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Investigation of the biosynthesis pathway to give *cis*-jasmone

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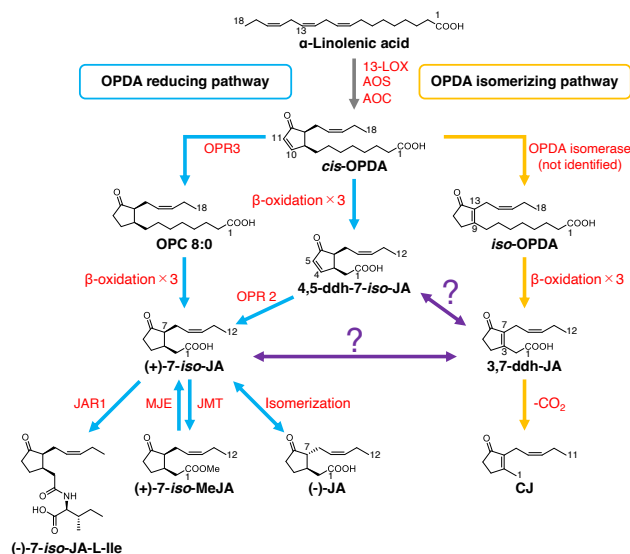
Abstract: There is a report that (+)-7-*iso*-jasmonic acid ((+)-7-*iso*-JA) is an intermediate in the production of *cis*-jasmone (CJ), although the biosynthetic pathway of CJ has still not been fully described. It has been reported that CJ, a substructure of pyrethrin II produced by pyrethrum (*Tanacetum cinerariifolium*), is not biosynthesized via this proposed biosynthetic pathway. To clarify this ambiguity, the stable isotope-labeled jasmonates were synthesized, and some compounds were administered to apple mint (*Mentha suaveolens*) via air propagation. The results showed that (+)-7-*iso*-JA is not an intermediate that yields *cis*-jasmone, and 3,7-didehydro-JA (3,7-ddh-JA) is not an intermediate that gives (+)-7-*iso*-JA, although 3,7-didehydro-JA and 4,5-didehydro-7-*iso*-JA were converted into CJ and JA, respectively.

Introduction

The biosynthesis of jasmonate begins with a release of α -linolenic acid in chloroplasts, and the released α -linolenic acid undergoes peroxidation by lipoxygenase (LOX), epoxidation by allene oxide synthase (AOS), and cyclization by allene oxide cyclase (AOC) to produce *cis*-12-oxo-phytodienoic acid (*cis*-OPDA). *cis*-OPDA is then transported to peroxisomes, where it is reduced by OPDA-reductase 3 (OPR3) to form 3-oxo-2-(2'-[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC 8:0), followed by three β -oxidation steps to produce (+)-7-*iso*-jasmonic acid ((+)-7-*iso*-JA). (+)-7-*iso*-JA is isomerized to (-)-JA by keto-enol tautomerization in vivo, which has *trans* conformation of side chains at C-7 and C-3. Furthermore, (+)-7-*iso*-JA was conjugated with L-isoleucine via Jasmonate amido synthase (JASMONATE RESISTANT1: JAR1) to give (-)-7-*iso*-JA-L-isoleucine ((-)-7-*iso*-JA-L-Ile), which controls defense responses against insects, necrotrophic pathogens, and other environmental stresses, such as the production of proteinase inhibitors to suppress feeding by pests^[1]. One of the volatile forms of jasmonates, methyl (+)-7-*iso*-JA ((+)-7-*iso*-MeJA), can induce defense responses in neighboring plants by air transmission. (+)-7-*iso*-MeJA is produced from (+)-7-*iso*-JA by JA carboxyl methyltransferase

(JMT)^[2], whereas (+)-7-*iso*-MeJA is hydrolyzed to (+)-7-*iso*-JA by MeJA-esterase (MJE)^[3]. In addition, there is a supplemental pathway for JA biosynthesis that uses 4,5-didehydro-7-*iso*-JA (4,5-ddh-7-*iso*-JA) as an intermediate. In this pathway, 4,5-ddh-7-*iso*-JA is generated from *cis*-OPDA by three β -oxidation steps, and then reduced by *cis*-OPDA reductase 2 (OPR2) to form (+)-7-*iso*-JA^[4]. The pathways via OPR3 and OPR2 were collectively named as the OPDA-reducing pathway (Scheme1) in the report by Miyawaki *et al.*^[5].

