Contribution of Mevalonate and Methylerythritol Phosphate Pathways to Polyisoprenoid Biosynthesis in the Rubber-Producing Plant *Eucommia ulmoides* Oliver

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The biosynthetic origin of isopentenyl diphosphate in the polyisoprenoid biosynthesis of the rubber-producing plant *Eucommia ulmoides* Oliver was elucidated for the first time by feeding experiments using ^{13}C-labeled isotopomers of (*RS*)-mevalonate, 1-deoxy-D-xylulose-3,4,5-triacetate, 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate, and pyruvate. After ^{13}C-labeled isotopomers were fed to the young seedlings, the polyisoprenoid fractions were prepared and analyzed by ^{13}C NMR. The NMR data showed that the isoprene units of polyisoprenoid derived from isopentenyl diphosphate, which was biosynthesized using both mevalonate and 1-deoxy-D-xylulose-5-phosphate in *E. ulmoides*. It is assumed that the cross-talk of isopentenyl diphosphate, derived from both pathways, occurs during the biosynthesis of polyisoprenoid; therefore, it was observed in the formation of low-molecular weight isoprenoids.

Key words: Polyisoprenoid, Isopentenyl Diphosphate, *Eucommia ulmoides*

Introduction

Following the discovery of the dual biosynthetic origin of isopentenyl diphosphate (IPP) by Rohmer *et al*. (1993), reanalysis of the biosynthetic pathway of IPP has been conducted in many organisms. The previous results strongly suggested that IPP is independently biosynthesized via the mevalonate pathway in cytosol and via the methylerythritol phosphate (MEP) pathway in plastids (Lichtenthaler, 1999). However, recent studies strongly proposed the existence of a cross-talk in which IPP translocates between the plastidial and cytosolic spaces (Kasahara *et al*., 2002; Hemmerlin *et al*., 2003). Therefore, the biosynthetic pathway of isoprenoids in plants needs to be studied again because the magnitude of "IPP cross-talk" may have a significant role to play in plant physiology.

Rubber (polyisoprene) is one of the most important biomasses whose application has spread widely to industries. Generally, natural rubber exhibits to the *cis*-form rubber produced by the "para-rubber tree" (*Hevea brasiliensis*). A few exotic plant species such as the "hard rubber tree" (*Eucommia ulmoides,* Eucommiaceae) are known to produce *trans*-form rubber. Although the economic benefits of rubber are significantly large, their biosynthetic mechanisms have not been completely elucidated. To date, both *cis*- and *trans*-form rubber are assumed to be biosynthesized via IPP as a crucial unit. Rubber is considered to be

Abbreviations: IPP, isopentenyl diphosphate; MEP, methylerythritol phosphate; GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; ME-4Ac, 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate; DX-3Ac, 1-deoxy-D-xylulose-3,4,5-triacetate; DX, 1-deoxy-D-xylulose; ME, 2*C*-methyl-D-erythritol; DXP, 1-deoxy-D-xylulose-5-phosphate; TMS, trimethylsilyl; SIM, single ion monitoring.

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biosynthesized using allyl diphosphate as a starting material, and IPP is added by a specific prenyltransferase to yield a high-molecular weight polymer (Lynen and Henning, 1960; Archer *et al.*, 1961) (Fig. 1). However, no experimental evidence has been reported for the pathway through which IPP would be biosynthesized for rubber.

Cultured cells instead of real plants are usually employed as living materials for labeling experiments involving a ^{13}C-labeled biosynthetic intermediate because feeding experiments are easier to perform with cultured cells (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997; Disch *et al.*, 1998). However, real plants should be used for the rubber biosynthesis experiments because cell culture systems cannot produce rubber. Comprehensive preliminary experiments are required to decide the appropriate conditions for the uptake of biosynthetic intermediates by plant organs such as leaves, and roots. This study aimed to reveal the biosynthetic mechanism of *trans*-form rubber formation in *E. ulmoides*. *E. ulmoides* is native to the southeastern part of China and is widely distributed in the temperate zone. It is known to produce a fibrous rubber (*EU*-rubber) in various organs such as leaf, bark, root, and fruit coat (Bamba *et al.*, 2001). Rubber is produced even in the plant seedlings, one month after germination. After several preliminary experiments, we succeeded in labeling the *EU*-rubber with isotopic intermediates in both biosynthetic pathways – "mevalonate pathway" and "MEP pathway". Hence, we present the results of the feeding experiments where the seedlings were cultured under aseptic conditions.

Material and Methods

Chemicals and plant materials

[2-^{13}C] Mevalonolactone and [2-^{13}C] sodium pyruvate were purchased from ISOTEC (Miamisburg, OH, USA). [1-^{13}C] 1-Deoxy-D-xylulose-3,4,5-triacetate ([1-^{13}C] DX-3Ac) and [5-^{13}C] 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate ([5-^{13}C] ME-4Ac) were synthesized from commercially available sugar and $^{13}CH_3MgI$ (Hoeffler *et al.*, 2000; Okumoto and Katto, 2003).

E. ulmoides seeds were collected at the Hitachi Zosen Corporation Experimental Station (Habu 2264-1 Innoshima, Hiroshima, Japan). The seeds were sterilized with 5% sodium hypochlorite and then aseptically transferred to test tubes containing MS medium (containing 2% sucrose and 0.24% gelite, pH 5.7). The test tubes were incubated in a growth chamber at 25 ºC with a light/dark cycle of 16 h/8 h. 40- to 50-day-old *E. ulmoides* seedlings were used for the feeding experiments.

