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Behavior of Monolignol Glucosides in Angiosperms

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To examine the behavior of cinnamaldehyde and cinnamyl alcohol glucosides in lignifying tissues, angiosperms were subjected to tracer experiments using radioisotope-labeled and stable isotope-labeled glucosides. The aglycone from coniferaldehyde glucoside was efficiently incorporated into guaiacyl and syringyl lignin as a cinnamyl alcohol unit. The aglycone from coniferin was also incorporated into guaiacyl and syringyl lignin. However, some of the coniferin-derived aglycone that was incorporated into lignin passed through a cinnamaldehyde form prior to dehydrogenative polymerization. When coniferin was administered together with coniferaldehyde glucoside, syringyl units were rarely synthesized from coniferin via the cinnamyl alcohol stage, whereas numerous syringyl units were synthesized from coniferaldehyde glucoside. These observations suggest that the coniferaldehyde form is crucial for the biosynthesis of syringyl lignin in angiosperms.

KEYWORDS: Monolignol biosynthesis; monolignol glucoside; coniferaldehyde; tracer experiments; lignin; *Magnolia kobus*; *Nerium indicum*

INTRODUCTION

Lignin, a common secondary metabolite in vascular plants, plays an important role in conferring physical strength to plants, facilitating the transport of moisture (*1*), and protecting plants against the external environment (*2*). Biosynthetic studies of monolignol, a precursor for lignin synthesis, have revealed that the biosynthesis of lignin is regulated in a complex manner in lignifying tissues (*3*−*9*). There are many unresolved questions about the process of lignin biosynthesis, the answers to which would provide the means to synthesize and use lignin. At present, two major unknown factors in the biosynthesis of monolignol are (i) how a guaiacyl unit is converted to a syringyl unit and (ii) how monolignol is transported to the site of polymerization. Regarding the former factor, it is becoming widely accepted that the conversion of a guaiacyl to a syringyl unit proceeds via a cinnamaldehyde/cinnamyl alcohol stage (*10*−*15*). However, the precise mechanism is yet to be resolved. Regarding the latter factor, although the localization of enzymes that are involved in the biosynthesis of monolignol has been studied (*16*), more data are required to determine how and when monolignols are transported to the cell wall.

Coniferin is a putative storage form of monolignols (*18*). However, among woody plants, only gymnosperms and a small proportion of angiosperms contain a pool of endogenous coniferin. Therefore, it has been suggested that coniferin might not be involved in lignin biosynthesis. Although endogenous coniferin is found in a limited number of species, the lack of endogenous coniferin is not evidence that coniferin is not an intermediary metabolite that is involved in lignin biosynthesis. In recent studies, we prepared selective-labeled lignin precursors with isotopes as a strategy for tracing the molecular behavior of monolignol precursors during the process of lignification. The results of our study revealed that some of the coniferin that was administered to the shoots of magnolia and eucalyptus was oxidized to coniferaldehyde and rereduced to cinnamyl alcohol prior to being incorporated into lignin (*17*). In addition, the study in gymnosperm, *Ginkgo biloba* L., suggested that the aldehyde compound that resulted from coniferin by oxidation was probably coniferaldehyde glucoside (Tsuji et al., submitted). These results might imply a role of cinnamaldehyde form in monolignol biosynthesis. In light of the report that the transition to syringyl unit from guaiacyl unit proceeds in cinnamaldehyde stage (*13*−*15*), this is absorbing especially in lignin biosynthesis of angiosperm. Angiosperms evolved from gymnosperms, and it is thought that angiosperms that lack endogenous coniferin evolved an alternative system, by which lignin biosynthesis is regulated (*19*). Therefore, it is possible that the relatively small number of angiosperms that do contain a pool of endogenous coniferin might represent species that are in the process of evolving from gymnosperms (which contain coniferin) into angiosperms (which lack a pool of endogenous coniferin). Consequently, comparative studies of the behavior of coniferin in angiosperms that do, and do not, contain a pool of endogenous coniferin should shed some light on the process of the evolution of lignification. To obtain more information about the role of monolignol glucosides and cinnamaldehydes in lignification in angiosperms, we compared the behavior of cinnamaldehyde and cinnamyl alcohol glucosides in angiosperms that do, and do not, contain a pool of endogenous coniferin.

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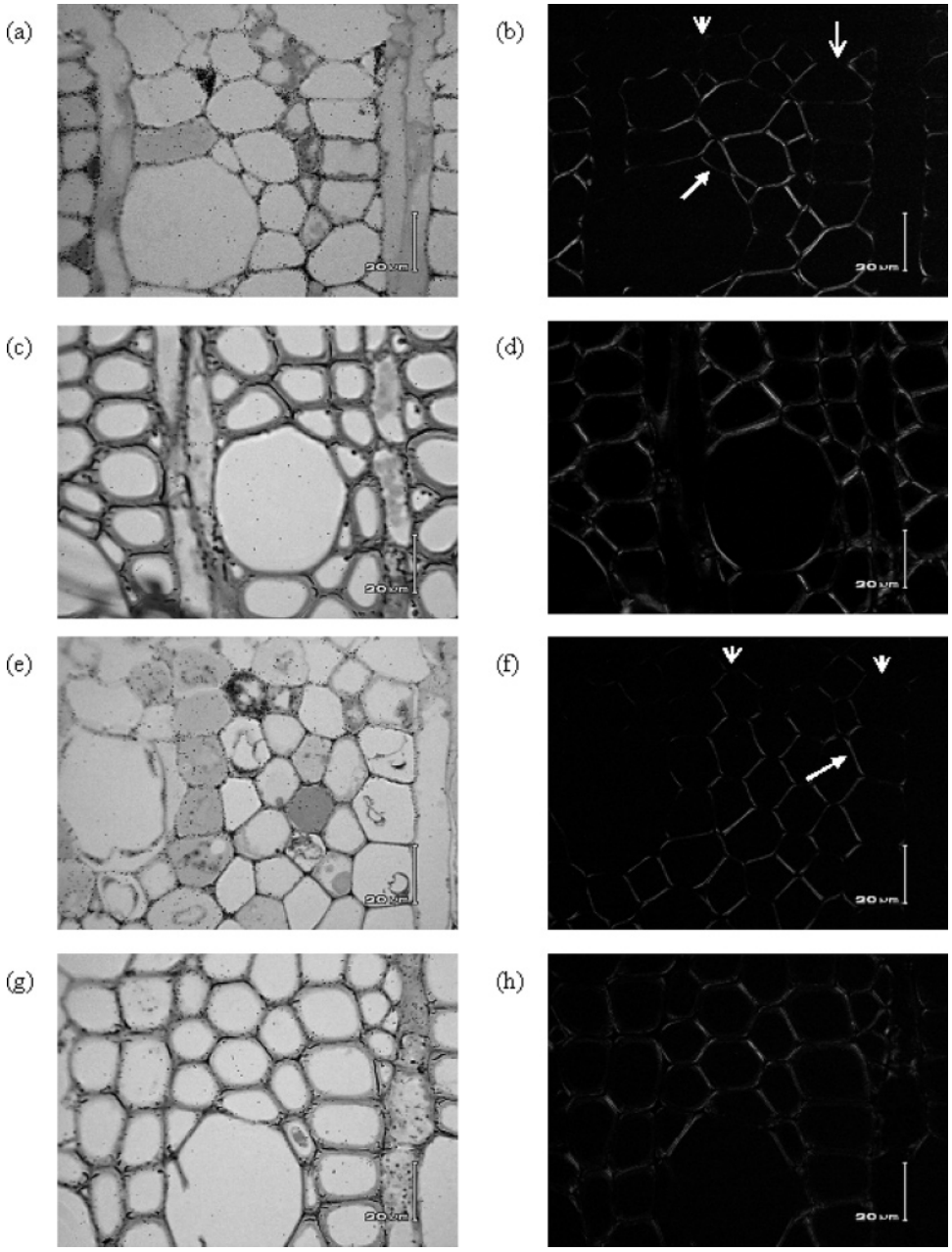

