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MECHANISM OF FORMATION OF SYRINGYL COMPONENTS IN LIGNIN

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Key Word Index—*Phyllostachys pubescens*; *Miscanthus sinensis*; Gramineae; biosynthesis; lignin; ferulic acid; methoxylation; demethoxylation.

Abstract—Various labelled compounds including ferulic acid–$O^{14}CH_3$ were administered to a bamboo and a grass to study the biosynthesis of syringyl lignin. It was found that ferulic acid was demethoxylated to *p*-coumaric acid in sliced bamboo tissues. However, the analytical data obtained by nitrobenzene oxidation and ethanolysis of the fed plant showed that ferulic acid–$O^{14}CH_3$ was incorporated into syringyl units as well as into guaiacyl units without rearrangement of the labelled methoxyl group. This finding supports the early indication that ferulic acid can serve as a precursor of syringyl lignin.

INTRODUCTION

IT IS presumed that ferulic acid (FA) is a natural intermediate of sinapic acid (SA) since both FA[1–3] and 5-hydroxy-ferulic acid (5-HFA)[4] were incorporated into syringyl components of lignin molecules. However, a number of problems still remain to be investigated. For example, the evidence for enzymatic conversion of FA to 5-HFA has not yet been provided. Also, it is still uncertain whether FA administered to plants is incorporated via 5-HFA into syringyl components of lignin with the methoxyl group retained intact or via both caffeic acid (CA) and 3,4,5-trihydroxycinnamic acid (THC) after demethylation and/or demethoxylation of FA (Scheme 1). In fact, the demethylation and demethoxylation of FA or SA fed to plants has been observed by many investigators. Kratzl and Billek[5] reported that radioactive syringin was incorporated into guaiacyl components of spruce lignin. Reznik and Urban[6,7] observed the conversion of FA–3-^{14}C to the CA moiety of chlorogenic acid in both wheat and red cabbage. Brown and Neish[3] recognized that SA-3-^{14}C was partly incorporated into guaiacyl components, isolated as dihydroconiferyl alcohol. Conversion of SA to FA was also reported by Higuchi and Brown.[4] El-Basyouni *et al.*[8] found radioactive vanillin and *p*-hydroxybenzaldehyde after feeding FA–3-^{14}C and SA-3-^{14}C to wheat plants. Recently, Steiner[9] has reported that demethylation of SA occurs in biosynthesis of delphinidin in petunia flowers. He also observed the conversion of SA to FA and to CA. These results show that the methyl ethers, FA and SA are easily degraded by plants. Again, THC may serve as a natural precursor for SA. Thus, bamboo *O*-methyltransferase[10,11] utilizes THC as a potent substrate, yielding 5-HFA and SA and Meier and Zenk[12] reported that THC might be formed from CA, showing that THC was

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efficiently incorporated into delphinidin than CA. Then, apart from a biosynthetic relationship between delphinidin and lignins, it is necessary to clarify the mechanism of formation of SA not only with FA labelled at the side chain or on the benzene ring but also with FA labelled at the methyl group. With the former labelled compounds, it is impossible to obtain the exact information on the removal of the methyl group of FA. However, FA-O$^{14}CH_3$ has not yet been tested in lignin biosynthesis, although Hess used it for studying the biosynthesis of anthocyanins in petunia.[13]

The present investigation has two aims. The first is to determine whether or not FA fed to bamboo shoot is converted to other hydroxycinnamic acids by demethylation or by demethoxylation.[6–9] The second is to see whether FA–O$^{14}CH_3$ is incorporated via 5-HFA into syringyl components of lignins with the methoxyl group intact.

RESULTS AND DISCUSSION

The R_f values of the hydroxycinnamic acids and the location of their radioactivities are shown in Table 1. Radiochromatographic patterns show that FA administered to sliced tissues of bamboo shoot was converted to PCA and probably to 5-HFA. However, no radioactive CA was obtained. Radioactive PCA isolated from the chromatogram was recrystallized from hot water after addition of 20 mg of cold PCA until the specific activity was constant (Table 2). Thus, FA–2-^{14}C was demethoxylated to PCA in bamboo tissues. Similar attempts to crystallize radioactive 5-HFA were unsuccessful.

TABLE 1. R_f VALUES OF HYDROXYCINNAMIC ACIDS AND LOCATION OF THEIR RADIOACTIVITY

Solvent	PCA	CA	FA	5-HFA	TCA
A	0·60	0·35	0·86	0·50	0·12
B	0·40	0·34	0·54	0·34	0·23
C	0·30	0·07	0·30	0·07	0·02
	Radioactivity located on the chromatograms				
A	+	−	+	+	−
B	+	−	+	+	−
C	+	+	+	+	−

A—TLC, $CHCl_3$–HOAc–H_2O (2:1:1); B—TLC, toluene–HCO_2Et–HCO_2H (5:4:1); C—PPC, Xylene-Me, Et, Ketone-HCO_2NH_2 (25:25:1).

