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Note

Capsinoid Is Biosynthesized from Phenylalanine and Valine in a Non-Pungent Pepper, *Capsicum annuum* L. cv. CH-19 Sweet

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The biosynthetic pathway of capsinoid in 'CH-19 Sweet' was investigated. [^{3}H]Valine and [^{14}C]phenylalanine were injected into the fruits of the intact plant. Both of radioactivities were detected in capsinoid fractions. ^{14}C radioactivity was observed in phenylpropanoid compounds, and in vanillin, vanillylamine, vanillyl alcohol, and vanillic acid. We confirmed that capsinoid is biosynthesized from phenylalanine and valine.

Key words: *Capsicum annuum*; CH-19 Sweet; biosynthetic pathway; tracer experiment; capsinoid

Recently, three homologs of nonpungent compounds, capsiate, dihydrocapsiate, and nordihydrocapsiate, have been isolated from the fruits of a nonpungent cultivar of *Capsicum annuum* cv. CH-19.[1,2] They were fatty acid esters of vanillyl alcohol named capsinoid.[2] We conjectured that capsinoid and capsaicinoid have a close relation in their biosynthetic pathway because capsinoid has a remarkable structural resemblance to capsaicinoid except for their center linkage: an amide moiety in capsaicinoid and an ester moiety in capsinoid. Moreover, 'CH-19 Sweet' plants have been selected and fixed from a pungent cultivar of *Capsicum annuum* cv. CH-19 which contains much capsaicinoid.[3]

In the present study, an *in vivo* experiment was performed by injection of radiolabelled valine and phenylalanine into 'CH-19 Sweet' fruits of intact plants. A further *in vivo* experiment administered radiolabelled vanillin into 'CH-19 Sweet' and pungent cultivar of *Capsicum annuum* cv. Takanotsume fruits.

Fruits 20–30 d from anthesis (DFA) were used for most experiments because the period of active accumulation of capsinoid is 15–30 DFA in summer.[4]

All chemicals were of analytical reagent grade. L-[3,4-^{3}H]Valine (sp. act., 30 Ci/mmol), L-[U-^{14}C]phenylalanine (sp. act., 396 mCi/mmol), and *S*-[methyl-^{14}C]-adenosylmethionine ([^{14}C]-SAM) (sp. act., 52.7 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). Vanillin and dithiothreitol (DTT) were purchased from Wako Pure Chemicals (Osaka, Japan). Capsiate and dihydrocapsiate were enzymatically synthesized in our laboratory.[5] The remaining chemicals were obtained from Sigma-Aldrich Japan (Tokyo, Japan).

For [^{14}C]vanillin synthesis, [^{14}C]-SAM (500 µl), 8 mM 3,4-dihydroxybenzaldehyde (50 µl), 50 mM DTT (30 µl), 1.6 M $MgCl_2$ (20 µl), catechol *O*-methyltransferase (0.3 mg, 300 U, Sigma), and 1 M phosphate buffer pH 9.7 (15 µl) were mixed into a 1.5 ml volume light-shielding Treff microtube. The mix solution was incubated at 37 °C at 100 rpm for 48 h. [^{14}C]Vanillin was purified by high performance liquid chromatography (HPLC), as described below, and gave 43% yield.

The experiment was carried out under 5,500 lux of fluorescent lamp illumination at 26 °C. [^{3}H]Valine and [^{14}C]phenylalanine, each 2 µCi, or [^{14}C]vanillin, 0.3 µCi, were injected directly into the loculus of fruits using a 100 µl Hamilton microsyringe (#710). The fruits were harvested at 1, 2, 3, 5, and 10 h after injection in the case of [^{3}H]valine and [^{14}C]phenylalanine, and 5 h after injection in the case of [^{14}C]vanillin, and were immediately freeze-dried.

The freeze-dried fruits were separated into placenta, pericarp, and seeds, and each tissue was ground in a mortar. The placenta was first extracted twice with 5 ml of ethyl acetate each time for 1 h because capsinoid has high solubility and stability against ethyl acetate.[6] Subsequently, the residue was extracted twice with 5 ml of 80% ethanol each time for 1 h to obtain phenolics. The pericarp and seed were extracted twice with 5 ml of 80% ethanol each time to obtain phenolics. Each extractant was evaporated to dryness, and placenta extracts were dissolved in 300 µl of 80% ethanol for HPLC. The pericarp and seeds extracts were dissolved

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Abbreviations: CAP, capsaicin; CST, capsiate; DC, dihydrocapsaicin; DCT, dihydrocapsiate; DTT, dithiothreitol; HPLC, high performance liquid chromatography; SAM, *S*-adenosylmethionine; VOH, vanillyl alcohol