The essential oil of jasmine has long been used in the perfume industry due to its excellent fragrance, and the compounds *cis*-jasmone (CJ) and (+)-7-*iso*-MeJA or (-)-MeJA have been identified in it^[6-7]. CJ is also found in the absolutes of neroli (*Citrus bigaradia*), jonquil (*Narcissus jonquilla* L.), and bergamot (*Citrus bergamia*) plants and in plants of the Pittosporum family^[8]. Today, CJ is an important industrial material used to produce many commodities, such as perfumes, cosmetics, and food products. The biological effects of CJ have been studied extensively to date. First, it was noted that CJ is released from flowers in many cases and functions as a mark that attracts olive flies^[8]. CJ has received



Scheme 1. Proposed biosynthesis pathway of CJ.

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attention as a wounding signal as well as a beneficial ingredient promoting the arrivals of pollinator insects. In fact, small amounts of CJ are released from cotton leaves during injury^[9]. CJ exterminates aphids by inviting natural enemies of the aphids such as aphid parasitoids^[10]. CJ acts as a signal to trigger plant defense responses, similar to MeJA. *Brassica* plants treated with CJ were less attractive to and less suitable for *Myzus persicae* aphids^[11], and CJ-treated tomato leaflets significantly reduced *Tuta absoluta* leaf-mining activity^[12]. Wheat treated with CJ accumulated phenolic acids, which act as defensive substances^[13]. Interestingly, CJ triggers a distinct signaling pathway from that of jasmonates. CJ-responsive genes are not affected by mutations in *COI1* or *JAR1*, which encode critical signaling proteins in the JA response^[14].

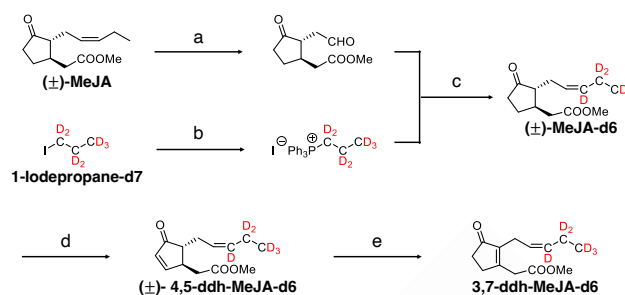
As of now, two pathways have now been proposed for CJ biosynthesis. One is the OPDA-reducing pathway as mentioned above. In another, a part of chemical structure of *cis*-OPDA is isomerized from C11-C10 carbons double bond to that spanning C13-C9 carbons to give *iso*-OPDA, which were collectively named as the OPDA-isomerizing pathway (Scheme1) in the report by Miyawaki *et al.*^[5].

Inspired by the commonality of the chemical structure in CJ with that of jasmonate, Koch *et al.*^[15] offered deuterium-labeled ($\pm$)-JA and 3,7-ddh-MeJA to lima beans (*Phaseolus lunatus*), willow (*Salix alba*) and jasmine (*Jasminum racemiflorum*), which found that the labeled compounds were converted to deuterium-labeled CJ. From the results of the experiment, it could be determined that CJ was biosynthesized via the OPDA-reducing pathway. On the while, the OPDA-isomerizing pathway to produce CJ via *iso*-OPDA was proposed by Dabrowska *et al.*^[8], who showed conversion of deuterium-labeled methyl *iso*-OPDA to deuterium-labeled CJ in several plants. Recently, it was reported that in *Tanacetum cinerariifolium*, CJ composing a partial structure of pyrethrin II is biosynthesized via the OPDA-isomerizing pathway rather than the OPDA-reducing pathway^[16]. Therefore, the OPDA-isomerizing pathway may well be the pivotal route for CJ biosynthesis.

We have been studying whether CJ is biosynthesized via the OPDA-reducing or the OPDA-isomerizing pathway in plant. In this study, we used apple mint (*Mentha suaveolens*) as an experimental plant and proved that CJ is biosynthesized via the OPDA-isomerizing pathway. Furthermore, we proved that 3,7-ddh-JA, on the OPDA-isomerizing pathway is not converted to (+)-7-*iso*-JA.

