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ARTICLE

Biosynthesis of Vanillin via Ferulic Acid in *Vanilla planifolia*

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^{14}C-Labeled phenylalanine, 4-coumaric acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, ferulic acid, and methionine were applied to disks of green vanilla pods 3 and 6 months after pollination (immature and mature pods), and the conversion of these compounds to vanillin or glucovanillin was investigated. In mature green vanilla pods, radioactivities of 11, 15, 29, and 24% from ^{14}C-labeled phenylalanine, 4-coumaric acid, ferulic acid, and methionine, respectively, were incorporated into glucovanillin within 24 h. In the incorporation processes of methionine and phenylalanine into glucovanillin, some of the ^{14}C labels were also trapped by the unlabeled ferulic acid. However, ^{14}C-labeled 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol were not converted to glucovanillin. On the other hand, in immature green vanilla pods radioactivities of the above six compounds were not incorporated into glucovanillin. Although 4-coumaric acid, ferulic acid, 4-hydroxybenzaldehyde, and 4-hydroxybenzyl alcohol were converted to the respective glucose esters or glucosides and vanillin was converted to glucovanillin, their conversions were believed to be from the detoxication of the aglycones. These results suggest that the biosynthetic pathway for vanillin is 4-coumaric acid → → ferulic acid → → vanillin → glucovanillin in mature vanilla pods.

KEYWORDS: ***Vanilla planifolia*; biosynthesis; vanillin; glucovanillin; ferulic acid**

INTRODUCTION

Vanillin is a compound with a sweet smell that has been used as a flavor and fragrance since ancient times. Vanillin is accumulated as a glucoside in the green pods of vanilla (*Vanilla planifolia*). The pleasant aroma is released only after injury or by fermentation, called "curing", when the glucoside of vanillin, glucovanillin, is hydrolyzed by glucosidase in vanilla pods (*1*). Vanillin was isolated in 1858 (*2*) and the chemical structure determined in 1874 (*3*). Large-scale chemical synthesis of vanillin has been achieved from materials such as guaiacol, eugenol, and safrole. Nowadays, synthetic vanillin is prepared by the oxidation of lignin contained in pulp wastes and is widely used in industry and as a fragrance and medicine as well as flavor (*4*).

For the formation of vanillin in vanilla pods, Anwar (*5*) proposed that vanillin is synthesized from coniferin, a precursor of lignin, by shortening of the C_3 side chain and hydrolysis of the glucoside, whereas Zenk (*6*) proposed that vanillin is formed from ferulic acid with shortening of the side chain by β-oxidation. Tokoro et al. (*7*) and Kanisawa (*8*) proposed a pathway to form glucovanillin from the glucoside of the 4-hydroxybenzyl alcohol after analysis of glucosides in green vanilla pods. On the other hand, Yazaki et al. (*9*) reported the existence of a nonoxidative reaction for shortening of the phenylpropanoid side chain and the formation of 4-hydroxybenzaldehyde from 4-coumaric acid (C_6-C_3 compound) in cell-free extracts of *Lithospermum erythrorhizon* cell cultures. Furthermore, Podstolski et al. (*10*) reported an enzyme involved in the reaction that converts 4-coumaric acid to 4-hydroxybenzaldehyde in tissue culture of vanilla. However, the complete biosynthetic pathway of vanillin from phenylpropanoids has not yet been demonstrated (*4*, *11*). To clarify the biosynthetic pathway for vanillin, we carried out pulse-chase experiments with ^{14}C-labeled compounds in disks of green vanilla pods.

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MATERIALS AND METHODS

General Experimental Procedures. NMR spectra were measured with a JNM-A400 spectrometer (JEOL, Japan). HPLC analysis was performed with a Shimadzu LC-10A system liquid chromatograph (Shimadzu Corp., Japan) and a reversed-phase C_{18} column (250 mm × 4.6 mm i.d., 5 μm, TSK-gel ODS 80Ts) (Tosoh Corp., Japan). The separation of compounds related to vanillin biosynthesis was carried out according to our previous paper (*12*). Eluates were collected for every 1 min and mixed well with 4 mL of scintisol EX-H (Dojindo, Tokyo, Japan), and their radioactivities were measured with a Beckmann LS-6500 scintillation counter. Bis[(β-D-glucopyranosyloxy)benzyl]-2-isopropyltartrate (glucoside A) and bis[(β-D-glucopyranosyloxy)benzyl]-2-(2-butyl)tartrate (glucoside B) were supplied from Takasago International Corp. [U-^{14}C]-L-Phenylalanine (Phe, 360 mCi/mmol), [U-^{14}C]-L-tyrosine (Tyr, 360 mCi/mmol), and [*methyl*-^{14}C]-L-methionine (Met, 54 mCi/mmol) were purchased from Moravek Biochemicals, Inc., Brea, CA. [^{14}C]-Methyl iodide (7.6 mCi/mmol) was a product of DuPont, Boston, MA. The purities of all ^{14}C precursors were confirmed by HPLC.