Figure 1 shows the time courses of formation of PCA and compound X (5-HFA?) during incubation of the bamboo tissue with FA–2-^{14}C. Both compounds were found to be formed rather rapidly, showing that about 20% of FA fed was converted to them after 100

TABLE 2. CRYSTALLIZATION OF RADIOACTIVE *p*-COUMARIC ACID FORMED FROM FERULIC ACID–2-^{14}C

No. of crystallizations	Specific activity of PCA (cpm/mg × 10^3)
1	1
2	0·90
3	1
Background	0·06

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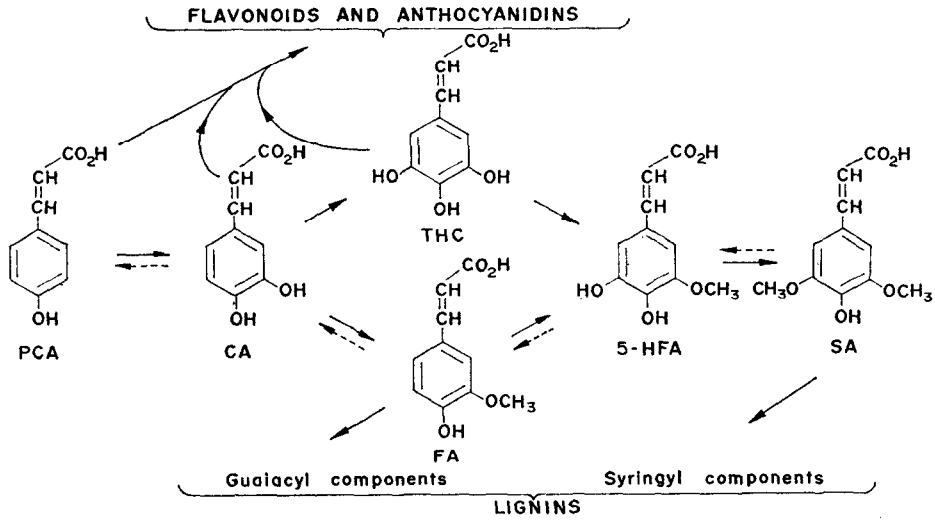

SCHEME 1. METABOLIC PATHWAYS OF HYDROXYCINNAMIC ACIDS IN FORMATION OF LIGNINS AND FLAVONOIDS.

min incubation. The fact that PCA was obtained from FA is in good agreement with the results of El-Basyouni *et al.*[8] and Steiner.[9] Although the mechanism of this 'demethoxylation' is unknown, it seems that hydroxycinnamic acids (Scheme 1) are relatively interconvertible. Therefore, the demethoxylation of FA indicated the importance of using the tracer compound labelled at the methyl group, i.e. FA–O$^{14}CH_3$, for biosynthetic studies on lignins. This labelled compound was prepared from CA and *S*-adenosylmethionine-$^{14}CH_3$ with bamboo *O*-methyltransferase. Identification of FA–O$^{14}CH_3$ was achieved by a recrystallization to constant specific activity (Table 3).

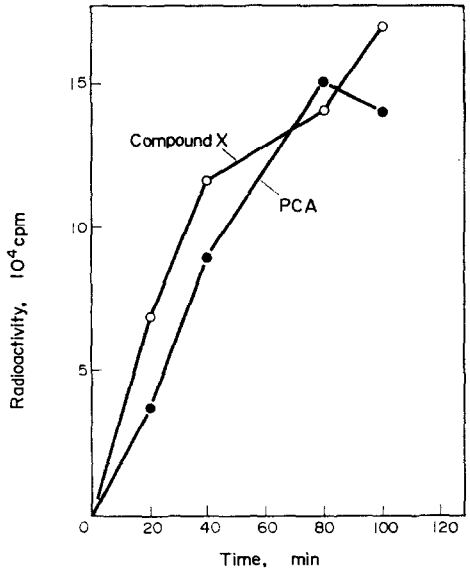

FIG. 1. TIME COURSE OF FORMATION OF *p*-COUMARIC ACID AND COMPOUND *X* FORMED FROM FERULIC ACID.

TABLE 3. CRYSTALLIZATION OF FA–O-$^{14}CH_3$ PREPARED WITH BAMBOO *O*-METHYLTRANSFERASE

No. of crystallization	Specific radioactivity of FA–O-$^{14}CH_3$ (cpm/mg × 10^3)
1	48
2	49
3	49
Back ground	0·05

After feeding the various labelled compounds to a grass, *Miscanthus sinensis*, vanillin (V) and syringaldehyde (S) were isolated by nitrobenzene oxidation. The yields, specific activities and dilution values for the aldehydes are given in Table 4. The ratios of the specific activities, S/V, obtained with FA–O$^{14}CH_3$ was found to be 0·33, which is nearly equal to the value obtained with phenylalanine (Phe)–U-^{14}C, indicating that the incorporation rate of FA–O$^{14}CH_3$ into syringyl components was as great as that of Phe–U-^{14}C.