Figure 1. Incorporation of tritiated coniferin and coniferaldehyde glucoside into magnolia xylem. Panels **a**, **c**, **e**, and **g** are microautoradiographs. Panels **b**, **d**, **f**, and **h** are polarization microphotographs of the same fields as those in panels **a**, **c**, **e**, and **g**. (**a**) Early stage (after S_1 formation) of differentiating xylem fed with coniferin [9-$^3H_2$]. (**c**) Later stage (after S_3 formation) of differentiating xylem fed with coniferin [9-$^3H_2$]. (**e**) Early stage of differentiating xylem fed with coniferaldehyde glucoside [9-^{3}H]. (**g**) Later stage of differentiating xylem fed with coniferaldehyde glucoside [9-^{3}H]. Arrow, S_1 layer; downward arrow in **b** and **f**, starting point of S_1 formation.

MATERIALS AND METHODS

Synthesis of Radioisotope-Labeled Precursors. Coniferin [9-$^3H_2$] and syringin [9-$^3H_2$] were synthesized using the same procedure as that used to synthesize monolignol glucoside [9-D_2] (*20*). We used tritiated sodium borohydride rather than sodium borodeuteride. Coniferaldehyde glucoside [9-^{3}H] was synthesized as follows. First, 4-*O*-[2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl] coniferyl alcohol [9-$^3H_2$] was synthesized from tetra-*O*-acetyl-α-D-glucopyranosyl bromide and vanillin using the method of Matsui et al. (*21*). Between 200 and 250 mg of 4-*O*-[2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl] coniferyl alcohol [9-$^3H_2$] was dissolved in chloroform and oxidized with activated manganese(IV) oxide for 16 h while being stirred in an atmosphere of nitrogen. Excess activated manganese(IV) oxide was removed from the reaction mixture by filtration, and the filtrate was condensed under reduced pressure. The residue was applied to a silica gel column with a mixture of acetone:*n*-hexane (1:2, v/v) as a developer, and purified tetra-*O*-acetyl-*β*-D-glucopyranosyl coniferaldehyde [9-^{3}H] was recovered. Deacetylation was carried out with sodium methoxide, according to the method of Matsui et al. (*21*). The reaction mixture was evaporated, and recrystallization from hot water gave colorless crystals of coniferaldehyde glucoside [9-^{3}H] (1.18 MBq/mg). Sinapaldehyde glucoside [9-^{3}H] (58 mg, 90 KBq/mg) was synthesized in a manner similar to that used to synthesize the corresponding coniferaldehyde glucosides by replacing vanillin with syringaldehyde.

Synthesis of Stable Isotope-Labeled Precursors. We synthesized two kinds of labeled coniferaldehyde glucosides, [9-^{13}C, D, 3-OCD_3] and [9-^{13}C, 3-OCD_3], and two kinds of labeled coniferin, [8-^{13}C, 3-OCD_3] and [9-D_2, 8-^{13}C, 3-OCD_3]. All compounds were synthesized from tetra-*O*-acetyl-*β*-D-glucosyl vanillin [3-OCD_3], which was synthesized from 3,4-dihydroxybenzaldehyde (2.8 g) and tetra-*O*-acetyl-α-D-glucopyranosyl bromide (3.15 g) instead of benzylbromide using the method of Umezawa et al. (*22*) for 4-benzyloxy-3-[OCD_3] meth-

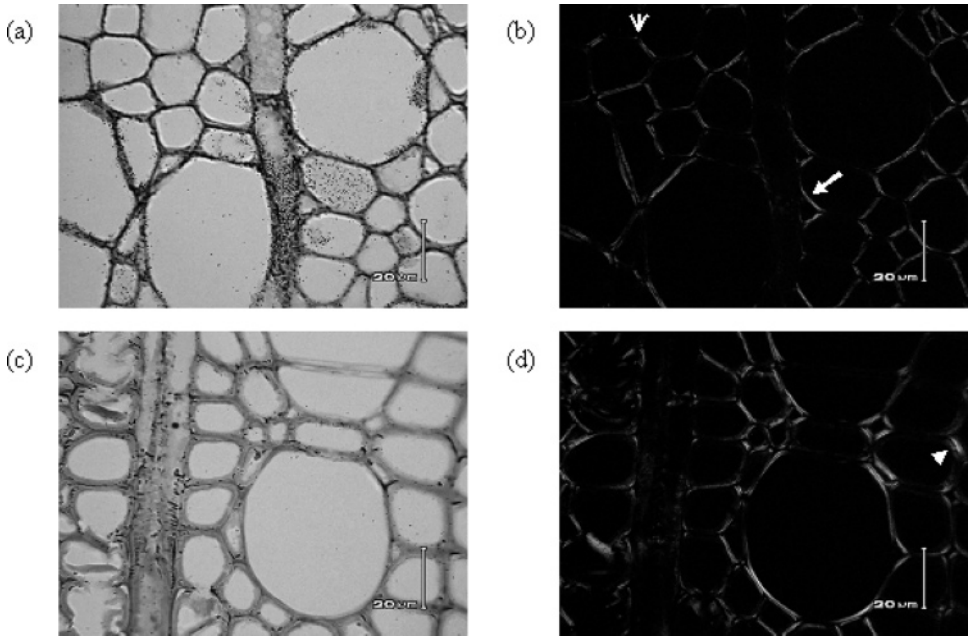

Figure 2. Incorporation of syringin [9-$^3H_2$] into magnolia xylem. Panels **a** and **c** are microautoradiographs. Panels **b** and **d** are polarization microphotographs of the same fields as those in panels **a** and **c**. (**a**) Early stage (after S_1 formation) of differentiating xylem fed with syringin [9-$^3H_2$]. (**c**) Later stage (after S_3 formation) of differentiating xylem fed with syringin [9-$^3H_2$]. Arrow, S_1 layer; arrowhead, S_3 layer; downward arrow in **b**, starting point of S_1 formation.

