into syringyl lignin. The mechanisms by which monolignols are stored and transported are not clear, and the behavior of coniferin and coniferyl alcohol in monolignol biosynthesis needs to be explored further.

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