J Wood Sci (2003) 49:333–338
DOI 10.1007/s10086-002-0477-7

ORIGINAL ARTICLE

Katsuyoshi Hamada · Yuji Tsutsumi
Kazuchika Yamauchi · Kazuhiko Fukushima
Tomoaki Nishida

Treatment of poplar callus with ferulic and sinapic acids I: incorporation and enhancement of lignin biosynthesis

Received: May 17, 2002 / Accepted: September 4, 2002

Abstract Ferulic acid (FA), tetradeuteroferulic acid (DFA), sinapic acid (SA), or heptadeuterosinapic acid (DSA) was exogenously supplied to poplar (*Populus alba* L.) callus. Administration of FA or SA increased the lignin content of the callus to about twice that of the control callus. Gas chromatographic analysis of the alkali hydrolysate of the cell wall residue revealed that only a trace amount of SA was bound to the cell wall, and the amount of FA was less than 2% of the total callus lignin. Thioacidolysis of the DFA-treated callus indicated that DFA is effectively converted to both coniferyl and sinapyl alcohols and then incorporated into the corresponding lignin. Incorporation of DSA into syringyl lignin or guaiacyl lignin was not observed, but yields of syringyl lignin thioacidolysis products were markedly increased by DSA treatment of the callus. These results suggest that SA may not be a precursor of sinapyl alcohol and syringyl lignin per se, but it may induce or enhance the biosynthesis of syringyl lignin in poplar callus.

Key words Ferulic acid · Lignin biosynthesis · *Populus alba* L. · Sinapic acid · Syringyl lignin

K. Hamada[1] · T. Nishida
Department of Forest Resources Science, Faculty of Agriculture, Shizuoka University, Shizuoka, 422-8529, Japan

Y. Tsutsumi (✉)
Systematic Forest and Forest Product Sciences, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
Tel. +81-92-642-4282; Fax +81-92-642-4282
e-mail: y-tsutsu@agr.kyusyu-u.ac.jp

K. Yamauchi · K. Fukushima
Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

[1]K. Hamada is a Ph.D. student of The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1112, Japan

Introduction

Plant phenylpropanoids and their related metabolites, such as lignin, lignans, flavonoids, and suberins, are derived from cinnamic acid analogues via a common general pathway.[1–3] Lignin, the most abundant of a large number of plant phenylpropanoids, is largely deposited in secondary xylem cell walls. Angiosperm lignin is composed of monomeric guaiacyl and syringyl units, whereas gymnosperm lignin consists almost solely of guaiacyl units.[3,4] Many of the enzymes required for lignin biosynthesis have been characterized, but the mechanism of biosynthesis of syringyl lignin remains to be fully elucidated.[2,5]

One of the controversial aspects is whether the syringyl lignin monomer, sinapyl alcohol, is synthesized from sinapic acid (SA) through the action of 4-coumarate: coenzyme A (CoA) ligase (4CL, EC 6.2.1.12) followed by reduction by cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase.[2,6] 4CL catalyzes the conversion of cinnamic acid derivatives to their corresponding thioesters, which are central intermediates in pathways leading to a number of plant phenolics.[7,8] Therefore, it has been proposed that distinct 4CL isoenzymes acting on various substrates may play an important role in directing the metabolic flux to specific biosynthetic pathways of phenylpropanoids in plants.[9–12] 4CL isoenzymes and recombinant 4CL proteins have also been studied in the context of directing carbon flux into syringyl and guaiacyl lignin biosynthesis.

Results of recent investigations have shown that 4CL activity with SA as the substrate is extremely low or nondetectable.[9,11,13–17] These observations have led to the hypothesis that the route of biosynthesis of sinapyl alcohol may be not via 4CL action from SA. In addition to these observations, it has been reported recently that conversion of the guaiacyl nucleus to the syringyl nucleus through hydroxylation followed by methylation at the 5 position of the guaiacyl nucleus is catalyzed by 5-hydroxylase at the stage of cinnamyl aldehyde or alcohol.[18–21] These findings strongly suggest that an alternative 4CL-independent pathway for sinapyl alcohol biosynthesis may exist and call into question

whether SA is a true intermediate in the biosynthesis of sinapyl alcohol and syringyl lignin.