oxybenzaldehyde. Tetra-*O*-acetyl-*β*-D-glucopyranosyl coniferyl alcohol [9-D_2, 8-^{13}C, 3-OCD_3] and −[9-D_2, 3-OCD_3] and −[9-^{13}C,D, 3-OCD_3] and −[9-^{13}C, 3-OCD_3] were synthesized from tetra-*O*-acetyl-*β*-D-glucosyl vanillin [3-OCD_3], according to the method of Matsui et al. (*21*), except that malonic acid and sodium borohydride [^{3}H] were replaced with the following regents. To introduce ^{13}C into C8, malonic acid [2-^{13}C] acid (99 atom % ^{13}C; Sigma Aldrich, St. Louis, MO) was used. Malonic acid [1, 3-$^{13}C_2$] (min. 99 atom % ^{13}C; Isotec) was used to incorporate ^{13}C into C9. Sodium borodeuteride was used to substitute two deuteriums for two hydrogens at C9. Tetra-*O*-acetyl-*β*-D-glucopyranosyl coniferyl alcohol [9-D_2, 8-^{13}C, 3-OCD_3] and −[9-D_2, 3-OCD_3] were deacetylated to generate coniferin [9-D_2, 8-^{13}C, 3-OCD_3] and −[9-D_2, 3-OCD_3], respectively, using the method of Matsui et al. (*21*). Tetra-*O*-acetyl-*β*-D-glucopyranosyl coniferyl alcohol [9-^{13}C,D, 3-OCD_3] and −[9-^{13}C, 3-OCD_3] were converted to corresponding tetra-*O*-acetyl-*β*-D-glucosyl coniferyl aldehydes using activated manganese(IV), as described for the synthesis of coniferaldehyde glucoside [9-^{3}H] (see above). Deacetylation of the aldehydes yielded coniferaldehyde glucoside [9-^{13}C,D, 3-OCD_3] and −[9-^{13}C, 3-OCD_3]. Sinapaldehyde glucoside [8-^{13}C, 3-OCD_3] was synthesized in a manner that was similar to the synthesis of coniferaldehyde glucoside [9-^{13}C, 3-OCD_3], except that malonic acid [1,3-^{13}C] was used instead of malonic acid [2-^{13}C].

Plant Material and Administration of Radioisotope-Labeled Precursors. Two-year-old shoots of magnolia (*Magnolia kobus* DC) and oleander (*Nerium indicum* Mill.) were harvested in July on the campus of Nagoya University, Japan. The shoots were cut into 10-cm-long segments. Solutions of coniferin [9-$^3H_2$], coniferaldehyde glucoside [9-^{3}H], syringin [9-$^3H_2$], sinapaldehyde glucoside [9-^{3}H], and sinapaldehyde glucoside [8-^{14}C] were administered, respectively, to wedged slits that had been made in the shoots, so that the solutions reached the differentiating xylem. After 12 h, tissue blocks that adjoined the slits were cut from the shoots and fixed in a solution of 3% glutaraldehyde and 1% osmic acid. Thereafter, the tissue blocks were dehydrated through a graded series of ethanol before being embedded in epoxy resin (a mixture of Quetol 812, MNA, and DMP-30). The embedded tissue blocks were then subjected to microautoradiography.

Plant Material and Administration of Stable Isotope-Labeled Precursors. We used 2-year-old shoots from the plants that were described above. The upper region of each shoot was removed, and small wells were attached to the top of the remaining stem using plastic sheets (*12*). The wells were filled with 30 mL of a solution that contained one or more of the labeled precursors (see below) dissolved in a mixture of 0.067 M KH_2PO_4 and 0.067 M Na_2HPO_4 (4:6, v/v; pH 7.1). We prepared the following precursor solutions: (I) 2 mM coniferaldehyde glucoside [9-^{13}C, D, 3-OCD_3]; (II) 2 mM coniferin [8-^{13}C, 3-OCD_3]; (III) a mixture of 2 mM coniferin [9-D_2, 8-^{13}C, 3-OCD_3] and 2 mM coniferaldehyde glucoside [9-^{13}C, 3-OCD_3]; (IV) 2 mM sinapaldehyde glucoside [8-^{13}C, 3-OCD_3]; and (V) a mixture of 2 mM coniferin [9-D_2, 8-^{13}C, 3-OCD_3] and 2 mM sinapaldehyde glucoside [8-^{13}C, 3-OCD_3]. Only solutions II and III were administered to the oleander shoots. After 1 month, the shoots were harvested for the chemical analysis of lignin.

Microautoradiography. Transverse sections (2 μm) were cut with a glass knife on a rotary microtome (Leica RM2155, Leica, Germany) (*23*). The sections placed onto glass slides were covered with autoradiography emulsion (Konica NR-M2, Konica, Tokyo, Japan). After exposure at 4 °C for 2 months, the emulsion on the slides was developed with Rendol (Fuji, Tokyo, Japan) and fixed with Renfix (Fuji). The sections were stained with toluidine blue O and observed with a microscope (BX50, Olympus) that was equipped with a digital camera (DP12, Olympus).

Chemical Analysis of Lignin. We used the "derivatization followed by reductive cleavage" (DFRC) method (*24*) to degrade the lignin that had been biosynthesized within magnolia and oleander shoots, which had been administered solutions that contained the labeled precursors. Tangential sections (50 μm) were cut from the periphery of the bark-free xylem with a sliding microtome, and some sections were milled and subjected to lignin chemical analysis after successive extractions with ethanol/benzene (1:2, v/v) and hot water. The acetylated degradation products of lignin were dissolved in dichloromethane before being subjected to gas chromatography−mass spectroscopy (GC−MS). Mass spectra were recorded at 70 eV with a mass spectrometer (Mstation JMS 700) that was coupled to a gas chromatograph (HP 6890, Hewlett-Packard, Wilmington, DE), which was equipped with a fused silica capillary column (TC1, 30 m × 0.25 mm i.d.; GL Sciences, Tokyo, Japan). Two microliters of each sample was injected at 220 °C. The temperature was programmed to increase from 150 to 300 °C at 3 °C/min, and the carrier gas was helium.