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Vanilla Pods. Green vanilla pods (*V. planifolia*) 3 and 6 months after pollination, cultivated by a farmer in central Java, Indonesia, were imported by Takasago International Corp.

Syntheses of ^{14}C-Precursors. (*a*) *[U-^{14}C]-4-Coumaric Acid.* ^{14}C-Labeled 4-coumaric acid was prepared by the deamination of [U-^{14}C]-L-Tyr with phenylalanine ammonia lyase (PAL) as follows. The reaction mixture of [U-^{14}C]-L-Tyr (50 μCi/500 μL), 100 μL of 0.2 M TAPS-KOH buffer (pH 8.5), 283 μL of water, and 27 μL of PAL from *Rhodotorula glutinis* (0.33 unit/0.9 mL) (Sigma Chemicals) was incubated at 30 °C for 3.5 h. After the addition of 10 μL of 3 N HCl, [U-^{14}C]-4-coumaric acid was extracted with 1 mL of diethyl ether 5 times. The diethyl ether in the extract was removed, and the resulting 40 μCi of [U-^{14}C]-4-coumaric acid was 98% pure. The specific radioactivity of this compound was diluted to 50 mCi/mmol by the addition of unlabeled 4-coumaric acid.

(*b*) *[U-^{14}C]-4-Hydroxybenzaldehyde.* ^{14}C-Labeled 4-hydroxybenzaldehyde was prepared by the ozonolysis of [U-^{14}C]-4-coumaric acid with a DMO-10BDF ozone generator (Ishimori Co., Ltd., Tokyo, Japan). A dry methanol solution (500 μL) of [U-^{14}C]-4-coumaric acid (50 μCi; specific radioactivity = 50 mCi/mmol) was cooled to −78 °C in an acetone−dry ice bath, and ozone−oxygen gas was introduced into the solution for 30 s. After removal of the ozone from the solution by passing dried nitrogen gas through the solution, the ozonides were destroyed by the addition of 10 μL of methanol solution of dimethyl sulfide (10%). The solution was concentrated on a rotary evaporator. The product was purified by chromatography on a silica gel column (1 × 15 cm) by chloroform/methanol (95:5) to obtain 38 μCi of [U-^{14}C]-4-hydroxybenzaldehyde with a radiochemical purity of 95%.

(*c*) *[U-^{14}C]-4-Hydroxybenzyl Alcohol.* This compound was obtained by the reduction of [U-^{14}C]-4-hydroxybenzaldehyde as follows. [U-^{14}C]-4-Hydroxybenzaldehyde (35 μCi) in 500 μL of anhydrous THF was treated with a small amount of $NaBH_4$ for 1 h at room temperature. After the solution had been neutralized with 0.1 N HCl, it was concentrated and developed on a silica gel column (1 × 15 cm) by chloroform/methanol (90:10) to obtain 21 μCi of [U-^{14}C]-4-hydroxybenzyl alcohol with a radiochemical purity of 96%.

(*d*) *[O-methyl-^{14}C]-Ferulic Acid.* ^{14}C-Labeled ferulic acid was synthesized by the condensation of malonic acid and vanillin that was prepared by methylation of 3,4-dihydroxybenzaldehyde with $^{14}CH_3I$ via the modification of the 4-position with a benzyl group (*13*) as follows. Liquid $^{14}CH_3I$ (250 μCi) was added to a mixture of 4-benzyl-3-hydroxybenzaldehyde (37.5 mg), methyl ethyl ketone (3 mL), and K_2CO_3 (60 mg), and the solution was kept for 1 h at room temperature, followed by further incubation for 16 h at 60 °C. After concentration of the reaction mixture, the reaction mixture was incubated with 20 mL of 6 N HCl for 2 h at 120 °C under a stream of nitrogen gas to eliminate the benzyl group. Extraction with diethyl ether and successive chromatography on a silica gel column (1 × 15 cm) with a solvent of benzene/ethyl acetate (3:1) produced 267 μCi of [*O*-$^{14}CH_3$]-vanillin. In the next step, 267 μCi of [*O*-$^{14}CH_3$]-vanillin, 100 μL of pyridine, 4 μL of aniline, and 20 mg of malonic acid were incubated at 65 °C for 12 h, and 134.7 μCi of [*O*-$^{14}CH_3$]-ferulic acid (specific radioactivity of 7.6 mCi/mmol; radiochemical purity of 82.1%) was obtained by chromatography with a silica gel column (1 × 15 cm) and a solvent of $CHCl_3$/MeOH/AcOH (95:5:1).