Results and Discussion

In this study, some deuterium labeled JA-skeleton analogs were synthesized to investigate the CJ biosynthesis pathway, such as (10,11,11,12,12,12-²H₆)-($\pm$)-MeJA (($\pm$)-MeJA-d₆), (10,11,11,12,12,12-²H₆)-($\pm$)-4,5-ddh-MeJA (($\pm$)-4,5-ddh-MeJA-d₆),



Scheme 2. Synthesized deuterium-labeled compounds. (a) RuCl₃ aq, NaIO₄, 1,2-dichloroethane, H₂O, rt, 1 h, 91%, (b) Ph₃P, CH₃CN, reflux, 16 h, 94%, (c) NaHMDS, CH₂Cl₂, -78 °C → rt, 16 h, 60%, (d) Palladium (II) acetate, Diethylallyl phosphate, Na₂CO₃, DMF, 80 °C, 24 h, 38%, (e) CH₃ONa, MeOH, 60 °C, 12 h, 60%.

a)	Plant species	Organ	Production of CJ
	<i>Jasminum sambac</i>	flower	n.d.
	<i>Nicotiana attenuata</i>	flower	n.d.
	<i>Rudbeckia laciniata</i>	flower	n.d.
	<i>Linaria vulgaris</i>	flower	n.d.
	<i>Allium tuberosum</i>	flower	n.d.
	<i>Rosa canina</i>	flower	n.d.
	<i>Impatiens textori</i>	flower	n.d.
	<i>Tanaxacum officinale</i>	flower	n.d.
	<i>Allium caeruleum</i>	flower	n.d.
	<i>Lonicera japonica</i>	flower	○
	<i>Mentha longifolia</i>	flower, leaf	○
	(n.d.; not detected)		

b)	Plant Species	CJ (μg/g FW)
	<i>Mentha longifolia</i>	32.63 ± 4.15
	<i>Mentha suaveolens</i>	46.50 ± 10.12
	<i>Mentha spicata</i>	0.76 ± 0.08
	<i>Thymus vulgaris</i>	n.d.
	<i>Origanum vulgare</i>	n.d.
	<i>Salvia officinalis</i>	n.d.
	<i>Salvia rosmarinus</i>	0.89 ± 0.04
	<i>Ocimum basilicum</i>	n.d.
	<i>Lavandula angustifolia</i>	n.d.
	<i>Perilla frutescens</i>	n.d.
	(n.d.; not detected)	

Table 1. Screening of plants with the ability to produce CJ. (a) List of examined plants. (b) List of examined Lamiaceae plants.

2,12-²H₆)-3,7-ddh-MeJA (3,7-ddh-MeJA-d₆) starting from commercially available ($\pm$)-MeJA according to the reported method^[17] (Scheme 2). In addition, we synthesized (1,1,1,3,3,3,4,4-²H₇)-CJ (CJ-d₇) to quantify the endogenous amount of CJ in plants according to the reported method^[18]. Experimental procedures to synthesize compounds are described in the supporting information.

CJ is biosynthesized in flowers in many cases, and its production has been reported in a variety of plants, including Honeysuckle (*Lonicera japonica*)^[19] and Jasmine (*Jasminum sambac*)^[20]. We first screened for plants having higher ability to produce CJ, which enabled us to detect CJ using GC-MS system through one step of extraction using ethyl acetate (EtOAc) (Table 1). Schematic illustration to analyze compounds using GC-MS is described in