RESULTS

Histological Examination of Labeled Precursor Incorporation. **Figure 1** shows the microautoradiograms of magnolia xylem in shoots that were administered coniferin [9-$^3H_2$] and coniferaldehyde glucoside [9-^{3}H]. Polarized images of the same field

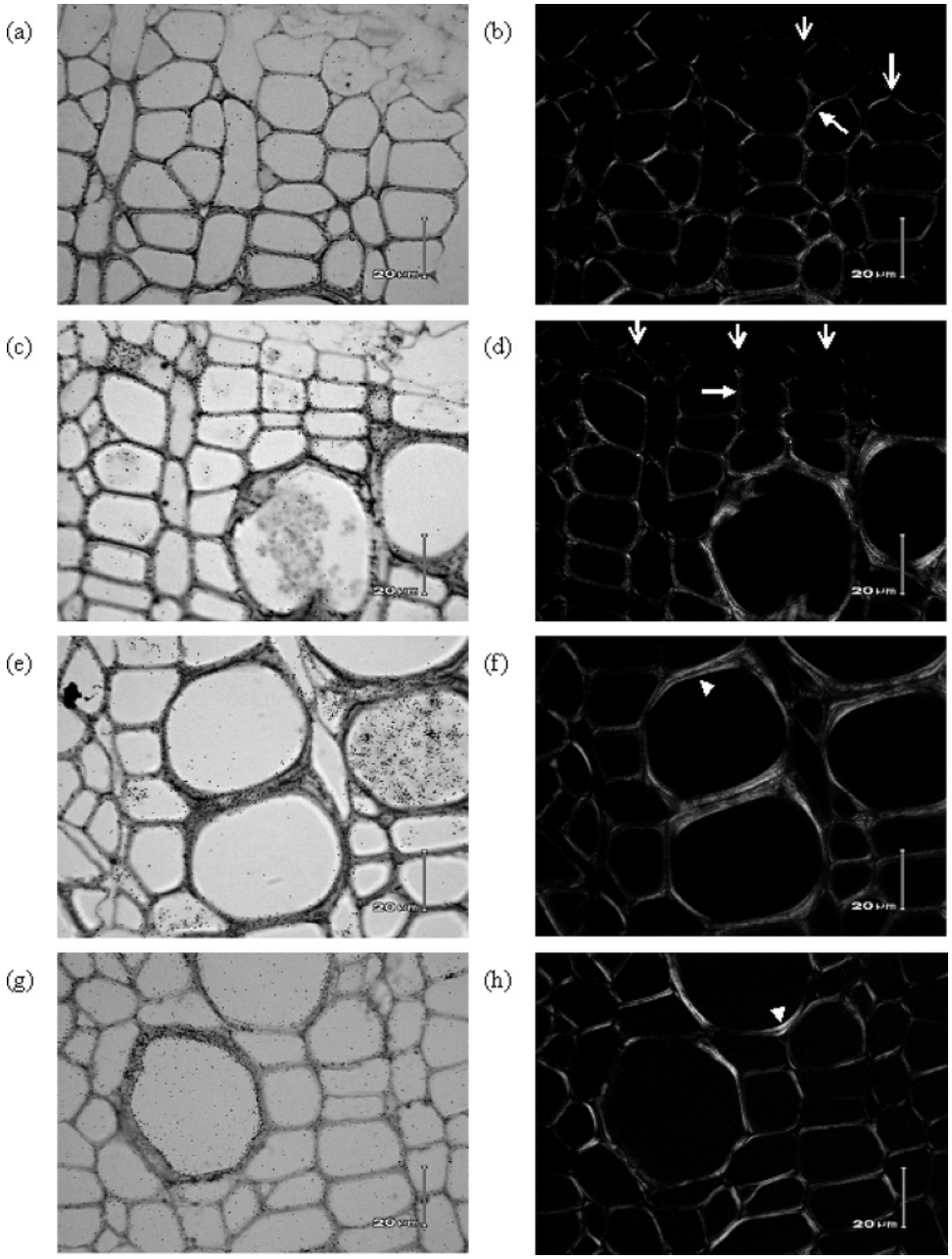

Figure 3. Incorporation of tritiated glucosides into oleander xylem. Panels **a**, **c**, **e**, and **g** are microautoradiographs. Panels **b**, **d**, **f**, and **h** are polarization microphotographs of the same fields as those in panels **a**, **c**, **e**, and **g**. (**a**) Early stage (after S_1 formation) of differentiating xylem fed with coniferin [9-$^3H_2$]. (**c**) Early stage (after S_1 formation) of differentiating xylem fed with coniferaldehyde glucoside [9-^{3}H]. (**e**) Later stage of differentiating xylem fed with syringin [9-$^3H_2$]. (**g**) Later stage of differentiating xylem fed with sinapaldehyde glucoside [9-^{3}H]. Arrow, S_1 layer; arrowhead, S_3 layer; downward arrow in **b** and **d**, starting point of S_1 formation.

are also presented to show the deposition of the S_1 and S_3 layers (outer and inner layer of the secondary wall, respectively). In the early stages of lignification, labels derived from coniferin [9-$^3H_2$] were incorporated efficiently into the compound middle lamella (**Figure 1a**,**b**). In the late stage of lignification, during which cellulose is deposited in the S_3 layer, a few labels that were attributable to coniferin [9-$^3H_2$] were distributed within the secondary walls (**Figure 1c**,**d**). **Figure 1e** shows the distribution of labeling that was derived from coniferaldehyde glucoside [9-^{3}H] in the early lignification stage, during which the S_3 sublayer is absent (**Figure 1f**). At this stage, labels from coniferaldehyde glucoside [9-^{3}H] were incorporated efficiently into the compound middle lamella, as was the case for coniferin [9-$^3H_2$]. At the stage at which the S_3 layer develops, incorporation of labels derived from coniferaldehyde glucoside [9-^{3}H] was observed mainly within the secondary wall (**Figure 1g**,**h**). The number of labels that were derived from coniferaldehyde glucoside [9-^{3}H] was greater than that from coniferin [9-$^3H_2$] during the stage at which syringyl-rich lignin is accumulated within the secondary walls, even though the specific activity of coniferaldehyde glucoside [9-^{3}H] was much lower than that of coniferin [9-$^3H_2$] (data not shown). These results suggest that the aglycone that was derived from coniferaldehyde glucoside was incorporated into the walls of cells.