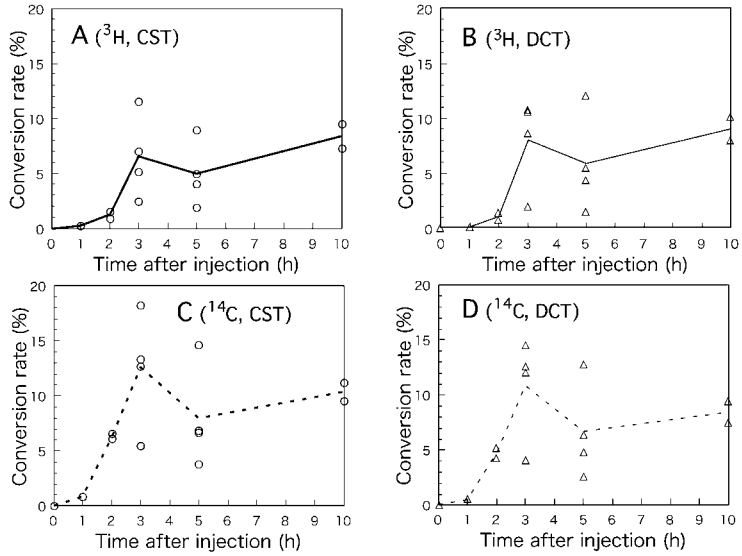

Fig. 1. The Conversion Rate (%) of [^{3}H]Valine and [^{14}C]Phenylalanine into Capsiate (CST) and Dihydrocapsiate (DCT) in Placenta Tissue *in Vivo*. A, ^{3}H CST; B, ^{3}H DCT; C, ^{14}C CST; D, ^{14}C DCT. Lines show the average of data. Number of experiments: 1 h, 1; 2–10 h, 2–4. ○, CST; ▲, DCT.

in 500 µl of 80% ethanol for liquid scintillation counting. To obtain the lignin-like substance fraction, the residues of each tissue were extracted with a mixture of 40 ml distilled water, 100 µl gracial acetic acid, and 250 mg $NaClO_2$ at 70 °C for 6 h.[7)]

HPLC was performed using a Shimadzu SCL-6B liquid chromatograph system with a Fluofix IEW 425 column (250 mm × 4.6 mm i.d., Wako Pure Chemicals) at 40 °C. A cold standard sample was injected simultaneously with a hot sample to clarify the retention times of compounds. Fractions of L-phenylalanine, cinnamic acid, *p*-coumaric acid, ferulic acid, vanillin, capsiate, and dihydrocapsiate were obtained under the following conditions: eluent, acetonitrile and 0.1% aqueous trifluoroacetic acid; gradient, 0–30 min, 7% acetonitrile, 30–70 min, 7–100% acetonitrile linear gradient; flow rate 0–30 min, 0.5 ml/min, 30–70 min, 1 ml/min; detection, 0–50 min UV 236 nm, 50–70 min UV 280 nm. Fractions of caffeic acid, vanillylamine, vanillyl alcohol, and vanillic acid were obtained under the following conditions: eluent, acetonitrile and 0.1% aqueous trifluoroacetic acid; gradient 0–15 min, 0% acetonitrile, 15–30 min, 0–30% acetonitrile; flow rate, 1 ml/min; detection, UV 236 nm.

The HPLC fractions, the pericarp and seed extracts, and lignin-like substance extracts were dissolved in 5–10 ml of Ultima Gold liquid scintillation counter cocktail (Perkin Elmer Japan, Yokohama, Japan) and their radioactivities were measured by LSC-5100 (Aloka, Tokyo, Japan).

Radioactivities in organic solvent-soluble and lignin-like substance fractions of each tissue of 'CH-19 Sweet' fruits after injection of [^{3}H]valine and [^{14}C]phenylalanine were measured with a liquid scintillation counter. Recovery of radioactivity at 1–10 h varied widely among the various tissues of the fruits, but total recovery was in the same range of 45–55% in ^{3}H and ^{14}C. The total recovery in the fruits did not reach 100%. It is thought that [^{3}H]valine and [^{14}C]phenylalanine moved into other plant parts. But, some radioactivity of ^{3}H was detected in the lignin fraction of parts of leaves and stems of 'CH-19 Sweet' plant (data not shown).

The conversion rate of [^{3}H]valine and [^{14}C]phenylalanine into capsinoid, *i.e.*, capsiate (CST) and dihydrocapsiate (DCT), was calculated by the radioactivity of capsinoid fraction per total radioactivity detected in the placenta (Fig. 1). The conversion rate of ^{3}H and ^{14}C into CST and DCT increased up to 3 h after injection, and showed the same values at 10 h. The higher conversion rate of ^{14}C than of ^{3}H at all time points except for that into DCT at 10 h after injection suggests that [^{14}C]phenylalanine is taken more easily in capsinoid fraction than [^{3}H]valine. Although the natural ratio of CST and DCT in the fruits of 'CH-19 Sweet' is about 2:1,[2)] the incorporation ratio of [^{3}H] and [^{14}C] into CST was lower than that of DCT in this *in vivo* experiment. Iwai *et al.*[8)] have reported that dihydrocapsaicin (DC) content became higher than capsaicin (CAP) as an effect of exogenously added vanillylamine and isocapric acid in *in vivo* experiments, but that the natural abundance ratios of CAP and DC were about 2:1. Therefore, exogenously added precursors probably have some effect on capsinoid biosynthesis.