Syntheses of 4-Coumaroyl Glucose and Feruloyl Glucose. 4-Coumaroyl-β-D-glucose (*14*) was prepared by the following four steps:

(*a*) *Preparation of Acetyl-4-coumaric Acid Chloride.* 4-Coumaric acid (1.0 g) and pyridine (1.2 mL) were dissolved in 4 mL of acetic acid and held overnight at room temperature. The reaction mixture was slowly dropped into 200 mL of water with ice, and the resulting white crystals were filtered with a glass filter. The crystals (1.1 g) were dried thoroughly in a desiccator under vacuum and then refluxed with thionyl chloride (3.35 g) at 100 °C for 3 h. After the solvent of the reaction mixture had been evaporated under vacuum, the reaction mixture was further dried in a desiccator under vacuum. Crude crystals were recrystallized from benzene to obtain 0.36 g of acetyl-4-coumaric acid chloride.

(*b*) *Preparation of 2,3,4,6-Tetraacetyl-β-D-glucose* (***15***). Acetobromoglucose (10 g) was dissolved in dry acetone (15 mL) and 5.63 g of $AgCO_3$ in 0.28 mL of water at 0 °C. The reagents were stirred for 30 min, and the reaction mixture was heated to 50 °C and filtered. The filtrate was concentrated at room temperature to obtain crystals. After the crystals had been dissolved in hot diethyl ether, the solution was cooled and the resulting crystals were filtered. Recrystallization with ether−ligroin yielded 2.47 g of 2,3,4,6-tetraacetyl-β-D-glucose.

(*c*) *Glucosylation* (***16***). Acetyl-4-coumaroyl chloride (1.0 g) was allowed to react with 2,3,4,6-tetraacetyl-β-D-glucose (1.21 g) in a solution of chloroform (2.6 mL) and pyridine (0.4 mL) at room temperature for 20 h. Afterward, the reaction mixture was washed with 2 N H_2SO_4 and 2 N $NaHCO_3$ and twice further with water. The chloroform layer was evaporated and recrystallized from EtOH to obtain 1.63 g of acetyl-4-coumaroyl-2,3,4,6-tetraacetyl-β-D-glucose.

(*d*) *Deacetylation* (***17***). The acetyl-4-coumaroyl-2,3,4,6-tetraacetyl-β-D-glucose (0.8 g) was dissolved in 15 mL of dry MeOH, and 0.1 N CH_3ONa−MeOH solution (19.4 mL) was added dropwise over 15 min; the reaction mixture was stirred under cooling with ice for 1 h. The reaction mixture was passed through a Dowex 50W-H8 (H^+) column (1 × 5 cm), equilibrated with MeOH, and the 4-coumaroyl-β-D-glucose was eluted with MeOH. The eluate was evaporated to dryness to obtain 0.43 g of crude material. The crude material was washed with 9 mL of chloroform and recrystallized from water to obtain 0.15 g of 4-coumaroyl-β-D-glucose. The structure was confirmed by NMR spectroscopy (*18*).

Feruloyl-β-D-glucose was prepared in the same manner as 4-coumaroyl-β-D-glucose. Crude feruloyl-β-D-glucose (0.42 g) was obtained by the deacetylation of acetyl-4-feruloyl-2,3,4,6-tetraacetyl-β-D-glucose (0.6 g). Further purification by HPLC with a TSK-gel ODS-80Ts column (4.6 × 250 mm) and elution with 18.5% MeOH gave 15 mg of feruloyl-β-D-glucose.

Feeding Experiments of ^{14}C-Labeled Compounds into Green Vanilla Pods. [U-^{14}C]-4-Coumaric acid (50 mCi/mmol), [*O*-methyl-^{14}C]-ferulic acid (7.6 mCi/mmol), [U-^{14}C]-4-hydroxybenzaldehyde (50 mCi/mmol), [U-^{14}C]-4-hydroxybenzyl alcohol (50 mCi/mmol), [U-^{14}C]-L-Phe (50 mCi/mmol), and [*methyl*-^{14}C]-L-Met (50 mCi/mmol) were used as ^{14}C-precursors in the feeding experiments. Green vanilla pods were washed with 0.02% chloramphenicol and cut into 2 cm length pieces (2 g). Subsequently, the 2 cm pieces of green pods were further sliced into 2 mm lengths to form 10 disks. After the 10 disks had been dipped into 0.02% chloramphenicol solution and wiped with a paper towel, they were separately placed on a Petri dish. Each disk was fed 10 μL of a water solution of ^{14}C-labeled compound (0.2 μCi), then 10 μL of unlabeled ferulic acid (0.04 μmol) in feeding experiments of ^{14}C-labeled Phe and Met, and incubated at 26 °C under an illumination of ca. 5300 lx in a growth cabinet. After 1, 3, 6, 12, and 24 h, each set of 10 disks was frozen and stored at −80 °C until the following extraction. Feeding experiments of ^{14}C-labeled 4-coumaric acid and ferulic acid into disks of 6-month-old vanilla pods were carried out in duplicate.