Figure 2 shows the microautoradiograms and polarized images of magnolia xylem in shoots that were administered syringin [9-$^3H_2$]. In the early stages of lignification, labels that were attributable to syringin [9-$^3H_2$] were detected mainly within ray parenchyma cells and proximal vessel walls (**Figure 2a**,**b**). This suggested that ray parenchyma cells may participate in

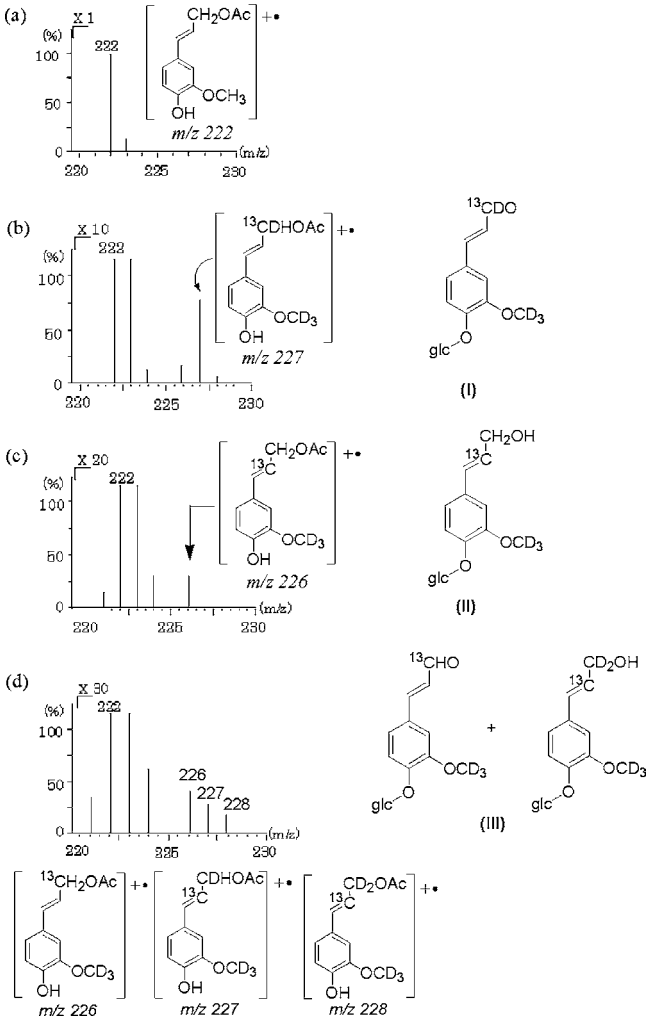

Figure 4. Precursors and partial masses of the guaiacyl DFRC derivatives from differentiating magnolia xylem administered stable isotope-labeled glucosides. (**a**) Control. (**b**) Fed with coniferaldehyde glucoside [9-^{13}C, D, 3-OCD$_3$] (I) only. (**c**) Fed with coniferin [8-^{13}C, 3-OCD$_3$] (II) only. (**d**) Fed with both coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (III).

the transportation of syringin [9-^{3}H$_2$], but it is unclear whether this observation reflects the transportation of endogenous syringin by these cells. In the late stage of lignification, few labels derived from syringin [9-^{3}H$_2$] were detected within the secondary walls (**Figure 2c**,**d**). When the magnolia shoots were administered sinapaldehyde glucoside [8-^{14}C], few labels were detectable across the xylem tissue during the early and late stages of lignification (data not shown).

Figure 3 shows the microautoradiograms and polarized images of oleander xylem that were administered radiolabeled coniferin, coniferaldehyde glucoside, syringin, and sinapaldehyde glucoside. Labels that were attributable to coniferin [9-^{3}H$_2$] were incorporated into the compound middle lamellae during the early stage of lignification (**Figure 3a**,**b**). Labels derived from coniferaldehyde glucoside [9-^{3}H] were also distributed within the cell wall at a relatively early stage of lignification (**Figure 3c**,**d**). Labels from syringin [9-^{3}H$_2$] (**Figure 3e**) and sinapaldehyde glucoside [9-^{3}H] (**Figure 3g**) were incorporated into vessel walls during the formation of the S$_3$ layer (**Figure 3f**,**h**). These results imply that during these stages of lignification, the vessel cell walls were primarily involved in accumulating syringyl lignin, although it is commonly believed that lignin within vessel walls is comprised primarily of a guaiacyl moiety.

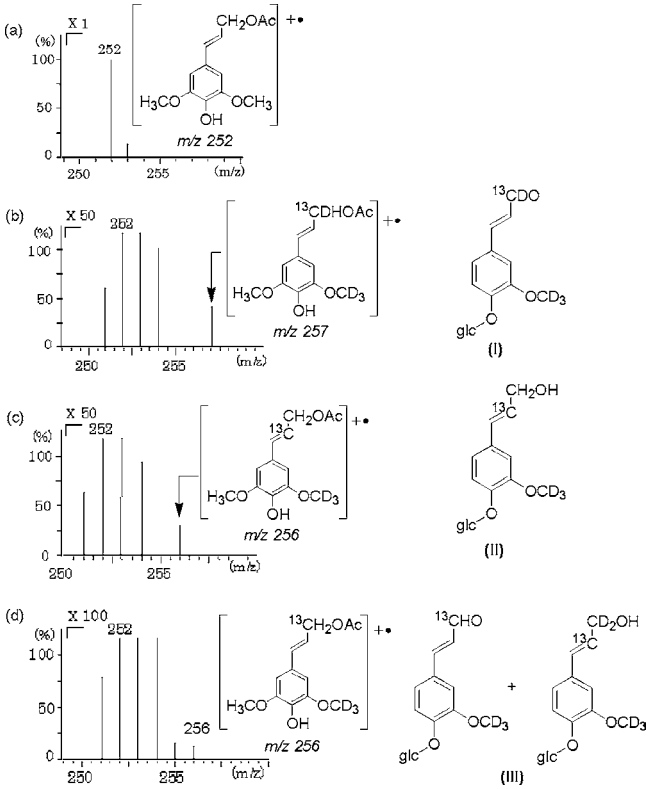

Figure 5. Precursors and partial masses of the syringyl DFRC derivatives from differentiating magnolia xylem administered stable isotope-labeled glucosides. (**a**) Control. (**b**) Fed with coniferaldehyde glucoside [9-^{13}C, D, 3-OCD$_3$] (I) only. (**c**) Fed with coniferin [8-^{13}C, 3-OCD$_3$] (II) only. (**d**) Fed with both coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (III).

Interestingly, the incorporation frequencies of syringin [9-^{3}H$_2$] and sinapaldehyde glucoside [9-^{3}H$_2$] were higher in the oleander shoots than in the magnolia shoots.