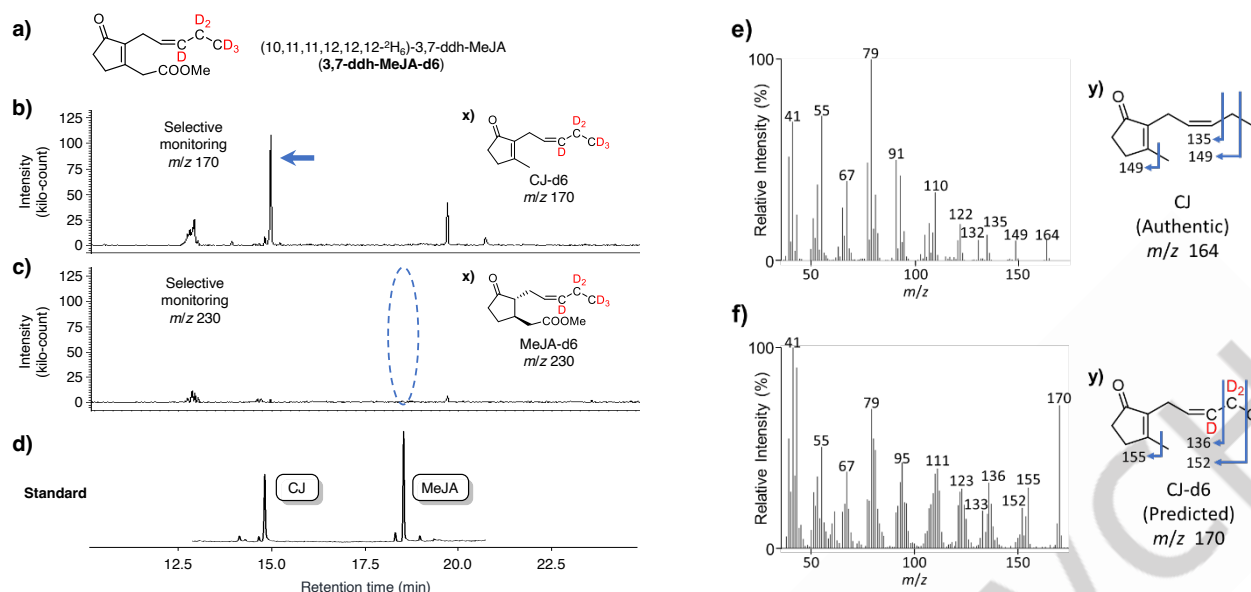


Figure 1. Feeding experiment using 3,7-ddh-MeJA-d6. (a) Chemical structure of 3,7-ddh-MeJA-d6, (b) Representative GC-MS chromatogram for detecting CJ-d6 using selected ion monitoring at *m/z* 170. (c) Representative GC-MS chromatogram for measuring MeJA-d6 using selected ion monitoring at *m/z* 230. (d) Representative GC-MS chromatogram for measuring authentic CJ and (±)-MeJA using total ion counting mode. (e) Representative EI mass spectra in GC-MS for analyzing authentic CJ. (f) Representative EI mass spectra in GC-MS for analyzing *M. suaveolens*-derived CJ-d6 using 3,7-ddh-MeJA-d6 as a substrate for the feeding experiment. (x) The chemical structure described in the inset is for that of the predicted metabolite and its molecular ion. (y) The chemical structure described in the inset is for that of the cleavage patterns and its molecular ion.