Extractions and Separations of ^{14}C-Labeled Metabolites. Ten disks of vanilla pods were homogenized in 60 mL of MeOH for 1 min with a Polytron (Kinematica AG, Switzerland) and held for 1 h at room temperature. After filtration of the mixture with a filter paper under suction, the residue was re-extracted with 60 mL of 80% MeOH for 1 h. Filtrates were combined, concentrated with a rotary evaporator, and lyophilized. The filtrate was dissolved in 30 mL of water and chlorophyll removed by extraction twice with 5 mL of *n*-pentane. ^{14}C-Labeled aglycones were extracted 10 times with 5 mL of diethyl ether, and the combined diethyl ether solution was concentrated and made up to 25 mL with MeOH, and the radioactivity of the solution was measured. The remaining water solution was concentrated to remove diethyl ether and made up to 50 mL. This solution was passed through an Amberlite XAD-2 column (2 × 25 cm) equilibrated with water after thorough washing with MeOH. The column was washed with 200 mL of water, followed by the elution of ^{14}C-labeled glucosides with 250 mL of MeOH. The MeOH eluate was concentrated and made up to 25 mL with MeOH, and the radioactivity of the solution was measured. The solutions containing aglycones or glucosides were concentrated and lyophilized. The lyophilized powders were dissolved in 50% MeOH, which were used to analyze the metabolites by HPLC.

Large-Scale Purification and Identification of Metabolites from ^{14}C-Labeled 4-Coumaric Acid and Ferulic Acid. After 4-coumaric acid (1 μCi, 0.05 mCi/mmol) had been fed to 170 disks of 3-month-old green vanilla pods (74.8 g) for 3 h, the extraction and separation procedures described above were carried out. The MeOH eluate

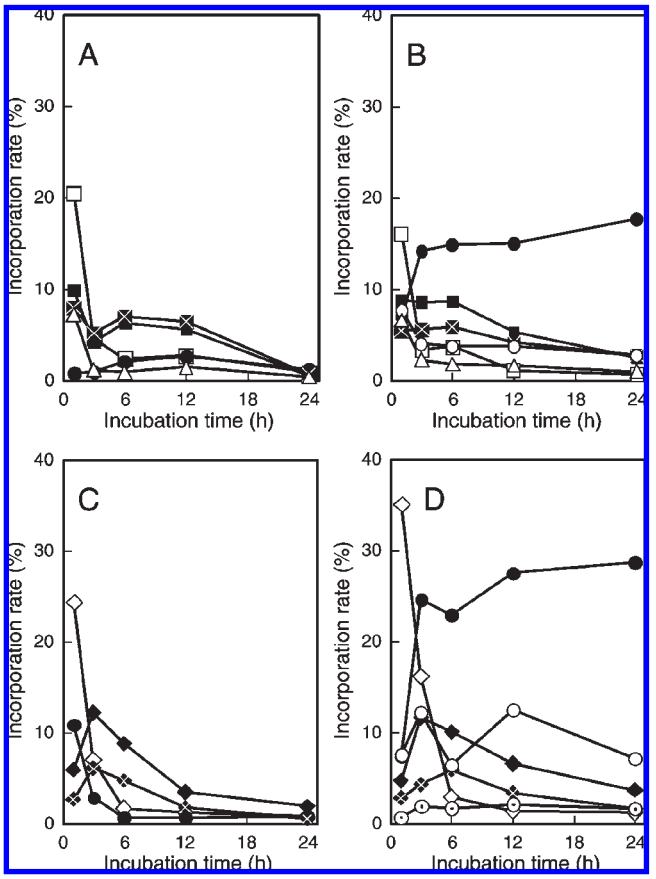

Figure 1. Feeding of ^{14}C-4-coumaric acid (**A**, **B**) and ^{14}C-ferulic acid (**C**, **D**) to 3-month-old pods (**A**, **C**) and 6-month-old pods (**B**, **D**): ○, vanillin; ●, glucovanillin; ⊙, vanillyl alcohol glucoside; □, 4-coumaric acid; ■, 4-coumaroyl glucose (*trans*); black box with cross, 4-coumaroyl glucose (*cis*); ◇, ferulic acid; ◆, feruloyl glucose (*trans*); black diamond with cross, feruloyl glucose (*cis*); △, 4-hydroxybenzaldehyde.