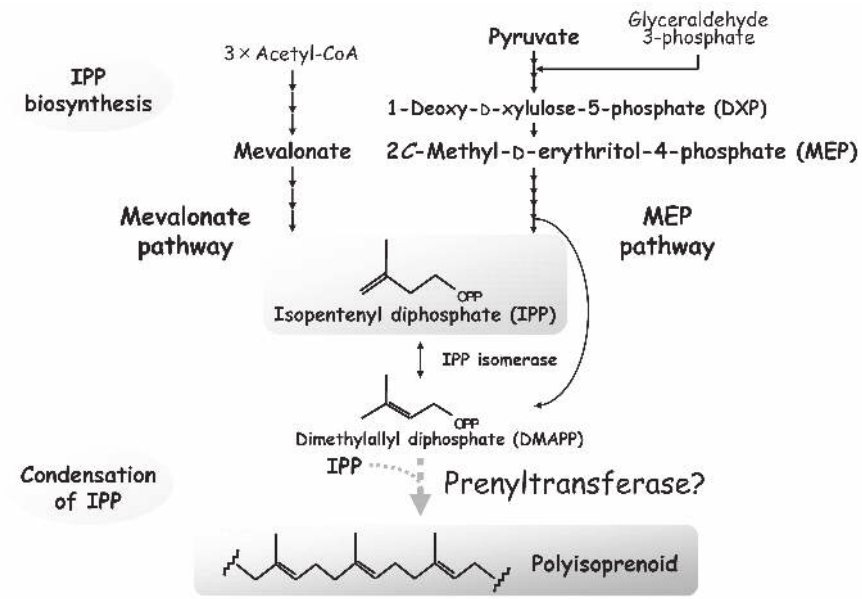

Fig. 1. Predicted polyisoprenoid biosynthetic pathway.

Feeding experiments

4 ml of 0.1% or 0.5% ^{13}C-isotopomers solution (containing 0.02% Tween 80) were aseptically added to the test tubes. The test tubes were incubated in a growth chamber at 25 ºC (16 h light/8 h dark) for 30 d. The test tubes were shaken once every two days, and the whole plant was drenched with the ^{13}C-isotopomer solution.

Preparation of isoprenoids from E. ulmoides seedlings

After being frozen and homogenized in liquid nitrogen, a low-molecular weight isoprenoid was first extracted by Soxhlet extraction with ethanol from the *E. ulmoides* seedlings. Next, a high-molecular weight isoprenoid was obtained by Soxhlet extraction with toluene.

The ethanol extract was treated with alkali (30% potassium hydroxide/ethanol/benzene 5:4:1, containing 2% pyrogallol) under reflux for 3 h. Next, the saponified lipid was extracted with *n*-hexane. Finally, the dried *n*-hexane extract was silylated for 15 min at 60 ºC using hexamethyldisilazane/trimethylchlorosilane/pyridine (2:1:10) for gas chromatography-mass spectrometry (GC-MS) analysis.

Analysis of polyisoprenoids

^{13}C NMR spectra were measured in benzene-d_6 using an ECP-400 NMR spectrometer (JEOL, Akishima, Japan) at 50 °C; tetramethylsilane (TMS) was used as an internal standard.

Analysis of β-sitosterol and phytol

The lipid fractions containing the trimethylsilyl (TMS)-derivatized *β*-sitosterol and phytol were analyzed by GC-MS using a TRACE GC gas chromatograph (Thermo Electron Co., San Jose, CA, USA) equipped with a fused-silica capillary column, DB-1 MS (30 mm × 0.25 mm I.D., df = 0.25 *μ*m; J & W Scientific, Folsom, CA, USA), and coupled with a TRACE DSQ mass spectrometer (Thermo Electron Co.). The temperature conditions were set as follows: 80 ºC to 240 ºC (25 °C/min), 240 ºC to 310 ºC (4 ºC/min), and 310 ºC for 5 min. The injector temperature was 260 °C. The temperatures of the transfer line and ion source were 310 °C and 200 °C, respectively. Ions representing *β*-sitosterol (*m/z* = 486, 487, 488, 489) and phytol (*m/z* = 353, 354, 355, 356) were analyzed using single-ion monitoring (SIM).

Results and Discussion

Incorporation of (RS)-[2-^{13}C] mevalonate

The feeding experiment was first performed using *(RS)*-[2-^{13}C] mevalonate in *E. ulmoides* seedlings (40–50 days after seeding). *(RS)*-[2-^{13}C] Mevalonate is incorporated as an intermediate of the mevalonate pathway; finally, only the fourth