Plant cell cultures have often been used as models for investigating physiological changes associated with metabolism[22] and the pathways involved in the biosynthesis of phenylpropanoids including lignin (for review see Dixon and Paiva[7] and Elland-Ivey and Douglar,[23] and the references therein) because the callus cultures are sensitive to many types of stress [e.g., ultraviolet (UV) irradiation, elicitation, wounding, and water stress], and the responses to these stimuli are synchronously inducible. Furthermore, the metabolites, enzyme activities, and mRNA transcripts produced during these responses are easily detected and quantified because of the extremely low background levels in untreated cell cultures. It is known that calli grown under optimum growth conditions neither accumulate as much lignin as that found in parent plants nor produce as much of the phenylpropanoids; and the syringyl lignin content in an angiosperm callus is much lower than that in the corresponding woody plant.[24–26] These observations suggest that expression of the enzyme activity specifically involved in syringyl monolignol biosynthesis, including 5-hydroxylase, might be suppressed in the callus. Therefore, incorporation of the labeled syringyl precursor exogenously provided in the callus lignin could be easily detected. To address the question of whether SA is a precursor of sinapyl alcohol and syringyl lignin, we investigated the incorporation of deuterated and nondeuterated SA and ferulic acid (FA) into lignin in poplar (*Populus alba* L.) callus culture. These results indicate that SA was not a precursor of syringyl lignin, but it may induce or enhance syringyl lignin biosynthesis in poplar callus.

Materials and methods

Plant materials and chemicals

The poplar (*Populus alba* L.) callus used in this study was induced to develop on Murashige and Skoog basal medium supplemented with 3% sucrose, 1.0 ppm 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 ppm kinetin, and 0.8% agar. The callus was maintained on the same medium at 25°C in the dark.[25] All chemicals, extra pure grade, were purchased and used without further purification except for FA and SA. Both FA and SA were used after purification by recrystallization using a hot methanol–water mixture. Tetradeuteroferulic acid (DFA) (β-D, ring 3-OCD_3) and heptadeuterosinapic acid (DSA) (β-D, ring 3,5-diOCD_3) were synthesized by a method reported elsewhere.[27]

Administration of labeled and unlabeled cinnamic acid derivatives to poplar callus

Unlabeled FA and SA were individually dissolved in 1,4-dioxane/water (9:1, v/v), and an aliquot (5 ml) of the FA or SA solution was added to the medium to obtain a final concentration of 0.10, 0.25, or 0.50 g/l. In the experiments examining the lignin content, 3- and 5-week culture periods were employed. In those involving administration of labeled DFA or DSA to the callus, the final concentration of DFA or DSA in the medium was 0.5 g/l, and a 3-week culture period was employed. In all cases, a specimen excised from a 4-week-old callus after subculture was transferred to the test medium and cultured under the conditions described above.

Quantification of lignin and cell wall-bound cinnamic acid derivatives

After the scheduled cultivation period, the callus was harvested and subjected to extraction with a 10-fold volume of methanol (w/w) for 24 h. The resulting cell wall residue was further analyzed. The lignin content of the callus cell wall was determined by the standard acetyl bromide method.[28] The calibration coefficient was determined from the absorbance at 280 nm versus the lignin content using extractives-free beech (*Fagus crenata*) wood meal.

The callus cell wall preparation was subjected to alkaline hydrolysis, and the hydrolysate was analyzed by gas chromatography (GC) to determine the amount of cinnamic acid derivatives bound to the cell wall via ester and ether linkages. The callus cell wall residue (ca. 100 mg) was treated with 10 ml of 4 N NaOH at 170°C for 2 h.[29] Acetoguaiacone-ethanol solution 1 ml (1 mg/ml) was added to the reaction mixture as an internal standard; then insoluble material was removed by filtration. The filtrate was acidified by adding HCl, saturated with NaCl, and then extracted three times with diethyl ether. The organic layer was recovered and dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. The residue was dissolved in pyridine, then silylated by treatment with *N*, *O*- bis(trimethylsilyl)trifluoroacetamide. The silylated sample was analyzed using a GC-14A gas chromatograph (Shimadzu, Japan) equipped with FID and a capillary column (TC-1, 0.25 mm i.d. × 30 m; GL Sciences). Helium was used as the carrier gas. The column temperature was raised from 200° to 250°C at a rate of 5°C/min, and the injection port temperature was 250°C.