Figure 2 shows the conversion rate of [^{14}C]phenylalanine into phenylpropanoid compounds, vanillin, vanillylamine, vanillyl alcohol, vanillic acid, CST, and DCT in placenta tissue. The radioactivity of [^{14}C]phenylalanine decreased gradually after injection. The conversion rate of all precursor candidates increased up to 5 h after injection.

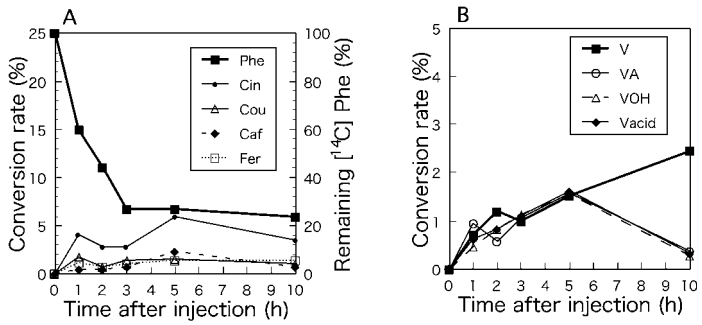

Fig. 2. The Conversion Rate (%) of [^{14}C]Phenylalanine into Phenylpropanoid (A) and Vanillyl Compounds (B) in Placenta Tissue *in Vivo*. Number of experiments: 1 h, 1; 2–10 h, 2–4. Phe, phenylalanine; Cin, cinnamic acid; Cou, *p*-coumaric acid; Caf, caffeic acid; Fer, ferulic acid; V, vanillin; VA, vanillylamine; VOH, vanillyl alcohol; Vacid, vanillic acid.

Table 1. The Conversion Rate (%) of [^{14}C]Vanillin into Vanillylamine (VA), Vanillyl Alcohol (VOH), Vanillic Acid (Vacid), Capsiate (CST), Dihydrocapsiate (DCT), Capsaicin (CAP) and Dihydrocapsaicin (DC) at 5 h in Placenta Tissue of 'CH-19 Sweet' or 'Takanotsume' Fruits *in Vivo*

Compound	CH-19 Sweet		Takanotsume	
	1	2	1	2
V	0.73	2.02	1.04	2.15
VA	nd	nd	3.38	7.25
VOH	10.03	0.58	15.27	8.70
Vacid	4.73	2.33	2.66	5.64
CST	3.77	12.03	nd	nd
DCT	3.35	5.14	nd	nd
CAP	nd	nd	0.13	8.97
DC	nd	nd	0.08	4.69

Duplicate data are shown as Nos. 1 and 2, separately.
nd, not detected.

As Fig. 1 and 2 show, capsinoid is synthesized from valine and phenylalanine as starting precursor of the biosynthetic pathway in a manner similar to capsaicinoid, and phenylpropanoid pathway compounds, *viz.*, cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid, participate in capsinoid synthesis. Furthermore, vanillin appears to be a key precursor as a branch point in the biosynthesis pathway between capsinoid and capsaicinoid.

To clarify the pathway from vanillin to capsinoid and the correlation between capsinoid and capsaicinoid in biosynthesis, an additional experiment was performed by injection of [^{14}C]vanillin into 'CH-19 Sweet' or pungent cultivar of *Capsicum annuum* 'Takanotsume' fruits of intact plants. [^{14}C]Vanillin was not incorporated into phenylpropanoid compounds at all in either 'CH-19 Sweet' or 'Takanotsume.' In 'CH-19 Sweet,' [^{14}C]vanillin was converted into vanillyl alcohol, vanillic acid, CST, and DCT, but not into vanillylamine or capsaicinoid. On the other hand, in 'Takanotsume,' [^{14}C]vanillin was converted into vanillylamine, vanillyl alcohol, vanillic acid, CAP, and DC fractions, but not into capsinoid. It is interesting that [^{14}C]vanillin was converted into vanillylamine only in pungent pepper fruits, whereas vanillyl alcohol was produced in both pungent and sweet pepper fruits. This result suggests that capsinoid biosynthesis in sweet fruit was caused by a defect in vanillylamine synthesis from vanillin. Since the radioactivity of ^{14}C was detected in the vanillylamine fraction in the [^{14}C]phenylalanine injection experiment, however, we are now trying to identify genes encoding capsinoid or capsaicinoid synthetase to clarify what gene mutation accounts for capsinoid production in 'CH-19 Sweet' pepper.

In conclusion, the early part of the biosynthetic pathway of capsinoid is the same as that of capsaicinoid.

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