TABLE 4. INCORPORATION OF FA–O-$^{14}CH_3$ AND PHENYLALANINE–U-^{14}C INTO GRASS LIGNIN

Compound administered	Yield of aldehydes* (μmol) V	S	Specific activity (μCi/mM) V	S	Dilution V	S
FA–O-$^{14}CH_3$ (1)	23·3	8·2	0·96	0·32	192	575
FA–O-$^{14}CH_3$ (2)	29·3	10·5	1·00	0·33	184	551
Phe–U-^{14}C	17·8	6·6	0·98	0·33	337	1115

* V—Vanillin; S—Syringaldehyde.

This result also indicates that the demethoxylation did not take place to such an extent as to lower the specific activity of S derived from FA–O$^{14}CH_3$. This assumption was further supported by the results obtained from an ethanolysis experiment (Table 5). The incorporation rate of FA–O$^{14}CH_3$ into vanilloyl methyl ketone(VMK) and syringoyl methyl ketone (SMK) was found to be slightly greater than any of those of FA–2-^{14}C, CA–2-^{14}C, and Phe–U-^{14}C. Yet no distinct differences in the ratio (SMK/VMK) were found, which is in good harmony with the results of nitrobenzene oxidation. The ratios (S/V) and (SMK/VMK) obtained in the present investigation are, as a rule, compatible with those

TABLE 5. INCORPORATION OF THE VARIOUS RADIOACTIVE COMPOUNDS INTO GRASS LIGNIN

Compound administered	Yield of ethanolysis products (μmol)* VMK	SMK	Specific activity (μCi/mM) VMK	SMK	Dilution VMK	SMK
FA–O-$^{14}CH_3$	13·6	7·6	1·52	1·16	121	159
FA–2-^{14}C	12·2	8·3	0·61	0·41	320	481
CA–2-^{14}C†	2·1	1·1	0·99	0·98	270	273
Phe–U-^{14}C†	2·5	1·7	0·97	0·58	340	572

* VMK—vanilloyl methyl ketone; SMK—Syringoyl methyl ketone.

† Most of the VMK and SMK was lost by accident. However, the values for the specific activity and dilution are valid for comparison with those obtained in other two sets of experiments.

(0·2–1·0) reported in early investigations.[1,2,4,14] It can therefore be concluded that most of FA administered to the two plants was incorporated into syringyl components of lignins without removal of the methyl group, although demethoxylation of FA might occur to some extent. Accordingly, the present results confirm the validity of FA as a natural intermediate of SA in biosynthesis of lignins.[13] Furthermore, THC is not involved in biosynthesis of lignins, in spite of the methylation of THC to SA by bamboo *O*-methyltransferase. Therefore, hydroxylation must have occurred at the stage of FA yielding 5-HFA, which is regarded as a diverging step on the biosynthetic pathways to lignins of angiosperms and gymnosperms.[11,15]

EXPERIMENTAL

Plant material. Sliced tissues from bamboo shoots (*Phyllostachys pubescens*) 1·5–2·0 m in height were used for the experiment on the demethoxylation of FA–2-^{14}C. Grass plants (*Miscanthus sinensis*) were used for the experiment on incorpoartion of various radioactive compounds into lignin molecules.

Radioactive compounds. L-Phenylalanine-U-^{14}C (405 mCi/mM) purchased from Daiichi Kagaku Co., was diluted with cold phenylalanine to 330 μCi/mM. *S*-Adenosylmethionine(SAME)-$^{14}CH_3$ with the specific activity of 52·3 mCi/mM was procured from New England Nuclear. CA–2-^{14}C (266 μCi/mM) and FA–2-^{14}C (195) μCi/mM) were synthesized according to the method of Neish.[16] FA–O-$^{14}CH_3$ (184 μCi/mM) was enzymatically prepared as described below.