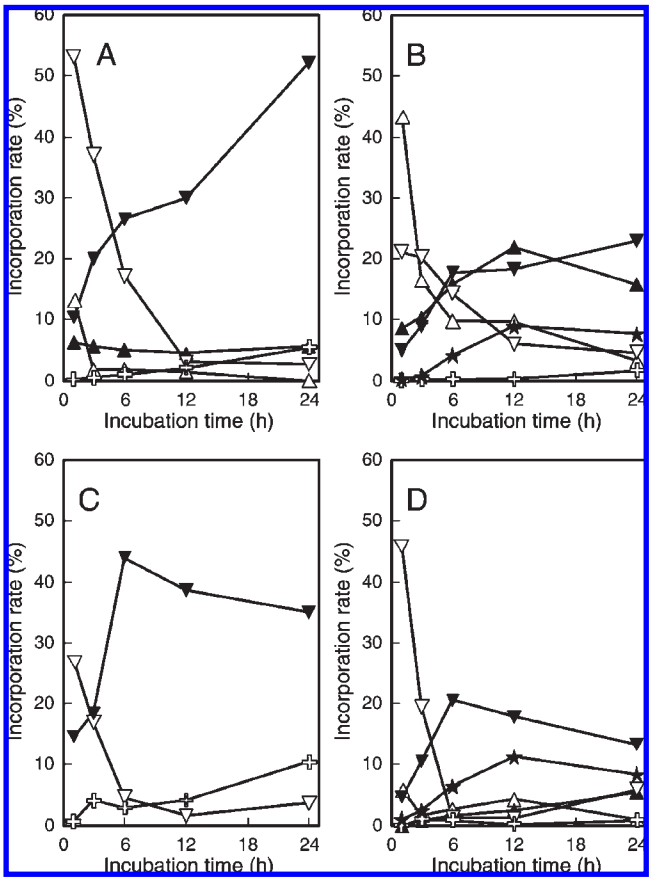

Figure 2. Feeding of ^{14}C-4-hydroxybenzaldehyde (**A**, **B**) and ^{14}C-4-hydroxybenzyl alcohol (**C**, **D**) to 3-month-old pods (**A**, **C**) and 6-month-old pods (**B**, **D**): △, 4-hydroxybenzaldehyde; ▲, 4-hydroxybenzaldehyde glucoside; ▽, 4-hydroxybezyl alcohol; ▼, 4-hydroxybezyl alcohol glucoside; outlined cross, glucosides A and B (bis[(β-D-glucopyranosyloxy)benzyl]-2-isopropyltartrate and bis[(β-D-glucopyranosyloxy)benzyl]-2-(2-butyl)tartrate, respectively); ★, unknown 1.

(0.48 μCi) from Amberlite XAD-2 column chromatography was further purified with a Toyopearl HW-40S column (2.5 × 35 cm) (Tosoh Corp.) equilibrated with MeOH/1% AcOH (5:95). Metabolites were eluted by a linear gradient of MeOH/1% AcOH from 5 to 30% MeOH. Eluates were monitored at 280 nm and their radioactivities measured. The fractions were purified by HPLC with an Inertsil prep ODS column (250 mm×6.0 mm i.d., 10 μm) (GL Science, Japan). The unknown fraction was hydrolyzed separately with 1 N NaOH and β-glucosidase to identify the metabolites by HPLC. These treatments revealed whether the metabolites were glucosides or glucose esters (*19*). Unknown metabolites from ^{14}C-ferulic acid were also purified and identified as well as ^{14}C-4-coumaric acid.

RESULTS AND DISCUSSION

Identification of Metabolites from ^{14}C-Labeled 4-Coumaric Acid and Ferulic Acid. In the feeding experiments with ^{14}C-labeled 4-coumaric acid and ferulic acid, several unknown metabolites were detected. We tried to identify the major unknown compounds from 4-coumaric acid and ferulic acid. After the application of ^{14}C-labeled 4-coumaric acid or ferulic acid to disks of 3-month-old vanilla pods (immature pods) for 3 h, the metabolites were extracted. The crude extracts were purified by Toyopearl HW-40S column chromatography. Peaks of unknown compounds were separated from others by a gradient of MeOH from 15 to 20%. Unknown compounds were further purified by HPLC and treated with base and β-glucosidase, and the products were analyzed by HPLC. Compounds prepared from the feeding experiments with ^{14}C-labeled 4-coumaric acid and ferulic acid were hydrolyzed to 4-coumaric acid and ferulic acid, respectively, by both treatments. The retention times of unknown compounds were the same as their synthesized glucose esters. These results indicate that the unknown metabolites are glucose esters (*19*). In addition, the cis and trans configurations of the two esters were observed by HPLC analysis (*19*).