Thioacidolysis of callus cell wall material

Thioacidolysis of callus cell wall material was performed as described by Matsui et al.[30] Approximately 10 mg of cell wall material was treated with 5 ml of dioxiane/ethanethiol (9:1, v/v) containing 0.2 N BF_3 etherate at 100°C for 4 h. After cooling, an aliquot (0.5 ml) of docosane dissolved in dichloromethane (29.5 μg/ml) was added as an internal standard. The reaction mixture was neutralized with 0.4 N $NaHCO_3$ to stop the reaction and adjusted to pH 3 by adding HCl. The mixture was extracted with dichloromethane. The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The silylated sample was subjected to analysis using a GC-14A gas chromatograph and a GC-mass spectrometer (GC-MS) with a TC-1 capillary column (GL Sciences). During both analyses, the column

temperature was programmed to increase from 180° to 280°C at 2°C/min. In the GC-MS analysis, mass spectra were recorded at 70 eV with a QP 5050 (Shimadzu) equipped with a GC-17A (Shimadzu). Selective ion monitoring was also performed during GC-MS analysis of the products.

Results

Lignin accumulation and cell wall-bound cinnamic acid derivatives in calli treated with ferulic or sinapic acid

The poplar calli were grown on culture medium supplemented with FA or SA at various concentrations. The calli were more brownish than the control callus, and the color become darker as the FA or SA concentration was increased. The appearance of the callus on the control medium containing dioxane was the same as that of the callus cultured in the absence of dioxane (data not shown).

Calli were extensively extracted with methanol to remove free substrate and then analyzed for polyphenolics by the acetyl bromide method. The resulting phenolic polymer content was taken as the lignin content of the callus (Table 1). The callus lignin content increased as the concentration of FA or SA increased in the medium (Table 1). After 3 weeks of incubation with FA or SA (0.5 g/l), the callus lignin contents reached 11.1% and 12.4% of the dry cell wall weight, respectively; similar results were obtained after 5 weeks of incubation.

Cinnamic acid derivatives are often detected in the cell walls of *Gramineae*, and these free acids are bound to either cell wall carbohydrates or lignin via ester and ether linkages.[31–33] During suppression of 4CL activity in transgenic tobacco[34,35] and *Arabidopsis*,[16] accumulation of hydroxycinnamic acid derivatives at extraordinarily high concentrations in the cell walls has been observed. In our experiments, exogenously supplied FA and SA could have accumulated as cell wall-bound cinnamic acid derivatives. To investigate this possibility, the callus cell wall material was treated with conditions (4 N NaOH at 170°C for 2 h) that release ester- or ether-linked cinnamic acid derivatives from the cell wall.[29] GC analysis of the samples after alkaline hydrolysis showed that the amounts of FA and SA bound to the cell walls in the SA-treated callus were as low as that in the control callus. FA liberated from the cell walls of the FA-treated callus increased compared to that in the control, but the amount was estimated to be only 1.6% based on the lignin content (Table 2). The amount of SA released was not significantly increased in FA-treated callus. The observation that treated calli have increased lignin content and low amounts of cell wall-bound cinnamic acid derivatives suggests that exogenously supplied cinnamic acid derivatives are converted to callus lignin in vivo. Confirmation of this interpretation was obtained from labeling studies, as described below.

Administration of DFA and DSA to poplar calli

We employed thioacidolysis in the analysis of lignin in the calli treated with cinnamic acid derivatives. The β-aryl ether linkage is the most abundant linkage among several formed by radical coupling of monolignols; it is estimated that roughly half of the total linkages in lignin are of this type.[36] Thioacidolysis involves two reactions: cleavage of the

Table 1. Lignin content of poplar calli

Culture period	Lignin (% by weight)[a]	
	3 weeks	5 weeks
Control	5.7 ± 0.1	6.3 ± 0.3
FA administered (g/l)		
0.10	7.6 ± 0.4	8.0 ± 0.1
0.25	9.4 ± 0.3	8.6 ± 0.5
0.50	11.1 ± 0.6	8.8 ± 0.6
SA administered (g/l)		
0.10	8.3 ± 0.2	8.6 ± 0.1
0.25	10.8 ± 0.2	10.5 ± 0.4
0.50	12.4 ± 0.5	11.7 ± 0.4