Enzymatic preparation of FA–O-$^{14}CH_3$. Bamboo *O*-methyltransferase was obtained according to the procedures previously reported.[17] A reaction mixture contained 3·0 ml of the enzyme solution (0·1 M phosphate buffer, pH 8·0) containing 33 mg protein, 0·5 ml of SAME-$^{14}CH_3$ (10 μCi), 0·1 ml of cold SAME (0·25 μmol), 0·2 ml of CA (0·5 μmol), 0·1 ml each of 0·1 M $MgCl_2$, 0·1 M NaN_3, and 0·1 M iso-ascorbate. The reaction mixture was incubated for 30 min at 30°. After addition of 10 mg of FA as a carrier and 2·0 ml of 5% HCl into the mixture, FA-O-$^{14}CH_3$ formed was extracted with ether. After evaporation of the ether the residue was dissolved in 10 ml of EtOH. The EtOH solution (9·5 ml) was evaporated to dryness and the residue used for feeding experiment. The remaining solution (0·5 ml) was analyzed in order to identify FA–O-$^{14}CH_3$ as follows: (*a*) The concentration of FA–O-$^{14}CH_3$ in the EtOH was determined by measurement of the absorbance at 323 nm. Alternatively, the radioactivity was measured with a Beckman LS-100 scintillation counter. From both values obtained the specific radioactivity (μCi/mM) of FA–O-$^{14}CH_3$ was calculated. (*b*) A portion of FA–O-$^{14}CH_3$ solution was submitted to PC with toluene–HOAC–H_2O (4:1:5) and TLC with $CHCl_3$–HOAc–H_2O (2:1:1) and with toluene–Et–HCO_2H (5:4:1). After scanning the radioactivity on the chromatograms by use of radiochromatogram scanner (Aloka PCS-4) or the Beckman scintillation counter, radioactive FA was located on the chromatograms. (*c*) To the residual portion of the FA–O-$^{14}CH_3$ solution, 20 mg of cold FA was added and crystallized from hot water to constant specific radioactivity.

Experiment on demethoxylation of FA. The tissue slices (5 g) from the bamboo shoots were infiltrated *in vacuo* with 0·5 ml of a solution containing each 10 μmol of FA–2-^{14}C (1·86 μCi), iso-ascorbate, cysteine, and NaN_3. The mixture was incubated for 1 hr at room temp. unless otherwise stated. The incubation was run in duplicate. After incubation, the tissues were homogenized with 95% EtOH and the homogenate filtered. The filtrate was evaporated and the resiiue was dissolved in 0·5 ml of 5% $NaHCO_3$, from which an acid fraction containing free hydroxycinnamic acids was obtained by the usual procedures. This fraction was submitted to TLC as described above. The radiochromatographs were obtained by measurement of radioactivities in silicagel which was scraped off at 5 mm intervals from the TLC plates.

Experiment on incorporation of the labelled compounds into lignin. The radioactive compounds such as FA–O-$^{14}CH_3$ (9·0 μCi, 9·5 mg), CA–2-^{14}C (15 μCi, 10 mg), FA–2-^{14}C (9·3 μCi, 10 mg) and phenylalanine–U-^{14}C (20 μCi, 10 mg) in 3·0 ml of water containing 0·2 ml of 5% $NaHCO_3$ were individually administered to four lots of plants in sunlight and metabolized for 30 hr after the complete inbibition of the solutions. The former two compounds were fed to 30 g of the fresh plants each and the latter to 15 g of the fresh plants. The plants in every lot was cut into small pieces, which were homogenized with hot 95% EtOH. The cell wall residues were used, after extraction with EtOH–benzene (1:1), for alkaline nitrobenzene oxidation and ethanolysis.

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Analyses of lignin aldehydes. The cell wall residues obtained from the plants fed with FA–O-$^{14}CH_3$ and phenylalanine–U-^{14}C was submitted to the alkaline nitrobenzene oxidation by the procedure of Stone *et al.*[18] The radioactive lignin aldehydes such as vanillin and syringaldehyde were isolated from the reaction mixture by alternate extractions with ether and 20% $NaHSO_3$. The aldehydes were purified by TLC, firstly with water-saturated isopropyl ether, then with $CHCl_3$–*n*-hexane (5:2) and again with the first solvent. The purified vanillin and syringaldehyde were eluted from the chromatograms and determined by the measurement of the absorbances in EtOH at 278 nm and 308 nm, respectively. At the same time, UV spectra of both aldehydes were taken and identified with those of the authentic specimens. Alternatively, the radioactivities of the aldehydes were measured with the liquid scintillation counter. Their specific activities were calculated from these data.

Analyses of ethanolysis products. Ethanolysis of the plant residues was carried out according to the procedure by Kratzl *et al.*[19] except that milled wood lignin (bamboo MWL) equal to 20% of the weight of the residue was added to each reaction mixture. Ethanol lignin and crude ethanolysis oil were separated from the reaction mixture. The crude oil was oxidized with $FeCl_3$ in EtOH. The diketones in the resulting mixture were separated by TLC, firstly with water-saturated iso-propyl ether and secondly with benzene. Vanilloyl methyl ketone and syringoyl methyl ketone in EtOH were determined by measurement of the absorbances at 326 and 320 nm, respectively. The UV spectra of the two diketones were taken at the same time and identified with those of the authentic specimens. The radioactivities of the diketones were determined with the scintillation counter. The specific activities were calculated from the values obtained.

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