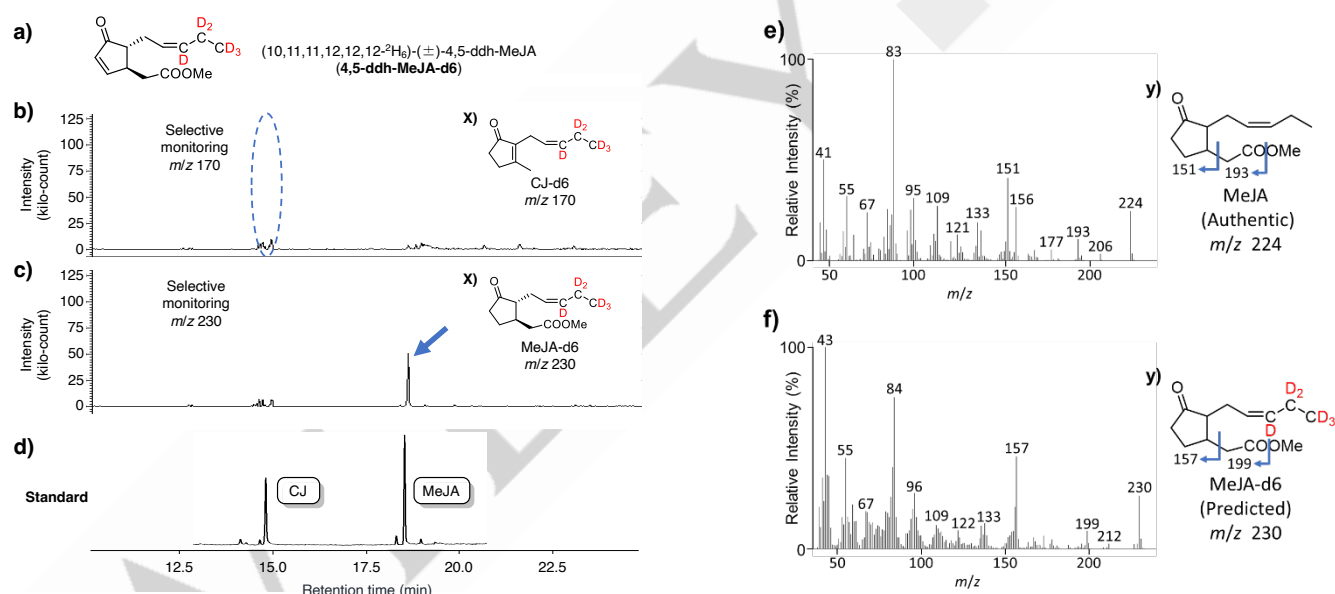


Figure 2. Feeding experiment using (±)-4,5-ddh-MeJA-d6. (a) Chemical structure of (±)-4,5-ddh-MeJA-d6, (b) Representative GC-MS chromatogram for detecting CJ-d6 using selected ion monitoring at *m/z* 170. (c) Representative GC-MS chromatogram for measuring MeJA-d6 using selected ion monitoring at *m/z* 230. (d) Representative GC-MS chromatogram for measuring authentic CJ and (±)-MeJA using total ion counting mode. (e) Representative EI mass spectra in GC-MS for analyzing authentic MeJA. (f) Representative EI mass spectra in GC-MS for analyzing *M. suaveolens*-derived MeJA-d6 using (±)-4,5-ddh-MeJA-d6 as a substrate for the feeding experiment. (x) The chemical structure described in the inset is for that of the predicted metabolite and its molecular ion. (y) The chemical structure described in the inset is for that of the cleavage patterns and its molecular ion.

the supporting information as Figure S1. CJ was detected only in the flowers of *L. japonica*. However, CJ was detected in both leaves and flowers of *Mentha longifolia* (Table 1a). We thought that leaves would be more suitable for experiments than flowers, as they are not seasonal and do not require much time to obtain the materials. For this reason, we focused on Lamiaceae plants, to which *M. longifolia* belongs. We further analyzed the leaf parts of several Lamiaceae plants (Table 1b), and in this experiment, CJ-d6 (10 µg) was added to the solution of leaf extract as an internal standard. CJ was detected in *M. suaveolens*, *M. spicata*,

and *Salvia rosmarinus*, and endogenous amounts of CJ were measured using GC-MS, revealing *M. suaveolens* as the highest producer of CJ (Table 1b).

The synthesized compounds were applied via airborne propagation as described in the reported method^[21-23]. *M. suaveolens* were placed in a semi-closed container with a filter paper, on which 1 mg compounds was loaded in each 1 L space. After 7 days, the aerial parts of the plants were extracted with EtOAc. The extracts were treated with CH₂N₂ and then subjected to GC-MS. The results of the feeding experiments using 3,7-ddh-

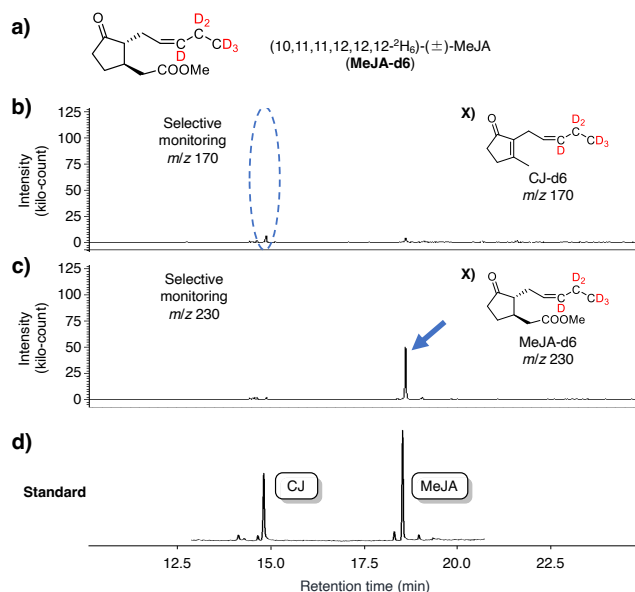


Figure 3. Feeding experiment using (±)-MeJA-d₆. (a) Chemical structure of (±)-MeJA-d₆, (b) Representative GC–MS chromatogram for detecting CJ-d₆ using selected ion monitoring at *m/z* 170. (c) Representative GC–MS chromatogram for measuring MeJA-d₆ using selected ion monitoring at *m/z* 230. (d) Representative GC–MS chromatogram for measuring authentic CJ and MeJA using total ion counting mode. (x) The chemical structure described in the inset is for that of the predicted metabolite and its molecular ion.