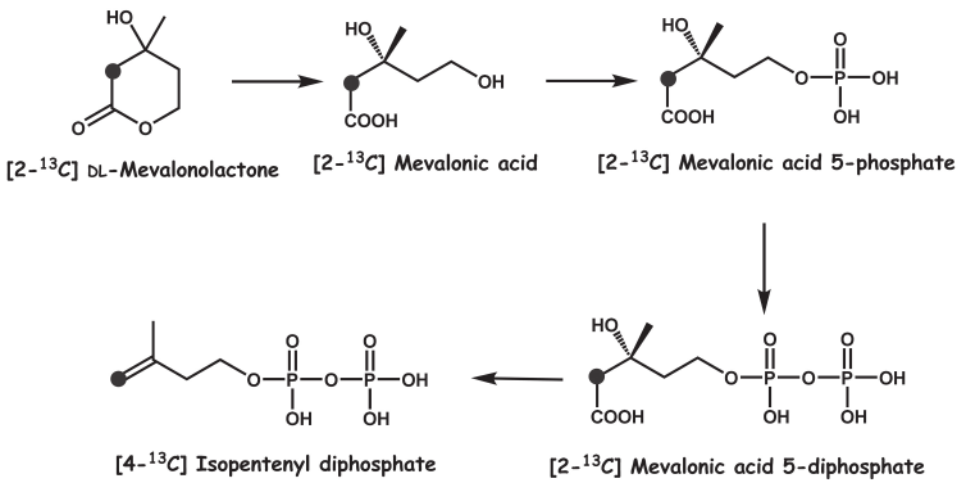

Fig. 2. Predicted labeling patterns of IPP and intermediates of the mevalonate pathway in the feeding experiment with [2-^{13}C] mevalonolactone.

position of IPP will be ^{13}C-labeled through each intermediate, as shown in Fig. 2. With regard to feeding the ^{13}C-isotopomers to a plant, methods involving their direct application onto a leaf or their addition to an agar medium were investigated. As a result, we attained a simple and effective method to conduct aseptically the feeding experiment in plants. Using this method, the ^{13}C-isotopomer solution was added so that the part of seedlings grown on the agar medium in a test tube sank. The test tubes were shaken once every two days, and the whole plant was drenched with the ^{13}C-isotopomer solution.

After 30 days of feeding, low- and high-molecular weight isoprenoids were obtained from the seedlings by Soxhlet extraction with ethanol and toluene, respectively. The toluene Soxhlet extract (*EU*-rubber fraction) was subjected to ^{13}C NMR analysis to identify the ^{13}C-labeled position. As a result, the signal intensity of the fourth position in the isoprene unit increased compared to those of the other positions (Fig. 3). Therefore, the incorporation of *(RS)*-[2-^{13}C] mevalonate as an intermediate of the mevalonate pathway was confirmed.

Incorporation of [1-^{13}C] 1-deoxy-D-xylulose-3,4,5-triacetate and [5-^{13}C] 2C-methyl-D-erythritol-1,2,3,4-tetraacetate

Next, the feeding experiment was performed using 1-deoxy-D-xylulose (DX), which is an intermediate of the MEP pathway. Since DX is a hydrophilic compound, its incorporation effi-

