Characteristic Incorporation of Fatty Acids into Lower Terpenoids in Cotyledons of *Perilla frutescens*

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Incorporation of ^{14}C-labeled sucrose, acetate, butyrate, and mevalonate into the terpenoid components of essential oils was examined using the detached cotyledons and foliage leaf discs of *Perilla frutescens*. Although the radioactivity of sucrose administered was incorporated into the mono- and sesquiterpenoids of both tissues, that of acetate or butyrate was hardly detectable in the lower terpenoids of any foliage leaves except cotyledons. These results suggest that the conversion of acetyl CoA derived from fatty acids into lower terpenoids in the cotyledons does not occur in the foliage leaves.

Keywords *Perilla frutescens*; Labiatae; essential oil; monoterpenoid; sesquiterpenoid; perillaldehyde; perillaketone; caryophyllene; [^{14}C]butyrate; tracer experiment

Introduction

In many tracer experiments on the biosynthesis of lower terpenoids in higher plants, a very low incorporation of labeled precursors into final metabolites has been observed. Especially, the incorporation ratios of labeled acetate and mevalonate (MVA) into monoterpenoids were as low as 0.01—0.1%,[1] except for a few cases reported for *Humulus lupulus*,[1] *Tanacetum vulgare*,[2] and rose.[3] Banthorpe *et al.* suggested that acetate would rarely be incorporated into monoterpenoids,[4] as it would mostly be used for the biosynthesis of diverse compounds. He also suggested the possibilities that 1) MVA administered externally may not readily penetrate the intracellular site of terpenoid synthesis, 2) it may be used exclusively for the synthesis of physiologically important steroids and carotenoids, and 3) a large quantity of MVA may well lead to the feedback inhibition of enzymes involved in terpenoid synthesis. In this paper, we report the existence of a marked qualitative difference in the incorporation pattern of precursors into monoterpenoids between cotyledons and leaves of *Perilla frutescens*.

Experimental

Plant Material Two chemotypes, PA and PK, of *Perilla frutescens* BRITTON, whose major monoterpenoid components of essential oils are perillaldehyde and perillaketone, respectively, had been self-pollinated for more than two generations to confirm that no genetic segregation for the oil components occurred in the progeny plants.

Administration of ^{14}C-Labeled Precursors [^{14}C(U)]Sucrose (4.8 mCi/mmol) was obtained from New England Nuclear Corp.; sodium [^{14}C(U)]acetate (53 mCi/mmol) was purchased from Amersham International plc. Sodium DL-[2-^{14}C]mevalonate (39.5 mCi/mmol) and sodium *n*-[3,4-^{14}C]butyrate (24.5 mCi/mmol) were obtained from Centre d'Etudes Nucleaires de Saclay. *Perilla* seedlings were cultivated in a growth chamber kept at a relative humidity of 60% and temp. of 25 °C under illumination (16 h/d) with white light (2.83 W/m^2) from fluorescent lamps (FL 20S PG, National). For administration of labeled sucrose, acetate, and MVA, six discs (6 mm in diameter) punched out from the second leaves of one-month-old plants cultivated in the greenhouse or eight pairs of cotyledons of one-week-old seedlings were placed in vials containing an aqueous solution of the labeled compound (5 μCi in 0.1 ml). Labeled butyrate (5.4 μCi in 0.1 ml) was administered to 12 pairs of cotyledons or six discs of the second leaves. The concentration of labeled or cold sucrose administered was 20 mM and that of MVA was 1.7 mM. After an incubation period of 24 h at 25 °C under illumination (2000 lux), the treated discs or cotyledons were mashed with a glass stick and extracted with 0.3 ml of Et_2O. The radioactivity of the Et_2O fraction was measured by a liquid scintillation counter (Aloka LSC-900); solvent system: toluene containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.1 g/l), sampling time: 5 min. Radioactive mono- and sesquiterpenoids in the Et_2O fraction were analyzed by radioisotope thin layer chromatography (RI-TLC) on a silica gel plate (Kieselgel 60F-254, Merck) using *n*-hexane–Me_2CO (19 : 1) as the solvent system, and the spots were detected under ultraviolet (UV) light (254 nm). The radioactivity of separated compounds was measured by a radiochromatoscanner (Aloka JTC-501). For identification of radioactive products, each part of the gel showing radioactivity was scraped off from the plate and extracted with Et_2O, and the extract was analyzed by radioisotope gas chromatography (RI-GC); glass column (3 mm × 3 m) packed with 17% PEG-6000 in Chromosorb W, column temperature: 180 °C, carrier gas: N_2 (50 ml/min). Radioactivity was measured with a radiogaschromatograph (Aloka RGC 212) using CH_4 (150 ml/min) as carrier gas. For examination of the participation of glyoxylate cycle, 12 pairs of cotyledons were placed in vials containing an aqueous solution of iodoacetic acid (0, 100, and 500 μM), and ^{14}C labeled sucrose (5 μCi in 0.1 ml) and butyrate (2.16 μCi in 0.1 ml) were added to each vial. In order to estimate the degree of decomposition of butyrate (1.08 μCi in 0.1 ml), $^{14}CO_2$ released from eight pairs of cotyledons or six discs of foliage leaves was collected in KOH aq. for 20 h after incubation and its radioactivity was counted by a liquid scintillation counter (LKB Wallac 1219 Rackbeta); solvent system: xylene–EtOH–ethylene glycol–toliton X100 (600 : 106 : 37 : 257) containing 4 g of 2,5-diphenyloxazole.