MeJA-d₆ are shown in Figure 1. In case of feeding 3,7-ddh-MeJA-d₆ via airborne propagation to the plants, a peak indicating the retention time (14.8 min, Figure 1b) in acceptable agreement with that of authentic CJ (Figure 1d) was detected in the GC chromatogram, although we did not detect a peak having the retention time (Figure 1c) in acceptable agreement with that of authentic (±)-MeJA (18.2 min, Figure 3d). The MS patterns for the peaks with retention time of 14.8 min in Figure 1c and 1d are analyzed in Figure 1e and 1f, respectively. In Figure 1e, the MS pattern for authentic CJ is shown, in which *m/z* 164 was determined as the molecular ion, *m/z* 149 as the ion derived from the methyl group missing ion from the parent ion, and *m/z* 135 as the ethyl group missing. In Figure 1f, the MS pattern is given for the peak assumed to be CJ-d₆. The ion with *m/z* 170 was determined to be the molecular ion of CJ-d₆, *m/z* 155 was determined to be the ion derived from the C-1 methyl group missing ion, *m/z* 152 was determined to be generated by cleavage of C-11 and C-10 carbons, and *m/z* 136 was determined to be generated by cleavage between C-10 and C-9 carbons. The coincidences of retention time in the chromatogram and MS pattern strongly supported that the extract originated from 3,7-ddh-MeJA-d₆ treated plant contained CJ-d₆. Based on these results, it was revealed that air-propagated 3,7-ddh-MeJA-d₆ was metabolized into CJ-d₆, but not into JA-d₆ in *M. suaveolens*.

In the case of feeding (±)-4,5-ddh-MeJA-d₆ via airborne propagation to the plants (Figure 2), a peak having the retention time (18.2 min, Figure 2c) in acceptable agreement with that of authentic (±)-MeJA (Figure 2d) was detected in the GC chromatogram, although we did not detect a peak having the retention time (Figure 2b) in acceptable agreement with that of authentic CJ (14.8 min, Figure 2d). The MS patterns for the peaks in Figure 2c and 2d with a retention time of 18.2 min are analyzed in Figure 2f and 2e, respectively. The features of Figure 2e and 2f

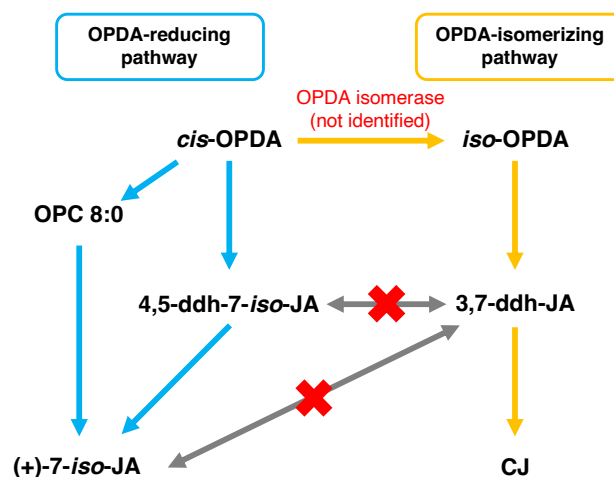
coincided well except for some peaks having deuterium atoms, such as *m/z* 230, 199, 157, and 84. Matsui *et al.*^[24] reported the GC–MS pattern of MeJA-d₆, and it also coincided well with that in Figure 2e. Based on these results, it was revealed that air-propagated (±)-4,5-ddh-MeJA-d₆ was metabolized into JA-d₆, but not into CJ-d₆ in *M. suaveolens*.