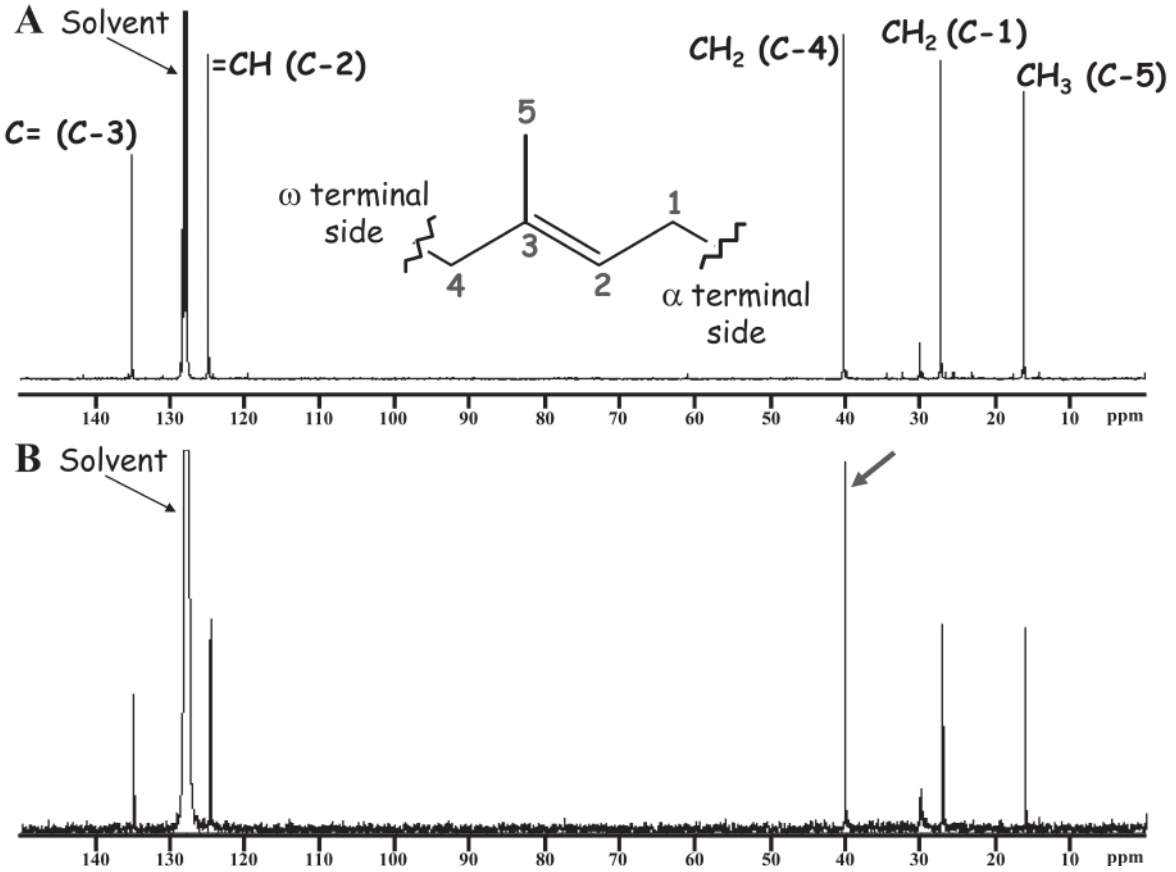

Fig. 3. ^{13}C NMR spectra of *EU*-rubber in the feeding experiment with [2-^{13}C] mevalonolactone (B) and control (A). The signal pointed by an arrow (C-4) increased compared to those of the other positions. Therefore, [2-^{13}C] mevalonolactone was incorporated as an intermediate of the mevalonate pathway in this feeding experiment (see Fig. 2).

ciency from the plant surface may not be high. Therefore, in this experiment, [1-^{13}C] 1-deoxy-D-xylulose-3,4,5-triacetate ([1-^{13}C] DX-3Ac) that increases the hydrophobicity by acetylation of the hydroxy group was used. Since [1-^{13}C] DX-3Ac was incorporated as an intermediate of the MEP pathway, only the fifth position of IPP was ^{13}C-labeled through each intermediate, as shown in Fig. 4. Using the procedure mentioned previously, a high-molecular weight isoprenoid was obtained from the seedlings and subjected to ^{13}C NMR analysis. As a result, the signal intensity of the fifth position increased compared to those of the other positions (Fig. 5). Therefore, the incorporation of [1-^{13}C] DX-3Ac as an intermediate of the MEP pathway was confirmed.

The feeding experiment was also performed using 2*C*-methyl-D-erythritol (ME), which is an intermediate of the MEP pathway, as well as DX. In this experiment, a tetra-acetylated ^{13}C-isotopomer, [5-^{13}C] 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate [(5-^{13}C] ME-4Ac) was synthesized and incorporated using the same procedure as that of the first experiment.

After [5-^{13}C] ME-4Ac was incorporated, only the fifth position of IPP was ^{13}C- labeled, as shown in Fig. 4. After extracting the high-molecular weight isoprenoid and subjecting it to ^{13}C NMR analysis, the change in signal intensity was not to that extent as observed in the control sample, *i.e.* ME-4Ac was not incorporated (Fig. 6).

Therefore, under deficiency of the intermediates by treatment with an inhibitor of the MEP pathway, *i.e.* the herbicide clomazone (dimetazone), that produces leaf bleaching by significant reduction in the levels of plastidial pigments such as carotenoids and chlorophylls (Lange *et al.*, 2001), the feeding experiment with [5-^{13}C] ME-4Ac was repeated. However, the high-molecular weight isoprenoid was not labeled by ^{13}C (data