Conversion of ^{14}C-Labeled 4-Coumaric Acid and Ferulic Acid to Glucovanillin. In 6-month-old vanilla pods (mature pods) 15% of ^{14}C-4-coumaric acid was incorporated into glucovanillin within 24 h (**Figure 1B**). Early in the feeding experiments higher incorporation of ^{14}C into 4-coumaroyl glucoses (*cis* and *trans*) was observed. Furthermore, 4-coumaric acid was also converted into vanillin and 4-hydroxybenzaldehyde. In 3-month-old pods (immature pods), ^{14}C was incorporated into 4-coumaroyl glucoses with only small amounts converted into glucovanillin (**Figure 1A**). A small amount of ^{14}C activity in ferulic acid was also detected when ^{14}C-4-coumaric acid was fed to disks of both mature and immature pods (data not shown). On the other hand, ^{14}C-ferulic acid labeled with a methyl group decreased rapidly within 6 h after feeding, but glucovanillin increased immediately to ca. 25% within 3 h in mature beans (**Figure 1D**). Incorporation of ^{14}C-ferulic acid into glucovanillin was 29% in 24 h. The *O*-Me group of ferulic acid appears not to be eliminated in this metabolism. Two glucose esters of ferulic acid similar to 4-coumaric acid metabolites were detected in both immature and mature pods (**Figures 1C**,**D**). Glucovanillin was also detected in immature pods, but not accumulated. These feeding experiments

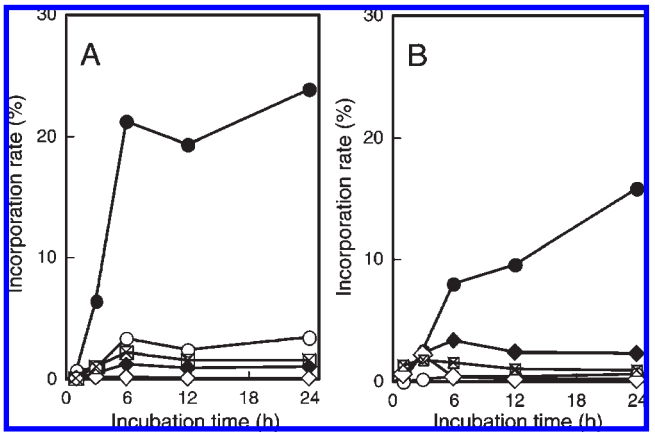

Figure 3. Feeding of ^{14}C-methionine to 6-month-old pods: (**A**) ^{14}C-Met (control); (**B**) ^{14}C-Met + unlabeled ferulic acid; ○, vanillin; ●, glucovanillin; ◇, ferulic acid; ◆, feruloyl glucoses (*cis* and *trans*); white box with cross, unknown 2. Decreases in fed ^{14}C-Met were not determined.

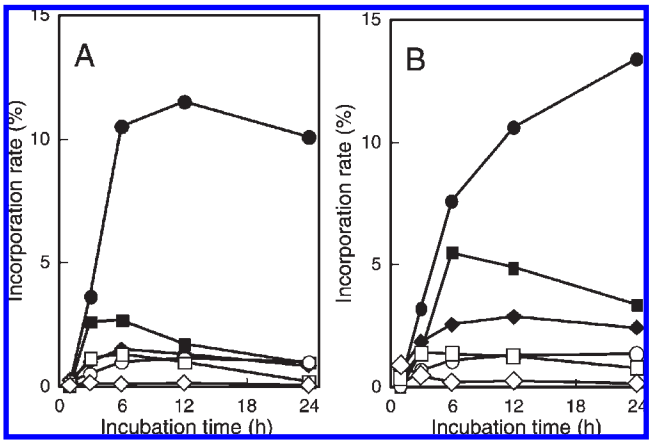

Figure 4. Feeding of ^{14}C-phenylalanine to 6-month-old pods: (**A**) ^{14}C-Phe (control); (**B**) ^{14}C-Phe + unlabeled ferulic acid; ○, vanillin; ●, glucovanillin; □, 4-coumaric acid; ■, 4-coumaroyl glucoses (*cis* and *trans*); ◇, ferulic acid; ◆, feruloyl glucoses (*cis* and *trans*). Decreases in fed ^{14}C-Phe were not determined.

with two compounds suggest that ferulic acid is the precursor closer to glucovanillin than 4-coumaric acid in the biosynthetic pathway of vanillin.