Molecular Behavior of Incorporated Stable Isotope-Labeled Precursors. To study the behavior of cinnamaldehyde and cinnamyl alcohol glucosides in the differentiating xylem of angiosperms, we used a combination of DFRC and selective labeling at the C9 position of the lignin precursor. The DFRC method is optimal for our experimental approach because it provides degradation products that produce C_6-C_3 base ion peaks retaining C9-deuteriums in GC-MS. The total ion chromatograms (TICs) of samples of shoots that were administered labeled precursors were indistinguishable from the TIC of the control sample. **Figure 4** shows partial mass spectra of guaiacyl monomers that were derived from a control sample and those of magnolia stems that were administered the labeled precursors. The guaiacyl unit has a base peak at *m*/*z* 222 (M$_g$) (**Figure 4a**). The ion peak at *m*/*z* 227 (M$_g$ + 5) (**Figure 4b**) indicates that the aglycone from coniferaldehyde glucoside [9-^{13}C, D, 3-OCD$_3$] (I) was incorporated into guaiacyl lignin. It is important to note that these ion peaks were detected in the largest of the DFRC degradation product peaks. This suggests that coniferaldehyde, the aglycone from coniferaldehyde glucoside, was incorporated into 8-*O*-4′ lignin after being reduced to cinnamyl alcohol. The peak at *m*/*z* 226 (M$_g$ + 4) (**Figure 4c**) demonstrates that the aglycones from coniferin [8-^{13}C, 3-OCD$_3$] (II) were incorporated into guaiacyl lignin. These results are clear evidence of the incorporation of the aglycones from coniferin and coniferaldehyde glucoside into 8-*O*-4′ structures in lignin. To compare the relative preference for coniferin and coniferaldehyde glucoside as lignin precursors, we administered simultaneously coniferin

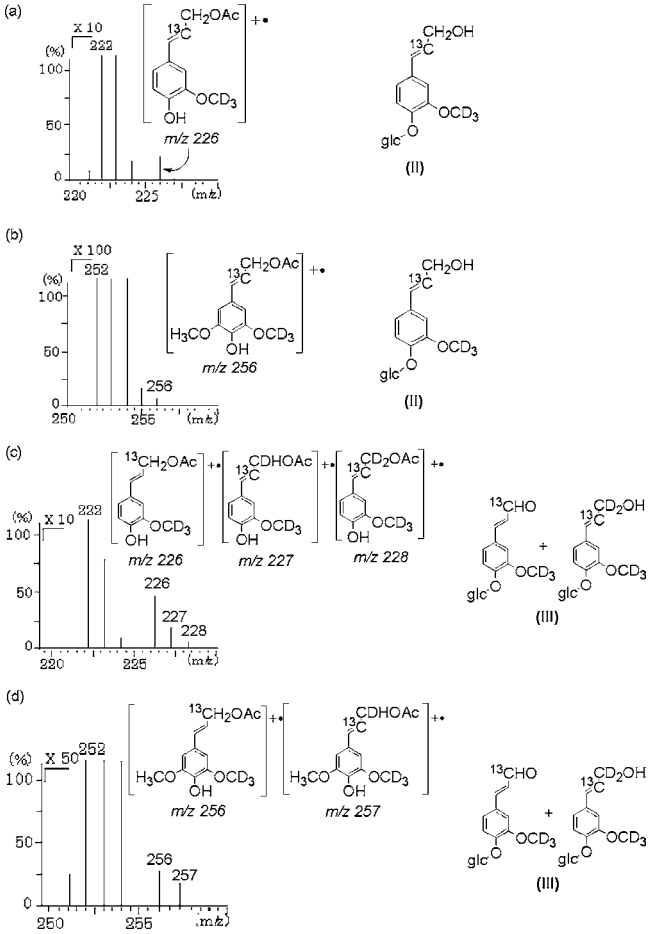

Figure 6. Precursors and partial masses of the DFRC products from differentiating oleander xylem administered stable isotope-labeled glucosides. (**a**) Partial masses of guaiacyl DFRC derivatives from the oleander fed with coniferin [8-^{13}C, 3-OCD$_3$] (II) only. (**b**) Partial masses of syringyl DFRC derivatives from the oleander fed with coniferin [8-^{13}C, 3-OCD$_3$] (II) only. (**c**) Partial masses of guaiacyl DFRC derivatives from the oleander fed with both coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (III). (**d**) Partial masses of syringyl DFRC derivatives from the oleander fed with both coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (III).

[9-D$_2$, 8-^{13}C, 3-OCD$_3$] and coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] (III) to shoots. The DFRC derivatives produced three ion peaks that were attributable to these two precursors (**Figure 4d**). Two of them were ion peaks detected at *m*/*z* 226 (M_g + 4) and *m*/*z* 228 (M_g + 6) that were derived from coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] (+4) and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (+6), respectively. Another significant peak was detected at *m*/*z* 227 (M_g + 5), which might be produced from coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] via the route that is illustrated in **Figure 7**. To compare the relative amount of each precursor that was incorporated into lignin, the area ratios of the *m*/*z* 226 (M_g + 4), *m*/*z* 227 (M_g + 5), *m*/*z* 228 (M_g + 6), and *m*/*z* 222 (M_g) signals were calculated using a selected ion-monitoring (SIM) function: the ratio of M_g + 4 (from coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$]) was higher than those of M_g + 5 and M_g + 6 (both from coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$]); the area ratio of M_g + 5 was greater than that of M_g + 6. This suggests that much of the coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (+6) that was administered was oxidized to the aldehyde form [9-D, 8-^{13}C, 3-OCD$_3$] (+5), which was subsequently rereduced to the alcohol form [9-D, 8-^{13}C, 3-OCD$_3$] (+5) prior to dehydrogenative polymerization (**Figure 7**).