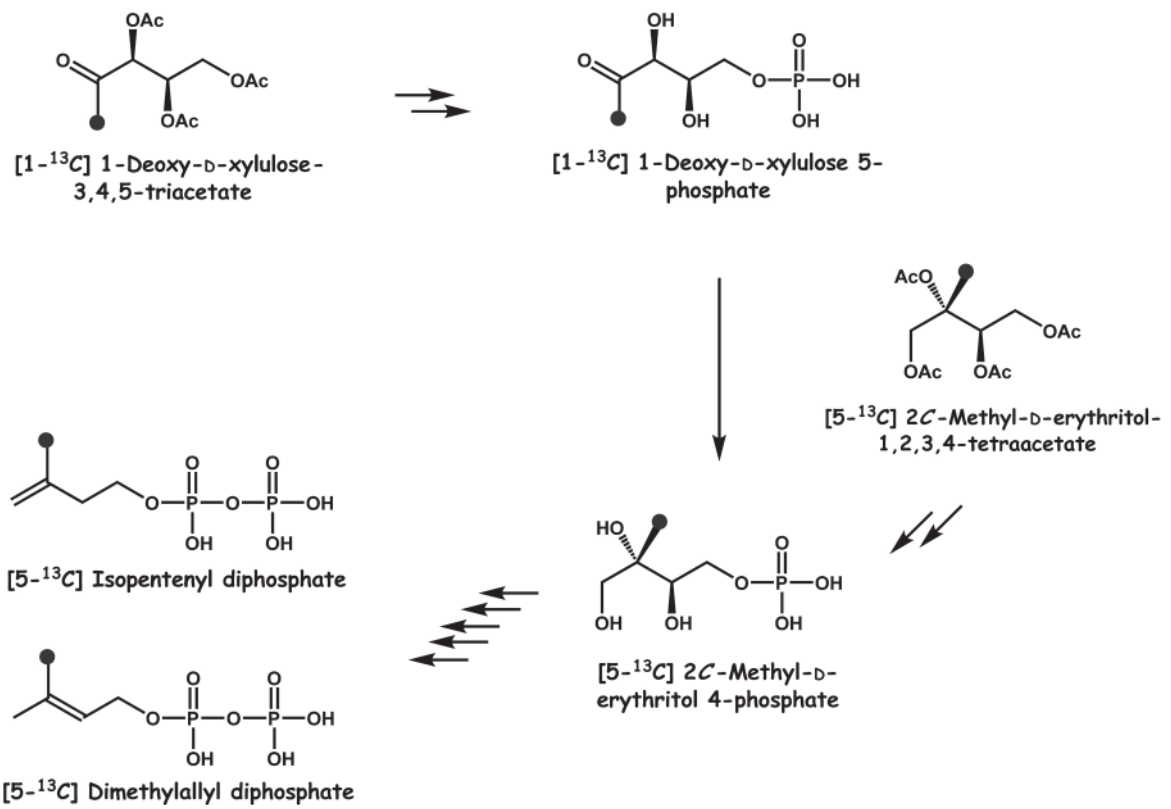

Fig. 4. Predicted labeling patterns of IPP and intermediates of the MEP pathway in the feeding experiment with [1-^{13}C] 1-deoxy-D-xylulose-3,4,5-triacetate and [5-^{13}C] 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate.

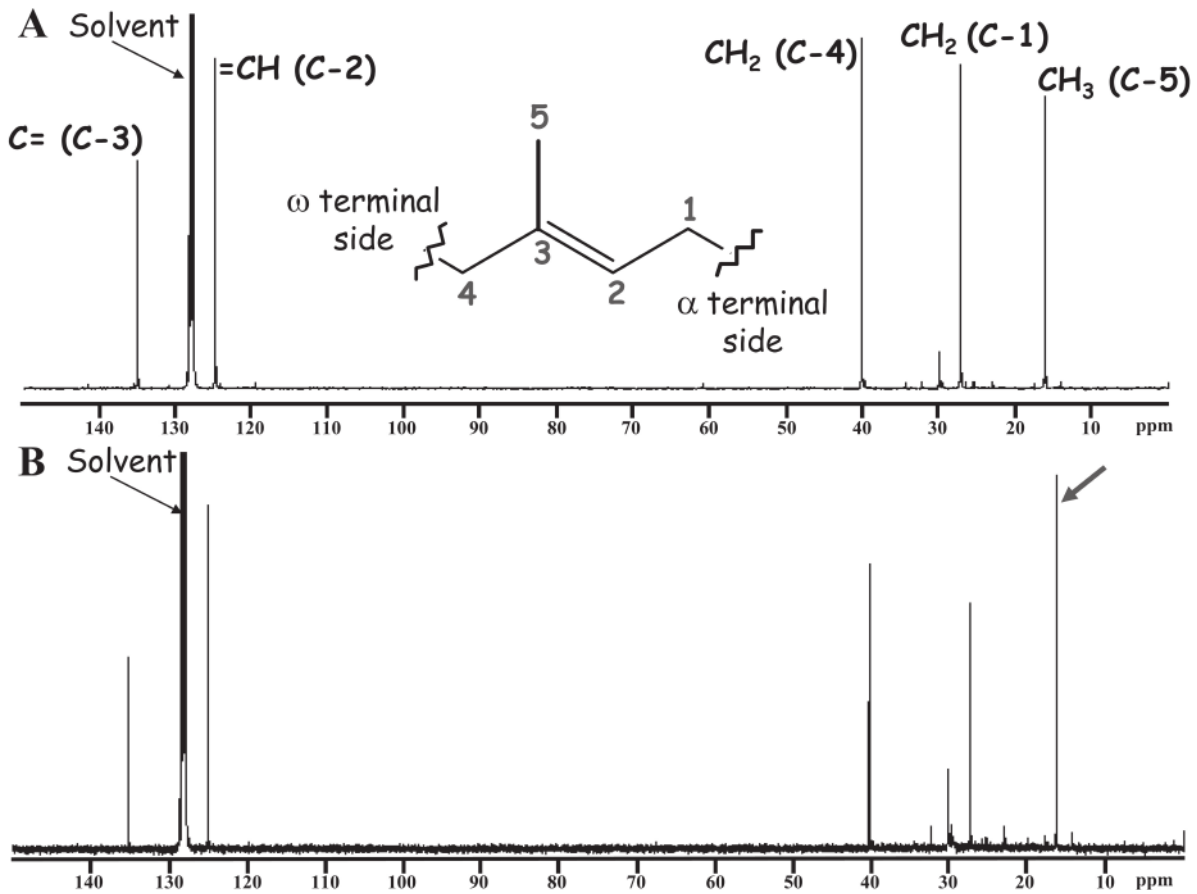

Fig. 5. ^{13}C NMR spectra of *EU*-rubber in the feeding experiment with [1-^{13}C] 1-deoxy-D-xylulose-3,4,5-triacetate (B) and control (A). The signal pointed by an arrow (C-5) increased compared to those of the other positions. [1-^{13}C] 1-Deoxy-D-xylulose-3,4,5-triacetate was incorporated as an intermediate of the MEP pathway in this feeding experiment (see Fig. 4).

not shown). Additionally, leaf bleaching was not observed to improve the addition of [5-^{13}C] ME-4Ac. From these results, it may be concluded that [5-^{13}C] ME-4Ac was not available as an intermediate of the MEP pathway.