The calli were grown on medium containing various concentrations of ferulic acid (FA) or sinapic acid (SA)

[a] Lignin content is expressed as a percentage of the total dry weight of cell wall material. The initial callus lignin content was 4.2% ± 0.3%. Data shown are means of duplicate analyses

Table 2. Yields of cell wall-bound cinnamic acid derivatives obtained from the callus through alkali treatment

Sample	Ferulic acid		Sinapic acid	
	μmol/g[a]	Percent of total lignin[b]	μmol/g[a]	Percent of total lignin[b]
Control	0.68 ± 0.62	0.24 ± 0.22	0.75 ± 0.05	0.31 ± 0.02
FA-treated callus	8.14 ± 0.67	1.61 ± 0.13	0.84 ± 0.37	0.19 ± 0.08
SA-treated callus	1.04 ± 0.87	0.20 ± 0.17	1.19 ± 0.12	0.26 ± 0.03

The calli were grown on medium containing ferulic acid or sinapic acid (0.5 g/l) for 3 weeks and extracted with methanol before alkaline treatment. In all cases the values shown are the mean ± SD for three samples

[a] The yield of FA or SA is expressed as micromoles per gram of methanol-extracted dry cell wall material

[b] The yield of FA or SA was calculated as a percentage of the total lignin in each callus

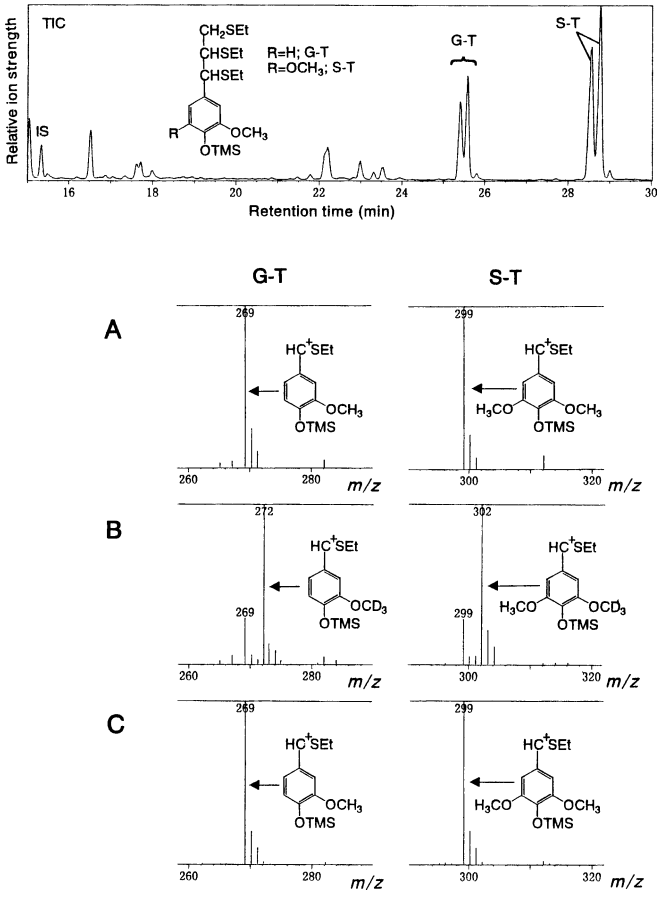

Fig. 1. Mass spectra of thioacidolysis products from poplar callus treated with deuterolabeled tetradeuteroferulic acid (DFA) or heptadeuterosinapic acid (DSA). Calli were grown on medium with or without (control) DFA or DSA (0.5 g/l) for 3 weeks and then extracted with methanol before gas chromatography-mass spectrometry (GC-MS) analysis. **A** Thioacidolysis products from poplar xylem. **B** Thioacidolysis products from a callus treated with DFA. **C** Thioacidolysis products from a callus treated with DSA. *G-T*, 1-guaiacyl-1,2,3-trithioethylpropane; *S-T*, 1-syringyl-1,2,3-trithioethylpropane; *TIC*, total ion chromatography

β-aryl ether (β-*O*-4) linkage and formation of thioether derivatives of the degradation products.[37] The monomers 1-guaiacyl-1,2,3-trithioethylpropane (G-T) and 1-syringyl-1,2,3-trithioethylpropane (S-T) are the principal degradation products generated by thioacidolysis from guaiacyl and syringyl subunits, respectively, in lignin.[37]