Because magnolia plants have a pool of syringin in addition to the pool of endogenous coniferin, sinapaldehyde glucoside [8-^{13}C, 3-OCD$_3$] was administered to magnolia shoots to determine whether the efficiency with which the aglycone from sinapaldehyde glucoside was incorporated into lignin was comparable to the efficiency with which coniferaldehyde glucoside was incorporated into lignin. When magnolia shoots were administered sinapaldehyde glucoside [8-^{13}C, 3-OCD$_3$] (IV), there were no ion peaks from the labeled precursors in the guaiacyl products (data not shown). This indicates that the syringyl unit could not be recycled into a guaiacyl unit, which is consistent with a previous report (*20*). **Figure 5** shows the partial mass spectra of syringyl monomers that were derived from the control sample and from magnolia shoots that were administered the labeled precursors. The syringyl unit has a base peak at *m*/*z* 252 (M_s) (**Figure 5a**). When the magnolia shoots were administered coniferaldehyde glucoside [9-^{13}C, D, 3-OCD$_3$] (I) or coniferin [8-^{13}C, 3-OCD$_3$] (II), there were ion peaks at *m*/*z* 257 or *m*/*z* 256 (**Figure 5b**,**c**), respectively. These results indicate that the aglycone from the glucosides was incorporated into syringyl lignin via sinapyl alcohol. However, the amount of the aglycone that was incorporated into syringyl lignin was less than that of guaiacyl lignin. When the magnolia shoots were administered simultaneously coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] (+4) and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (+6) (III), the only ion peak that was detected was at *m*/*z* 256 (M_s + 4) (**Figure 5d**), which suggested that the aglycone from coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] was incorporated into syringyl lignin, whereas little aglycone from coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] was incorporated. Tiny amounts of the aglycone from sinapaldehyde glucoside [8-^{13}C, 3-OCD$_3$] were incorporated into syringyl lignin when the magnolia shoots were administered sinapaldehyde glucoside [8-^{13}C, 3-OCD$_3$] (IV) (data not shown).

Figure 6 shows partial mass spectra of DFRC monomers that were derived from oleander shoots that were administered the labeled precursors. The aglycones from coniferin [8-^{13}C, 3-OCD$_3$] (II) were incorporated into guaiacyl lignin, whereas the incorporation into syringyl lignin was not active (**Figure 6a**,**b**). When the oleander shoots were administered simultaneously coniferaldehyde glucoside [9-^{13}C, 3-OCD$_3$] (+4) and coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$] (+6) (III), three ion peaks were detected in the guaiacyl products at *m*/*z* 228 (M_g + 6), *m*/*z* 227 (M_g + 5), and *m*/*z* 226 (M_g + 4) (**Figure 6c**). SIM analysis revealed that the relative area of the *m*/*z* 226 peak was the greatest, while the *m*/*z* 228 peak area was the smallest and the

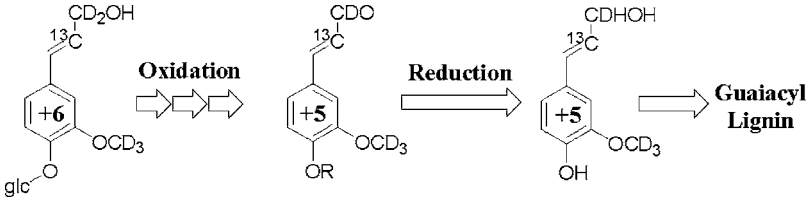

Figure 7. Possible pathway for the generation of +5-coniferyl alcohol [9-D, 8-^{13}C, 3-OCD$_3$] from coniferin [9-D$_2$, 8-^{13}C, 3-OCD$_3$]. R, glucose or hydrogen.

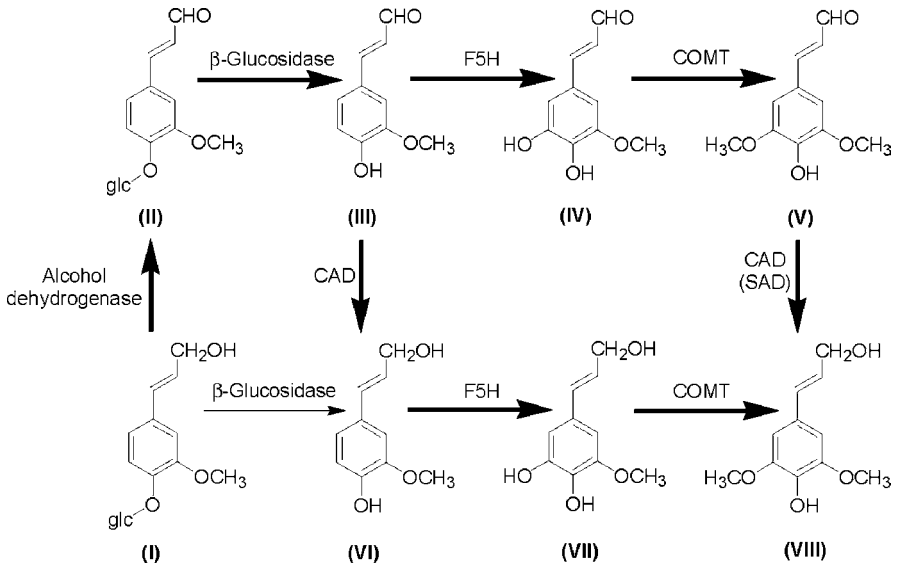

Figure 8. Proposed behavior of coniferin in differentiating angiosperm xylem. (I) Coniferin, (II) coniferaldehyde glucoside, (III) coniferaldehyde, (IV) 5-hydroxyconiferaldehyde, (V) sinapaldehyde, (VI) coniferyl alcohol, (VII) 5-hydroxyconiferyl alcohol, (VIII) sinapyl alcohol. F5H, ferulate 5-hydroxylase; COMT, caffeic acid/5-hydroxyferulic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase.

m/*z* 227 peak was intermediate. In syringyl products (**Figure 6d**), the peak of the coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] (+4) derivatives at *m*/*z* 256 (M_s + 4) was predominant. A small peak was detected at *m*/*z* 257 (M_s + 5), suggesting that only coniferin that passed through the aldehyde form was incorporated into syringyl lignin. These results suggest that coniferaldehyde glucoside is preferred over coniferin as a precursor for lignin and that coniferaldehyde is crucial for the biosynthesis of syringyl lignin.

DISCUSSION

In the present study, microautoradiography revealed that labels that were attributable to radiolabeled coniferaldehyde glucoside could be incorporated into the cell walls of angiosperms irrespective of whether they contained a pool of endogenous coniferin. In addition, we found that relatively less sinapaldehyde glucoside than coniferaldehyde glucoside was incorporated during lignification. These observations were consistent with the results of the feeding experiments in which we administered glucosides that were labeled with stable isotopes to magnolia and oleander shoots. Interestingly, labels from syringin [9-$^3H_2$] were detected in the ray parenchyma cells of magnolia shoots. It is accepted generally that parenchyma cells assume the role of storing nutrients and metabolites. Because coniferin is found in particularly high concentrations in cambial sap and differentiating xylem, it would appear that the transport of coniferin and/or syringin to lignifying cells occurs via ray parenchyma cells.

The results of the series of experiments that we carried out in the present study suggest that coniferaldehyde and/or coniferaldehyde glucoside are crucial for the biosynthesis of lignin (particularly syringyl lignin), while sinapaldehyde glucoside would appear to be less important. Because sinapaldehyde glucoside is not stored in magnolia, if any, this is likely an intermediate compound that is metabolized from syringin, which is stored. It has been reported that syringin is located exclusively within phloem and stored in winter in magnolia (*25*); this supports the suggestion that sinapaldehyde glucoside is not active in lignin biosynthesis.