Incorporation of [2-^{13}C] sodium pyruvate

Next, the feeding experiment using [2-^{13}C] sodium pyruvate, which is utilized in both the mevalonate and MEP pathways, was performed. [2-^{13}C] Sodium pyruvate was incorporated as an intermediate of the mevalonate pathway. The first and fifth positions of IPP were ^{13}C-labeled via the mevalonate pathway, while only the third position of IPP was labeled by ^{13}C via the MEP pathway (Fig. 7).

The toluene Soxhlet extract containing the high-molecular weight isoprenoid was subjected to ^{13}C NMR analysis and the ^{13}C-labeled positions were identified. The signal intensities of the first and third positions increased, and the increment in the intensity of the first position was greater than that of the third position (Fig. 8). This result suggests that polyisoprenoid was biosynthesized by IPP not only from the mevalonate pathway but also the MEP pathway.

GC-MS analysis of β-sitosterol and phytol from each feeding experiment sample

The ethanol Soxhlet extracts containing low-molecular weight isoprenoids were hydrolyzed with alkali and derivatized by the silylation rea-

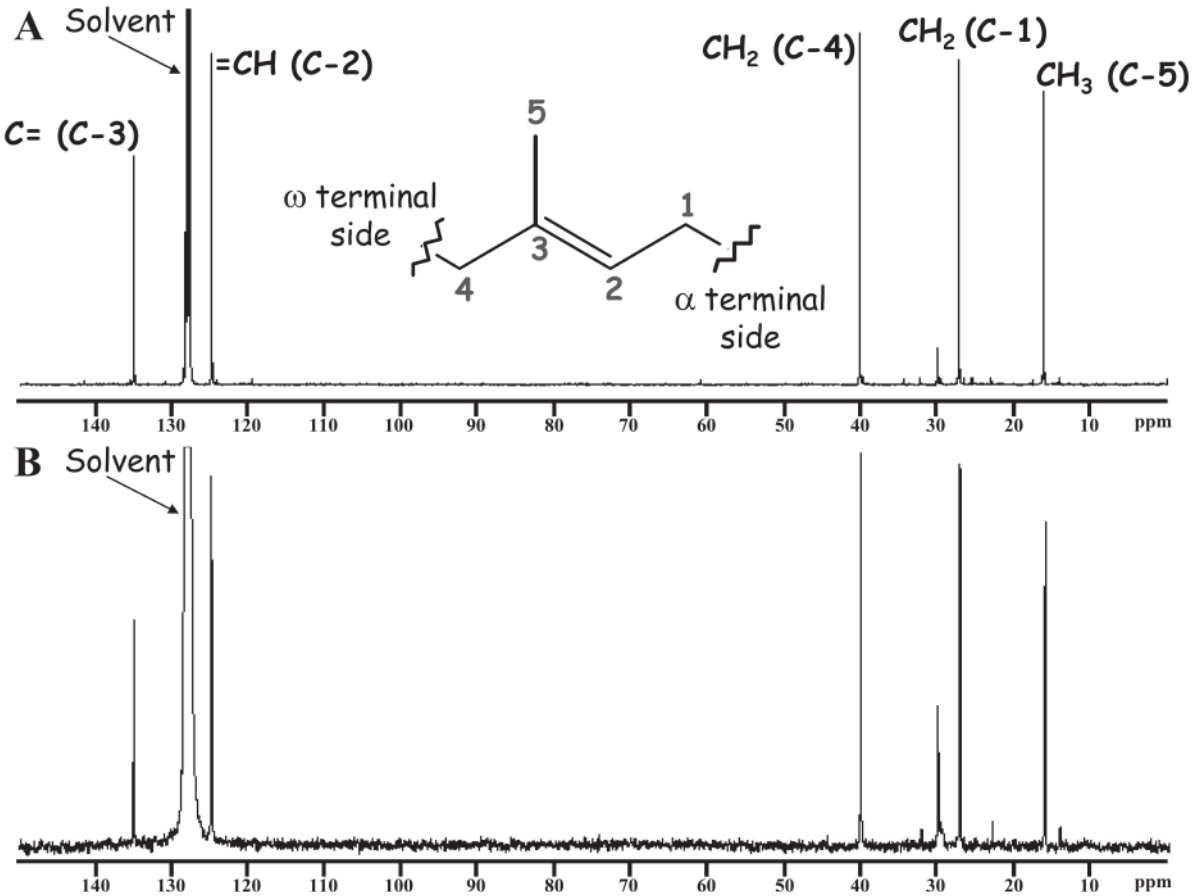

Fig. 6. ^{13}C NMR spectra of *EU*-rubber in the feeding experiment with [5-^{13}C] 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate (B) and control (A). The pattern of signal intensity did not change compared to that of the control. [5-^{13}C] 2*C*-methyl-D-erythritol-1,2,3,4-tetraacetate was not incorporated as an intermediate of the MEP pathway in this feeding experiment.

Table I. GC-MS analyses of *β*-sitosterol and phytol. Relative intensities (%) of the molecular ions of ^{13}C-labeled silanized *β*-sitosterol ($[M + n]^+ = 486 + n$) and phytol ($[M - CH_3 + n]^+ = 353 + n$) obtained from *E. ulmoides* fed with (*RS*)-[2-^{13}C] mevalonolactone, [1-^{13}C] DX-3Ac, [5-^{13}C] ME-4Ac, and [2-^{13}C] sodium pyruvate are summarized.