The thioacidolysis products from the calli treated with DFA or DSA were subjected to GC-MS analysis, and mass spectra of G-T and S-T from each callus were compared to those from poplar xylem (Fig. 1). In the case of poplar xylem, the base ion peaks, *m/z* 269 in G-T and *m/z* 299 in S-T (Fig. 1A), were attributable to the fragments as benzylthioethyl ions derived from G-T and S-T, respectively, by Cα-Cβ cleavage.[37] Thus, we tried to confirm the conversion of SA and FA by stable isotope labeling. The hydrogen atoms of the methoxyl group on the aromatic nucleus were almost fully substituted with deuterium. In the case of DFA administration, assuming that the DFA is incorporated into the callus lignin, the base ion peaks for G-T or S-T should be 3 mass units larger (*m/z* 272 and 302) than those for unlabeled G-T (*m/z* 269) or S-T (*m/z* 299). Similarly, if administered DSA was directly reduced to sinapyl alcohol and incorporated into syringyl lignin in the callus, the base-ion peak for the labeled S-T should appear at a position corresponding to 6 mass units larger (*m/z* 305) than that for unlabeled S-T. The labeling of two methoxyl groups in SA would help to identify the route by which administered DSA was converted to sinapyl alcohol. If the labeled DSA was converted to 5-hydroxyferulic acid and reconverted to a syringyl type of precursor, the base-ion peak for S-T from callus lignin should be 3 mass units larger (*m/z* 302) than that for unlabeled S-T. Alternatively, if the administered DSA was converted to FA and was incorporated into both guaiacyl and syringyl lignin, the base-ions for G-T and S-T from callus lignin should be 3 mass units larger (*m/z* 272 and 302) than those for unlabeled G-T and S-T, respectively.

The mass spectrum of G-T from DFA-treated callus has an obvious base ion at *m/z* 272 (Fig. 1B) that is attributable to a fragment from trideuterated G-T. Similarly, a base peak at *m/z* 302 was observed for S-T from DFA-treated callus (Fig. 1B). These results indicate that exogenous DFA was effectively converted to coniferyl and sinapyl alcohols, which were then incorporated into the callus lignin. In contrast, in the mass spectrum of S-T from DSA-treated callus, the base ion peak appeared at *m/z* 299 but no peak was observed at *m/z* 305 or *m/z* 302 (Fig. 1C). The mass spectrum of G-T yielded no detectable evidence of DSA incorporation into callus lignin (Fig. 1C). Thus, the mass spectra of S-T and G-T from DSA-treated callus indicate that exogenous SA was not converted to sinapyl alcohol. This finding appears to conflict with the results in Table 1, which indicate an increase in callus lignin as a result of SA administration.

To address this discrepancy, we compared the relative yields of G-T and S-T by the selective ion monitoring method (Fig. 2). In the case of DFA-treated callus, yields of G-T and S-T monitored at *m/z* 269 and 272 and at *m/z* 299 and 302, respectively, increased substantially compared to those for the control callus (Fig. 2A,B). A markedly higher yield of S-T, but not G-T, was observed for DSA-treated callus compared to the control callus (Fig. 2A,C). Therefore, we concluded that SA induces or enhances syringyl, but not guaiacyl, lignin biosynthesis in poplar callus. In contrast, exogenous DFA is converted to syringyl and guaiacyl lignins after enzymatic reduction to the corresponding monolignols in vivo.

Monomeric composition of lignin in calli treated with ferulic or sinapic acid

The yields of G-T and S-T were compared (Table 3) to determine the monomeric composition of the lignin in calli treated with cinnamic acid derivatives. G-T and S-T yields from the control callus were quite low compared to those from wood. The G-T and S-T yields from the callus treated with FA increased to 7.3 and 10.2 μmol/g, respectively, cor-