Results

Table I shows the major components of essential oils in the two different chemotypes of *Perilla*, PA and PK, used in the present study. In both chemotypes, the proportion of the main monoterpenoid (perillaldehyde in PA, perillaketone in PK) to that of sesquiterpenoid (caryophyllene) was higher in leaves than in cotyledons.

Table II shows the incorporation ratio of various precursors into major lower terpenoids in PA and PK chemotypes. ^{14}C labeled sucrose, acetate, MVA (5 μCi/0.1 ml, each), and butyrate (5.4 μCi/0.1 ml) were separately fed to six discs cut off from the second leaves of one-month-old plants. In both chemotypes, the radioactivity of only [^{14}C]sucrose was significantly incorporated into the monoterpenoids. Although MVA as well as sucrose was con-

TABLE I. Major Components of Essential Oils in the Cotyledons and the Second Leaves of Two Chemotypes of *Perilla frutescens*

Chemotype	Plant part	Essential oil components (%)		
		PA[a]	PK[b]	CR[c]
PA	Cotyledon	35.1	ND	20.5
	Leaf	67.5	ND	8.2
RK	Cotyledon	ND	79.9	8.3
	Leaf	ND	94.6	2.0

ND: not detected. *a*) Perillaldehyde. *b*) Perillaketone. *c*) Caryophyllene.

Table II. Incorporation of ^{14}C Labeled Precursors into the Main Components of Essential Oils in the Second Leaves of the PA and PK Chemotypes

Chemotype	^{14}C-Labeled precursor	Incorporation ratio (%)		
		PA[a]	PK[b]	CR[c]
PA	Sucrose	1.10	—	0.085
	Acetate	ND	—	ND
	Butyrate	ND	—	ND
	MVA	ND	—	0.078
PK	Sucrose	—	0.74	0.030
	Acetate	—	ND	ND
	MVA	—	ND	0.117

ND: not detected. *a*) Perillaldehyde. *b*) Perillaketone. *c*) Caryophyllene.

Table III. Incorporation of ^{14}C Labeled Precursors into the Main Components of Essential Oils in the Cotyledons of *Perilla* Seedlings

Chemotype	^{14}C-Labeled precursor administered	Incorporation ratio (%)		
		PA[a]	PK[b]	CR[c]
PA	Sucrose	0.37	—	0.059
	Acetate	0.35	—	0.047
	Butyrate	0.56	—	0.18
	MVA	ND	—	0.069
PK	Sucrose	—	0.67	0.046
	Acetate	—	0.72	0.045
	MVA	—	ND	0.105

ND: not detected. *a*) Perillaldehyde. *b*) Perillaketone. *c*) Caryophyllene.

verted into caryophyllene, the radioactivity of the two fatty acids was undetectable in the lower terpenoids.

In contrast to the leaves, the cotyledons of one-week-old seedlings of either chemotype were found to take up not only the radioactivity of [^{14}C]sucrose but also that of [^{14}C]acetate or butyrate into the mono- and sesquiterpenoids at similar rates (Table III). There was no incorporation of [^{14}C]MVA into the monoterpenoids, but sesquiterpenoid was detectable in the cotyledons as in the foliage leaves.