β-Sitosterol

m/z	Theoretical value	Control	^{13}C-Mevalonolactone	^{13}C-DX-3Ac	^{13}C-ME-4Ac	^{13}C-Pyruvate
486	100	100	100	100	100	100
487	40.4	39.8	41.8	45.3	41.4	49.9
488	11.4	11.3	12.9	15.6	11.3	18.4
489	2.3	2.2	5.5	4.8	2.1	5.8

Phytol

m/z	Theoretical value	Control	^{13}C-Mevalonolactone	^{13}C-DX-3Ac	^{13}C-ME-4Ac	^{13}C-Pyruvate
353	100	100.0	100.0	100.0	100.0	100.0
354	30.2	29.9	41.3	35.1	29.9	45.3
355	7.9	8.7	48.4	12.2	8.6	18.2
356	1.3	1.6	16.3	4.0	1.6	5.1

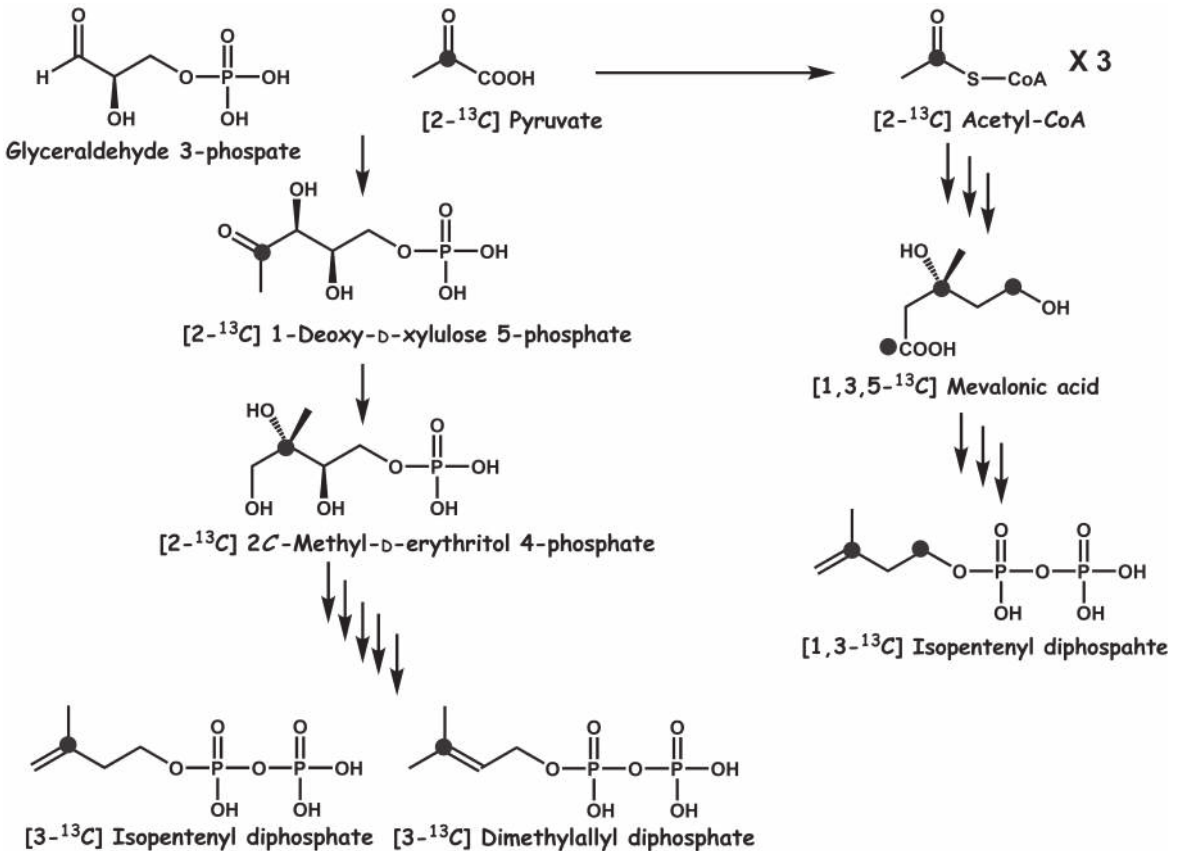

Fig. 7. Predicted labeling patterns of IPP and intermediates of the mevalonate and MEP pathways in the feeding experiment with [2-^{13}C] sodium pyruvate.

gent for GC-MS analysis. β-Sitosterol was biosynthesized from IPP by the mevalonate pathway and phytol was biosynthesized from IPP by the MEP pathway (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997; Disch *et al.*, 1998). The degrees of incorporation of ^{13}C-isotopomer in β-sitosterol and phytol were investigated by GC-MS.