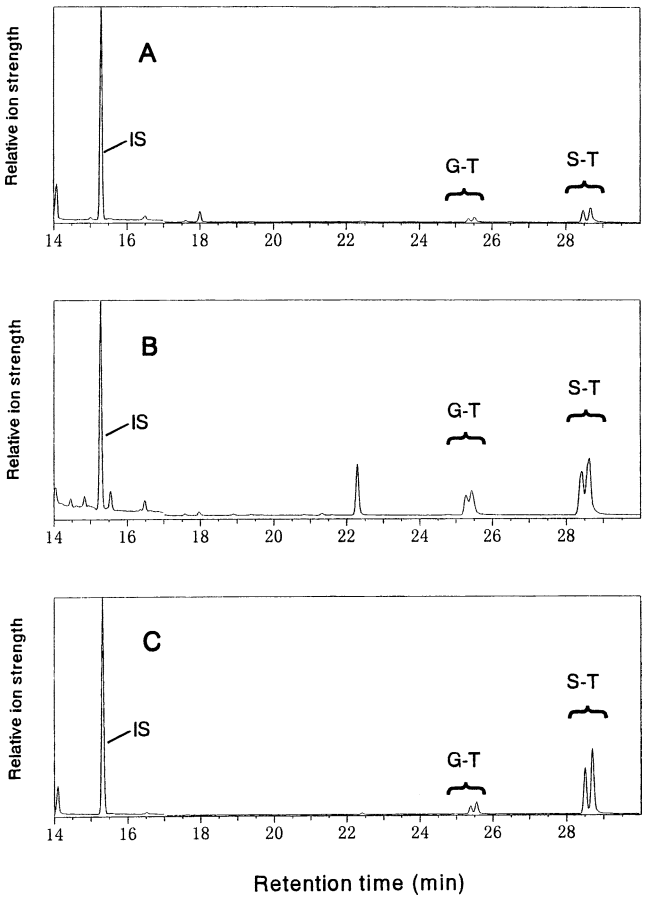

Fig. 2. GC-MS analysis of poplar callus thioacidolysis products from control (**A**), DFA-treated (**B**), and DSA-treated (**C**) calli. Calli were grown on medium with or without (control) DFA or DSA (0.5 g/l) for 3 weeks and then extracted with methanol before GC-MS analysis. For control and DSA-treated calli, selective ion monitoring at *m/z* 269 and 299 was employed for detection of G-T and S-T, respectively. For the DFA-treated callus, selective ion monitoring at *m/z* 269 and 272 was employed for detection of G-T and at *m/z* 299 and 302 for detection of S-T. In all cases, the internal standard was monitored at *m/z* 71. *IS*, internal standard (docosane)

Table 3. Yields of thioacidolysis products from poplar calli

Sample	G-T (μmol/g)	S-T (μmol/g)	S/G[a]
Control	0.46 ± 0.27	0.97 ± 0.54	2.1
FA-treated callus	7.31 ± 0.06	10.24 ± 2.11	1.4
SA-treated callus	1.91 ± 0.54	8.53 ± 2.86	4.5

Samples were the same as those shown in Table 2
Data are means ± SD of duplicate analyses
The yield is expressed as micromoles per gram of methanol-extracted dry cell wall material
G-T, 1-guaiacyl-1,2,3-trithioethylpropane; S-T, 1-syringyl-1,2,3-trithioethylpropane
[a] Ratio calculated as S-T/G-T

responding to about 16-fold and 11-fold the yield from each control callus. A markedly high yield of S-T (8.53μmol/g) from the callus treated with SA, which is ninefold that of the control, was also observed. This observation is consistent with the finding that the yields of G-T and S-T from DFA-treated callus increased, as did the yield of S-T from DSA-treated callus (Fig. 2). These quantitative results support our interpretation of the results of the experiments using deutero-labeled cinnamic acid derivatives, indicating that addition of SA resulted in an increase in only the syringyl subunits in the callus.

Discussion

Because CoA esters of hydroxycinnamic acid derivatives have been shown to be converted to the corresponding cinnamyl aldehydes and further to cinnamyl alcohols, cinnamoyl-CoA esters are thought to be intermediates produced during biosynthesis of cinnamyl alcohols.[2,5,6,38] In tracer experiments using deutero-labeled SA combined with GC-MS analysis of callus cell wall material, it was not converted to syringyl lignin in poplar callus. We did not detect conversion of labeled SA to guaiacyl lignin in the callus; therefore, in vivo conversion of SA to sinapyl alcohol or of SA to FA may not occur in poplar callus. Recent studies[19,39] have indicated that recombinant guaiacyl-5-hydroxylases have low affinity for FA compared to coniferyl aldehyde and coniferyl alcohol; hence, a different syringyl lignin biosynthetic pathway has been proposed that does not require SA as a precursor of sinapyl alcohol and syringyl lignin. Our results provide strong support for the view that this proposed pathway exists in poplar callus.