Conversion of ^{14}C-Labeled 4-Hydroxybenzaldehyde and 4-Hydroxybenzyl Alcohol to Glucovanillin. Panels **A** and **B** and panels **C** and **D** of **Figure 2** indicate conversions of 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol, respectively. In immature pods, 4-hydroxybenzaldehyde was converted rapidly to 4-hydroxybenzyl alcohol and further to its glucoside. Rapid glucosylation was recognized from the feeding experiments with 4-hydroxybenzyl alcohol (**Figure 2C**). On the other hand, similar amounts of ^{14}C labels from 4-hydroxybenzaldehyde were distributed to 4-hydroxybenzyl alcohol and their glucosides in mature pods (**Figure 2B**). In addition, conversion of 4-hydroxybenzyl alcohol to its glucoside appears to be easy (**Figures 2C**,**D**). Very small amounts of ^{14}C labels from 4-hydroxybenzyl alcohol were also incorporated into 4-hydroxybenzaldehyde and its glucoside. This conversion is in the opposite direction from immature pods. The conversion rate (about 10%) from 4-hydroxybenzyl alcohol to glucosides A and B, esters of tartaric acid derivatives and two molecules of 4-hydroxybenzyl alcohol glucoside (bis[(β-D-glucopyranosyloxy)benzyl]-2-isopropyltartrate and bis[(β-D-glucopyranosyloxy)benzyl]-2-(2-butyl)tartrate, respectively) (*7*, *8*), in immature pods was much higher than the others. However, the radioactivities of 4-hydroxybenzaldehyde and 4-hydroxybenzyl alcohol were not incorporated into glucovanillin.

Trapping of ^{14}C-Methionine by Unlabeled Ferulic Acid. To examine methylation in the pathway to vanillin, we investigated the effect of the addition of unlabeled ferulic acid (0.04 μmol/disk) to vanilla pod disks fed ^{14}C-methionine at the same time. As shown in **Figure 3B**, the rate of conversion from methionine to glucovanillin was slow (8−15%/6−24 h) compared to the control (22−24%/6−24 h) (**Figure 3A**), but glucovanillin gradually increased. We observed incorporation of much ^{14}C into ferulic acid at 3 h (ca. 3%) after feeding and into ferulic acid glucose ester during the incubation times from 3 to 24 h (3−4%). These results suggest that ferulic acid was directly converted to vanillin and further to glucovanillin, not via ferulic acid glucose esters because their decrease was slow. The slow conversion rates of glucose esters are shown in **Figures 1** and **4**. Furthermore, ^{14}C of methionine was observed in *S*-adenosylmethionine (SAM), a donor of the methyl group in methylation, and an unknown compound eluted earlier than SAM in HPLC. In immature pods, ^{14}C of methionine was not incorporated into glucovanillin (data not shown).

Conversion of ^{14}C-Labeled Phenylalanine to Glucovanillin with Addition of Unlabeled Ferulic Acid. The conversion of phenylalanine, a precursor of phenylpropanoids, to glucovanillin was examined. ^{14}C from phenylalanine was incorporated into 4-coumaric acid, ferulic acid, their glucose esters, vanillin, and glucovanillin (**Figure 4**). In the control experiment (**Figure 4A**), incorporation into glucovanillin was ca. 11% during 6−24 h, whereas by the addition of unlabeled ferulic acid (0.04 μmol/disk), the incorporation into glucovanillin was 7.6% within 6 h with an increase to 13.4% within 24 h (**Figure 4B**). More ^{14}C was found as ferulic acid in the early stages of incubation, and incorporation into 4-coumaroyl glucose and feruloyl glucose increased compared to the control (**Figure 4B**). The incorporation into glucovanillin reached a maximum in a short time (**Figure 4A**), whereas the amount continued to increase at 24 h (**Figure 4B**), which was similar to the incorporation of ^{14}C-methionine with added unlabeled ferulic acid. In the addition of ferulic acid, the ^{14}C-labeled glucose esters were greater than the control, but their decrease was small. The glucose esters do not appear to influence the ^{14}C incorporation into glucovanillin. The high incorporation of these amino acids into phenylpropanoids and glucovanillin indicates that the biosynthesis of vanillin in *V. planifolia* is very active.

Biosynthetic Pathway for Vanillin. It is known that vanillin is synthesized in vanilla pods (*4*, *6*, *8*, *11*), but it is difficult for a whole pod to take up ^{14}C-labeled compounds. Although Zenk (*6*) carried out feeding experiments by incubating the disks of pods in a solution of ^{14}C-labeled compound, we fed concentrated solutions to the disks so that the reactions proceeded securely. Zenk (*6*) determined the amount of vanillin by TLC after the hydrolysis of the incubated solutions. In addition, he proposed the pathway that vanillin is synthesized via vanilloyl-CoA from ferulic acid. On the other hand, we determined glucovanillin and other intermediates by HPLC to investigate the time course of the metabolite conversions in detail. The results of our feeding experiments are summarized as follows: phenylpropanoids from phenylalanine are precursors of vanillin, methionine is a donor of a methyl group to phenylpropanoids via *S*-adenosylmethionine, and ferulic acid is a good precursor of vanillin. These results are similar to those from Zenk (*6*); however, the rate of radioactivity incorporated into glucovanillin toward total radioactivity applied to disks by our method (**Figures 1**−**4**) was much higher than that by his method. Glucosylated metabolites were detected in feeding