According to each mass spectrum, the base ion peak of β-sitosterol (m/z = 486 [M$^+$]) and the isotope peaks at 487 ([M + 1]$^+$), 488 ([M + 2]$^+$), 489 ([M + 3]$^+$), and that of phytol (m/z = 353 [M – 15]$^+$) and the isotope peaks at 354 ([M – 15 + 1]$^+$), 355 ([M – 15 + 2]$^+$), 356 ([M – 15 + 3]$^+$) were quantitatively analyzed by SIM. The calculation results of relative intensities of the molecular ions of ^{13}C-labeled β-sitosterol ([M + n]$^+$ = 486 + n) and phytol ([M – CH_3 + n]$^+$ = 353 + n) from the peak area are presented in Table I. The isotope peaks of both compounds were greater in the feeding experiment samples containing *(RS)*-[2-^{13}C] mevalonate, [1-^{13}C] 1-deoxy-D-xylulose-3,4,5-triacetate, and [2-^{13}C] sodium pyruvate as compared to that of the control sample. These results strongly suggest the occurrence of a cross-talk of IPP between the cytosolic mevalonate and the plastidial MEP pathway in *E. ulmoides*. On the other hand, a change in signal intensity was not observed in the ME-4Ac feeding sample as compared with that of the control sample; therefore, ME-4Ac is not incorporated as effectively as in the case of polyisoprenoid.

Conclusion

As it is evident from the results of the feeding experiment of ^{13}C-isotopomers, polyisoprenoid was proved to be biosynthesized from IPP that is derived from both mevalonate and MEP pathways

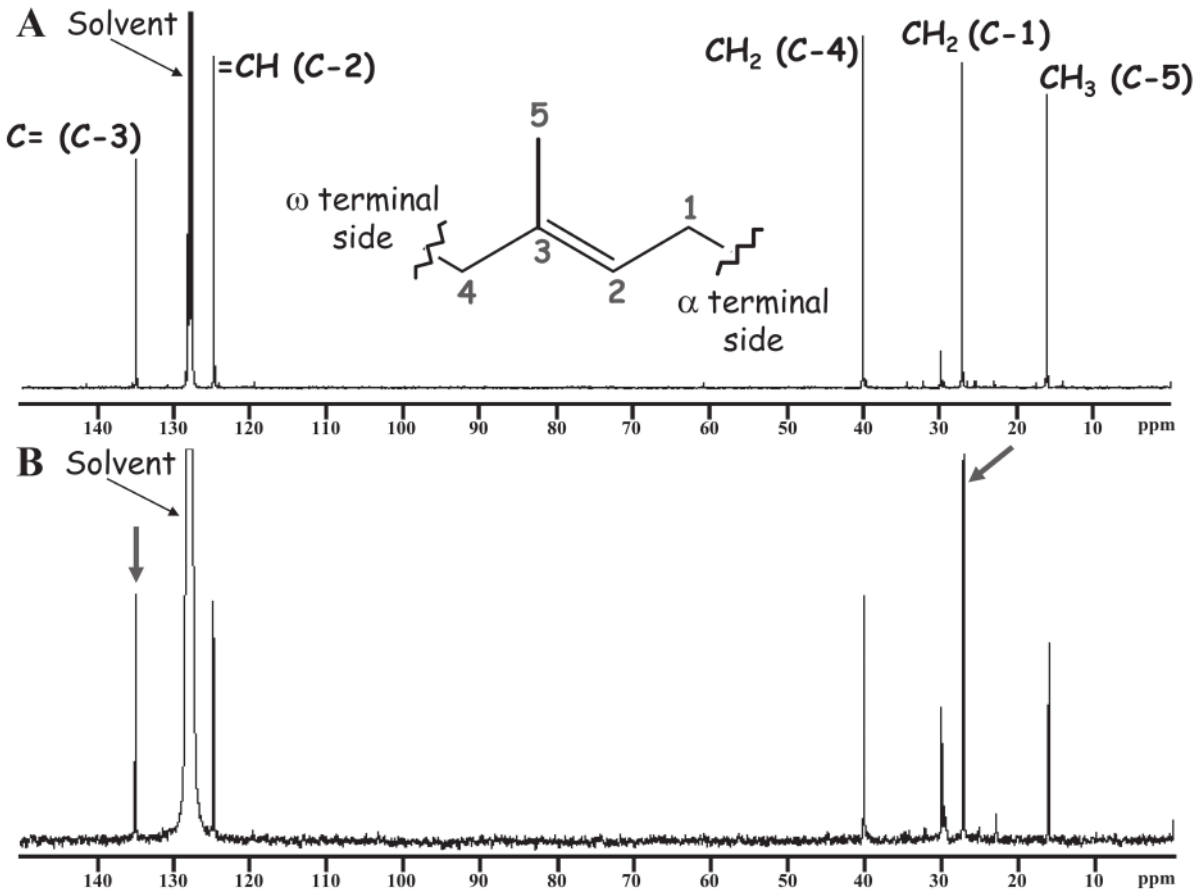

Fig. 8. ^{13}C NMR spectra of *EU*-rubber in the feeding experiment with [2-^{13}C] sodium pyruvate (B) and control (A). The signals pointed by arrows [(C-1) and (C-3)] increased compared to those of the other positions. [2-^{13}C] Sodium pyruvate was incorporated as an intermediate of both mevalonate and MEP pathways in this feeding experiment (see Fig. 7).

in *E. ulmoides*. However, this experiment could not clarify the primary pathway among these two. Since the cross-talk of IPP was observed in the biosynthesis of low-molecular weight isoprenoids, it should also occur in the biosynthesis of high-molecular weight isoprenoids. In future, we will consider that the supply pathway of IPP in the polyisoprenoid biosynthesis can be elucidated by an experiment with inhibitors, a short-term feeding experiment, etc.

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