Lignin is essential for all plants growing on land, so the lignin biosynthetic pathway might have high plasticity and might be connected by many metabolic grids. In fact, some routes for conversion of the guaiacyl nucleus to the syringyl nucleus at different stages, involving cinnamyl aldehydes or alcohols, have been proposed.[18–21,40] Regardless of the stage at which this conversion occurs, the essential enzymes are guaiacyl-5-hydroxylase and *O*-methyltransferase. GC-MS analysis of the cell wall material of poplar callus treated with DFA demonstrated that conversion of guaiacyl subunits to syringyl subunits readily occurs in poplar callus. This suggests that guaiacyl-5-hydroxylase and enzymes acting subsequently in the pathway for biosynthesis of sinapyl alcohol may be functioning sufficiently in the FA-treated callus. We also demonstrated that exogenously supplied SA is not used as a precursor of sinapyl alcohol per se but that it induces or enhances only syringyl lignin biosynthesis in the callus. In the present study, we easily detected the in vivo conversion of supplied FA or SA into callus lignin because of the low lignin content, particularly the low syringyl lignin content, of the callus. Callus culture is a good model system for our ongoing studies on lignin biosynthesis where changes in enzymes related to lignin biosynthesis as a result of adding cinnamic acid derivatives are being investigated, especially focusing on the induction of sinapyl alcohol biosynthesis.

Acknowledgment This study was supported in part by a grant-in-aid from the Scientific Research Fund (10760106) of the Ministry of Education, Science, Sports, and Culture of Japan.