Interestingly, the results of our microautoradiography study revealed that the incorporation of the radiolabel syringyl precursors, sinapaldehyde glucoside and syringin, was more active in oleander xylem than in magnolia. This suggests that the metabolism of these glucosides might differ among plant species. The feeding experiments in which glucosides were labeled with a stable isotope revealed that the aglycones from the labeled glucosides were incorporated as cinnamyl alcohol units into major lignin structures (8-*O*-4′). In a previous study (*17*), it was reported that some of the coniferin that was administered to angiosperms was transformed into an aldehyde form prior to being polymerized into lignin as cinnamyl alcohol units, but the manner of oxidation of coniferin during this process (i.e., whether coniferin was oxidized directly to coniferaldehyde glucoside or whether coniferyl alcohol released from coniferin was oxidized to coniferaldehyde) was not investigated. A previous study of *Ginkgo biloba* led to the suggestion that there is an alternative pathway from coniferin to coniferaldehyde via coniferaldehyde glucoside in gymnosperms (Tsuji et al., submitted). In the present study, the efficient incorporation of the aglycone from coniferaldehyde glucoside into lignin suggests that a similar pathway might exist in angiosperms. The most notable difference between gymnosperms and angiosperms is that the latter possess syringyl lignin in addition to guaiacyl lignin. In a study in which pentadeutero coniferin [9-D_2, 3-OCD_3] was administered to magnolia (*17*), the amount of the aglycone incorporated into lignin via an aldehyde form was much in syringyl lignin, not in guaiacyl lignin. This suggested that stored coniferin and the oxidation pathway in magnolia exists primarily for the synthesis of syringyl lignin rather than guaiacyl lignin. In the present study, however, when coniferin [9-D_2, 8-^{13}C, 3-OCD_3] (+6) and coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] (+4) were administered to magnolia simultaneously, the area of the ion peak at *m*/*z* 227 (M_g + 5) was greater than that of the peak at *m*/*z* 228 (M_g + 6) in the DFRC derivatives of guaiacyl lignin. This observation suggests that the oxidation process was also active for guaiacyl products. This apparent contradiction might be due to the coexistence of coniferin and coniferaldehyde glucoside. When coniferin [9-D_2, 8-^{13}C, 3-OCD_3] and coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] were administered simultaneously, the ion peak was derived exclusively from the coniferaldehyde glucoside in the syringyl products, rather than from coniferin.

In light of the conventional theory in which an enzyme β-glucosidase catalyzes the deglucosylation of coniferin to release coniferyl alcohol (*26*−*28*), we predicted that coniferyl alcohol [9-D_2, 8-^{13}C, 3-OCD_3] (+6) that had been released from coniferin [9-D_2, 8-^{13}C, 3-OCD_3] would be transferred to a syringyl unit at the cinnamyl alcohol stage and that an ion peak at *m*/*z* 258 (M_s + 6) would be detected. Indeed, in a previous study (*17*), the amount of syringyl products that had been synthesized via the cinnamyl alcohol stage was comparable to the amount of syringyl products that had been synthesized via an aldehyde form. In the present study, when coniferin [9-D_2, 8-^{13}C, 3-OCD_3] (+6) and coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] (+4) were administered to magnolia simultaneously, the ion peak (*m*/*z* 258) (M_s + 6) (corresponding to the product synthesized through the cinnamyl alcohol stage) was barely detectable for the syringyl products, whereas the peak at *m*/*z* 256 (M_s + 4) (corresponding to coniferaldehyde glucoside) dominated the syringyl products, which suggested that the cinnamaldehyde form was important for the synthesis of syringyl lignin. When coniferin [9-D_2, 8-^{13}C, 3-OCD_3] and coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] were administered simultaneously to oleander shoots, the ion peaks from coniferaldehyde glucoside [9-^{13}C, 3-OCD_3] and coniferin [9-D_2, 8-^{13}C, 3-OCD_3] that had been transformed via the cinnamaldehyde form [9-D, 8-^{13}C, 3-OCD_3] (+5) were dominant over the peaks from the coniferin [9-D_2, 8-^{13}C, 3-OCD_3] that had not been metabolized via cinnamaldehyde. The magnolia shoots possess a pool of endogenous coniferin in addition to the coniferin that was administered in the present study; therefore, we could not conclude that coniferaldehyde glucoside was preferred over coniferin as a precursor for lignin biosynthesis, because the labeled coniferin might have been diluted by the natural coniferin. By contrast, in oleander shoots, the coniferin that was administered was not diluted, because the oleander shoots lacked a pool of endogenous coniferin. Therefore, the results of the experiments in which oleander shoots were used suggest that coniferaldehyde glucoside is the preferred substrate for lignin biosynthesis and emphasize the importance of cinnamaldehyde in syringyl lignin biosynthesis.

Recent studies of lignin biosynthesis have not revealed whether the conversion of the guaiacyl moiety to the syringyl moiety occurs at the cinnamaldehyde or cinnamyl alcohol stage of biosynthesis. The results of the present study would appear to support the former. However, a small amount of syringyl lignin was synthesized via cinnamyl alcohol. Moreover, in the present study, it was not clear whether the coniferaldehyde that was produced finally by the process of oxidation had been metabolized to 5-hydroxy coniferaldehyde (see IV in **Figure 8**) or coniferyl alcohol (see VI in **Figure 8**) to be converted into the syringyl moiety.

The results of the present study and those of a previous study (Tsuji et al., submitted) suggest that coniferaldehyde glucoside is incorporated efficiently into lignin of gymnosperm (e.g., ginkgo) and angiosperms (e.g., magnolia and oleander), irrespective of whether the latter contain a pool of endogenous coniferin. In addition, it would appear that for coniferin to be incorporated into lignin, the conversion of coniferin to coniferaldehyde via coniferaldehyde glucoside is required for entry into the monolignol biosynthetic pathway (**Figure 8**). However, it should be considered that our results do not necessarily apply to plant in vivo secondary metabolism because of the artifactual condition, such as feeding experiments. Our results also suggested that coniferaldehyde is crucial for the biosynthesis of syringyl lignin. In studies using alfalfa (*29*, *30*), it was suggested that there were independent pathways to guaiacyl and syringyl monolignols, and the pathways intersected at coniferaldehyde, which is suggesting that coniferaldehyde is a reasonable compound for the synthesis of both guaiacyl and syringyl monolignols. Taken together, the role of coniferaldehyde in lignin biosynthesis deserves further study.

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