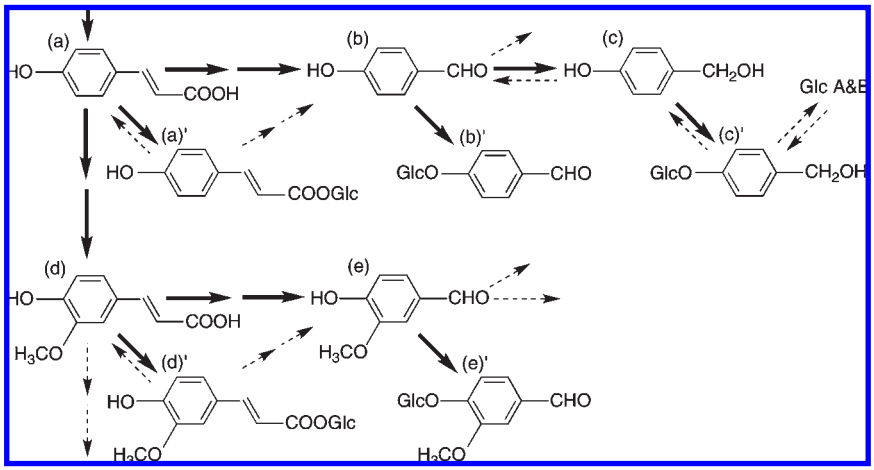

Figure 5. Proposed biosynthetic pathway for vanillin and related compounds from phenylpropanoids and formation of their glucosides and glucose esters in *Vanilla planiforia*: (a) 4-coumaric acid; (b) 4-hydroxybenzaldehyde; (c) 4-hydroxybenzyl alcohol; (d) ferulic acid; (e) vanillin; (a)′, (b)′, (c)′, (d)′, and (e)′ show the respective glucose esters or glucosides. Glc A and B are esters of tartaric acid derivatives and two molecules of (c)′ (bis[(β-D-glucopyranosyloxy)benzyl]-2-isopropyltartrate and bis[(β-D-glucopyranosyloxy)benzyl]-2-(2-butyl)tartrate, respectively). Bold and dotted arrows indicate identified and unidentified routes, respectively, in this study.

experiments with ^{14}C-labeled compounds (**Figure 1**) and in the analysis of vanilla pods by Tokoro et al. (*7*), suggesting that glucovanillin is synthesized via glucose esters of 4-coumaric acid and ferulic acid or the glucoside of 4-hydroxybenzaldehyde. Tokoro et al. (*7*) and Kanisawa (*8*) have presented a pathway via the glucosides to glucovanillin. However, the 4-hydroxybenzaldehyde glucoside was not metabolized to glucovanillin in this study (**Figure 2**). Furthermore, the formation of glucovanillin was very fast, with a maximum increase in 6 h after feeding ^{14}C-labeled compounds, and remained high. However, the glucose esters of ferulic acid and 4-coumaric acid slowly decreased (**Figure 1**). From these results the formation of vanillin via the glucose ester of ferulic acid may be excluded. In the feeding experiments with Met and Phe by the addition of unlabeled ferulic acid (**Figures 3** and **4**), the formations of glucovanillin slowed, but glucovanillin increased over time. In addition, ^{14}C was accumulated as ferulic acid, suggesting that vanillin is directly synthesized via ferulic acid and then immediately glucosylated to form glucovanillin. High levels of 4-coumaric acid, ferulic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, and vanillin are immediately glucosylated to become the respective glucose esters of C_6-C_3 compounds and glucosides of C_6-C_1 compounds (**Figure 5**), because they appear to be poisonous to the cells of vanilla plants (*11*). Large pools of 4-hydroxybenzaldehyde glucoside and glucovanillin accumulate in vanilla pods. The contents of 4-hydroxybenzaldehyde glucoside and glucovanillin in 6-month-old pods (mature pods) were 0.23 and 1.23 mmol/100 g of fresh weight, respectively. 4-Hydroxybenzaldehyde glucoside may be the end metabolite from 4-hydroxybenzaldehyde as well as glucovanillin or may be an intermediate of glucosides A and B (**Figures 2** and **5**). Immature and mature pods contain 1.19 and 1.02 mmol of glucosides A and B in 100 g of fresh weight, respectively. In conclusion, we propose the biosynthetic pathway for vanillin in **Figure 5**, which shows that vanillin is synthesized via ferulic acid from 4-coumaric acid and glucosylated to form glucovanillin in mature vanilla pods. The pathway from ferulic acid to vanillin may be supported by demonstrating the existence of an enzyme catalyzing the reaction for shortening of the phenylpropanoid side chain. As reported by Podstolski et al. (*10*), we also detected enzyme activity in cell-free extracts of vanilla pods. Further investigation to purify, isolate, and identify the key enzyme is in progress.

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