References

1. Baucher M, Monties B, Montagu MV, Boejan W (1998) Biosynthesis and genetic engineering of lignin. Crit Rev Plant Sci 17:125–197
2. Douglas CJ (1996) Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. Trends Plant Sci 1:171–178
3. Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41:455–496
4. Higuchi T (1997) Biochemistry and molecular biology of wood. Springer, Berlin Heidelberg New York, pp 93–96
5. Higuchi T (1997) Biochemistry and molecular biology of wood. Springer, Berlin Heidelberg New York, pp 131–236
6. Whetten RW, MacKay JJ, Sederoff RR (1998) Recent advantages in understanding lignin biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 49:585–609
7. Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097
8. Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Physiol Plant Mol Biol 40:347–469
9. Ehlting J, Buttner D, Wang Q, Douglas CJ, Somssich IE, Kombrink E (1999) Three 4-coumarate: coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. Plant J 19:9–20
10. Grand C, Boudet A, Boudet AM (1983) Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems properties and tissue distribution. Planta 158:225–229
11. Hu WJ, Kawaoka A, Tsai CJ, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). Proc Natl Acad Sci USA 95:5407–5412
12. Knobloch KH, Hahlbrock K (1975) Isoenzymes of *p*-coumarate:CoA ligase from cell suspension cultures of *Glycine max*. Eur J Biochem 52:311–320
13. Allina SM, Pri-Hadash A, Theilmann DA, Ellis BE, Douglas CJ (1998) 4-Coumarate:coenzyme A ligase in hybrid poplar: properties of native enzymes, cDNA cloning, and analysis of recombinant enzymes. Plant Physiol 116:743–754
14. Lee D, Douglas CJ (1996) Two divergent members of a tobacco 4-coumarate:coenzyme A ligase (4CL) gene family: cDNA structure, gene inheritance and expression, and properties of recombinant proteins. Plant Physiol 112:193–205
15. Lee D, Ellard M, Wanner LA, Davis KR, Douglas CJ (1995) The *Arabidopsis thaliana* 4-coumarate:CoA ligase (*4CL*) gene: stress and developmentally regulated expression and nucleotide sequence of its cDNA. Plant Mol Biol 28:871–884
16. Lee D, Meyer K, Chapple C, Douglas CJ (1997) Antisense suppression of 4-coumarate:coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition. Plant Cell 9:1985–1998
17. Zhang XH, Chiang VL (1997) Molecular cloning of 4-coumarate:coenzyme A ligase in loblolly pine and the roles of this enzyme in the biosynthesis of lignin in compression wood. Plant Physiol 113:65–74
18. Chen F, Yasuda S, Fukushima K (1999) Evidence for a novel biosynthetic pathway that regulates the ratio of syringyl to guaiacyl residues in lignin in the differentiating xylem of *Magnolia kobus* DC. Planta 207:597–603
19. Humphreys JM, Hemm MR, Chapple C (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc Natl Acad Sci USA 96: 10045–10050
20. Matsui N, Chen F, Yasuda S, Fukushima K (2000) Conversion of guaiacyl to syringyl moieties on the cinnamyl alcohol pathway during the biosynthesis of lignin in angiosperms. Planta 210:831–835
21. Osakabe K, Tsao CC, Li L, Popko JL, Umezawa T, Carraway DT, Smeltzer RH, Joshi CP, Chiang VL (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc Natl Acad Sci USA 96:8955–8960
22. Muhlbach HP (1998) Use of plant cell cultures in biotechnology. Biotechnol Annu Rev 4:113–176
23. Ellard-Ivey M, Douglas CJ (1996) Role of jasmonates in the elicitor- and wound-inducible expression of defense gene in parsley and transgenic tobacco. Plant Physiol 112:183–192
24. Fukuda H (1983) Studies on tissue culture of tree cambium. IX. Characterization of callus lignin from black locust. Mokuzai Gakkaishi 29:74–81
25. Tsutsumi Y, Sakai K (1993) Lignin biosynthesis in woody angiosperm tissues. I. Lignification and peroxidase activity stimulated in water-stressed *Populus* callus cultures. Mokuzai Gakkaishi 39:214–220
26. Venverloo CJ (1969) The lignin of *Populus nigra* L. CV "Italica": a comparative study of the lignified structures in tissue cultures and the tissues of the tree. Acta Bo Neel 18:241–314
27. Yamauchi K, Seiichi Y, Kazuhiko F (2002) Evidence for the biosynthetic pathway from sinapic acid to syringyl lignin using labeled sinapic acid with stable isotopeat both methoxy groups in *Robinia pseudoacacia* and *Nerium indicum*. J Agric Food Chem 50:3222–3227
28. Johnson DB, Moore WE, Zank LC (1961) The spectrophotometric determination of lignin in small wood samples. TAPPI 44:793–798
29. Lam TBT, Iiyama K, Stone BA (1992) Cinnamic acid bridges between cell wall polymers in wheat and phalaris internodes. Phytochemistry 31:1179–1183
30. Matsui N, Fukushima K, Kamada K, Nishikawa Y, Yasuda S (1994) On the behavior of monolignol glucosides. I. Synthesis of monolignol glucosides labeled with ^{2}H at the hydroxymethyl group of side chain, and polymerization of the labeled monolignol in vitro. Holzforschung 48:215–221
31. Grabber JH, Quideau S, Palph J (1996) *p*-Coumaroylated syringyl units in maize lignin: implications for β-ether cleavage by thioacidolysis. Phytochemistry 43:1189–1194
32. Iiyama K, Lam TBT, Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry 29:733–737
33. Scalbet A, Monties B, Lallemand J-Y, Guittet E, Roland C (1985) Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry 24:1359–1362
34. Kajita S, Hishiyama S, Tomimura Y, Katayama Y, Omori S (1997) Structural characterization of modified lignin in transgenic tobacco plants in which the activity of 4-coumarate:coenzyme A ligase is depressed. Plant Physiol 114:871–879
35. Kajita S, Katayama Y, Omori S (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate:coenzyme A ligase. Plant Cell Physiol 37:957–965
36. Lai YZ, Sarkanen KV (1971) Isolation and structural studies. In: Sarkanen KV, Ludwig CH (eds) Lignins. Wiley-Interscience, New York, pp 165–240
37. Lapierre C, Monties B, Roland C (1985) Thioacidolysis of lignin: comparison with acidolysis. J Wood Chem Technol 5:277–292
38. Baucher M, Monties B, Montagu MV, Boejan W (1998) Biosynthesis and genetic engineering of lignin. Crit Rev Plant Sci 17:125–197
39. Li L, Popko JL, Umezawa T, Chiang VL (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. J Biol Chem 275:6537–6545
40. Li L, Cheng XF, Leshkeich J, Umezawa T, Harding SA, Chiang VL (2001) The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13:1567–1585