To examine the possibility that in cotyledons the universally labeled fatty acids administered might be metabolized through the glyoxylate cycle to yield radioactive sucrose, [^{14}C]butyrate was fed to the cotyledons treated with iodoacetic acid, which is an inhibitor of glycolysis.[5] As shown in Table IV, 500 μM iodoacetic acid inhibited the incorporation of sucrose into perillaldehyde by 66% but not that of butyrate.

It is suspected that butyrate may not be incorporated into lower terpenoids by the leaves because of their inability to metabolize it to acetyl coenzyme A (CoA), so the following experiment was carried out. When 3,4-[^{14}C]butyrate was administered to leaves, 36.0% of its radioactivity was found in $^{14}CO_2$. This value was comparable to 34.6% obtained from the cotyledons treated with the same labeled compound. These results suggested that butyrate is equally decomposed in the leaves as in the cotyledons, in spite of the difference between the two organs in their incorporation of this compound into lower terpenoids.

To examine whether or not the exogenously administered MVA might be inhibitory to the monoterpenoid synthesis, [^{14}C]sucrose (20 mM) was administered to leaf discs simultaneously with cold MVA (1.7 mM). The results of this experiment showed that the incorporation of the radioactivity of [^{14}C]sucrose into the monoterpenoids was not hindered by adding exogenous cold MVA (Table V). Similarly, the conversion of [^{14}C]MVA into the monoterpenoids was not influenced by the simultaneous supply of cold sucrose.

Table IV. Incorporation of ^{14}C Labeled Sucrose and Butyrate into Perillaldehyde Existing with Iodoacetic Acid Using the Cotyledons of *Perilla frutescens*

Iodoacetic acid (μM)	Incorporation ratio (%)	
	^{14}C-Sucrose	^{14}C-Butyrate
0	0.810	0.876
100	0.804	0.923
500	0.276	1.243

Table V. Incorporation of ^{14}C Labeled Sucrose (20 mM) and MVA (1.7 mM) into Main Mono- and Sesquiterpenoids When Administered to the Second Leaves of the Chemotype PA

Precursors added			Incorporation ratio (%)	
Radioactive		Cold	PA[a]	CR[b]
Sucrose		—	1.01	0.18
Sucrose	+	MVA	1.02	0.15
MVA		—	ND	0.080
MVA	+	Sucrose	ND	0.062

ND: not detected. *a*) Perillaldehyde. *b*) Caryophyllene.

Discussion

It is well known that the cotyledons, which are considered to be the first leaves formed in the ontogeny of Spermatophyta, often show peculiar characteristics distinct from foliage leaves. The present experiments have clearly demonstrated that cotyledons of *Perilla* seedlings incorporated not only sucrose but fatty acids (acetate and butyrate) into lower terpenoids of essential oils, whereas in the foliage leaves the exogenously administered radioactive acetate or butyrate was not incorporated into the lower terpenoids. According to Leo *et al.*,[6] butyrate will more easily permeate a lipid membrane than mevalonate. Such a difference in permeability might be one of the reasons that the incorporation ratio of [^{14}C]butyrate was higher than that of mevalonate in the cotyledons. However, it seems difficult to account for the marked difference in the incorporation of [^{14}C]butyrate between the cotyledons and the foliage leaves unless the permeability of biomembranes to this fatty acid is different between them.

Rapid conversion of acetate to sugars *via* the glyoxylate cycle was reported only for the germinating seed of castor bean,[7] but the present study has suggested that positive participation of this cycle in the biosynthesis of lower terpenoids is unlikely in the cotyledons of *Perilla*. In the foliage leaves, the radioactivity of butyrate administered was not incorporated into lower terpenoids but *ca.* 35% of the activity was detected in the generating CO_2. These results suggest that the acetyl CoA derived from butyrate was not used for the synthesis of lower terpenoids by the foliage leaves.

In the cotyledons the conversion of fatty acids into lower terpenoids might be carried out in a special compartment which is lacking in the foliage leaves. According to Pauly *et al.*[8] the monoterpenoid synthesis is localized in leucoplasts of immature calamondin and satsuma fruits.

In the present experiments, [^{14}C]MVA was not incorporated into monoterpenoids but into caryophyllene in both cotyledon and foliage leaf. A similar phenomenon was reported for *Mentha piperita* by Croteau and Loomis,[9] that mono- and sesquiterpenoids are probably synthesized at different compartmentalized sites. The results of our study on *Perilla* apparently support their proposal. However, biochemical studies are needed to fully account for the lack of incorporation of MVA into monoterpenoids.

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