The results of feeding (±)-MeJA-d₆ via airborne propagation to the plants are presented in Figure 3. Comparing the retention times with those of the authentic compounds (Figure 3), we were not able to detect a peak corresponding to CJ-d₆, which revealed that air propagated (±)-MeJA-d₆ was not metabolized into CJ. In Figure 3b, there is a small peak with a retention time overlapping with that of CJ-d₆. However, the MS patterns for the peaks with retention times in this vicinity were analyzed one by one (data not shown), but none of them matched the MS pattern shown in Fig. 1b. Based on these results, it was revealed that air-propagated (±)-MeJA-d₆ was not metabolized into CJ-d₆ in *M. suaveolens*.

Conclusion

In this report, we synthesized some deuterium labeled JA-skeleton analogs (Scheme 2) to reveal CJ biosynthesis pathway. Furthermore, we found that some Lamiacea plants biosynthesize huge amount of CJ in their leaves (Table 1).

Feeding experiments revealed that (±)-MeJA-d₆ and (±)-4,5-ddh-JA are not metabolized to CJ in apple mint (*M. suaveolens*) (Figures 2 and 3). This finding is well consistent with the reports of Matsui *et al.*^[16] and Dabrowska *et al.*^[8], which found that CJ was biosynthesized via the OPDA-isomerizing pathway rather than the OPDA-reducing pathway. On the other hand, this finding is contradicted with the report of Koch *et al.*^[15] showing that (±)-JA was metabolized to CJ via 3,7-ddh-JA. We hypothesized that the discrepancy was due to the method how the compounds were supplied. Therefore, we performed an additional experiment based on the method of Koch *et al.*. We applied 3 mM (10,11,11,12,12,12-²H₆)-(±)-JA ((±)-JA-d₆) solution to *M. suaveolens* from the cut end of stem. After 24h, a portion of the EtOAc extract originated from the plant leaves were subjected to GC-MS analysis. As a result, CJ-d₆ was not detected (data not shown), although plant derived CJ was detected. In our



Scheme 3. Proposed biosynthesis pathway of CJ.

experiments, no conversion to the target compound was observed when the compound was supplied from the cut end of the stem as in Koch *et al.*^[15] We thought that the most compatible view might be the differences in plant species to give the contradicted results. Our results strongly suggested that JA was not metabolized to CJ.

In addition, feeding treatments revealed that 3,7-ddh-JA are not metabolized to 4,5-ddh-JA and JA (Figure 1). The result supported the findings reported by Miyawaki *et al.*^[5], which showed that feeding experiments of 4,5-ddh-JA and (-)-JA gave potato tubers using potato single node stems, although feeding experiments of 3,7-ddh-JA did not. These findings showed the OPDA-isomerizing and -reducing biosynthesis pathways should be independent, and the biosynthetic intermediates on OPDA-isomerizing and -reducing pathway do not complement each other, although *cis*-OPDA was employed as a common biosynthetic intermediate.

The findings obtained in this study would be useful for the industrial application of CJ production in plants to give higher efficiency. However, the identification of the isomerase that generates *iso*-OPDA from *cis*-OPDA, which has not yet been identified, will be indispensable to bring this concept even closer to reality. Future studies are eagerly awaited.

Experimental section

GC–MS conditions: GC–MS analysis of CJ and MeJA was completed on a Varian CP-3800 gas chromatograph with a Varian 1200 L quadrupole MS/MS in electron ionization mode. A summary of the analytical conditions is shown in Figure S1 in the supplementary section. Briefly, the injection temperature was 250 °C, and a fused-silica capillary column (TC-5; 30 m × 0.25 mm i.d., 0.25 µm film thickness; GL Sciences) was used. The temperature program started at 40 °C for 1 min and subsequently increased at 22 °C/min to 290 °C, which was maintained for 20 min. Helium was used as the carrier gas at a linear velocity of 1.2 mL/min, and all spectra were scanned within the *m/z* range of 10–520. Schematic illustration to analyse compounds using GC–MS is described in the supporting information as Figure S1.

Screening for the plants that produce CJ: Approximately 1 g of the plant was frozen, crushed, and extracted with EtOAc. The extract was filtered and concentrated under approximately 20 °C using a rotary evaporator to yield an oil. The resulting oil was redissolved with EtOAc (200 µL). A portion of the solution was subjected to GC–MS analysis. In the quantitative analysis of CJ in Lamiaceae plants, CJ-d7 (10 µg) was added to the EtOAc solution. For evaluation of the endogenous amount, area ratios of the ions of *m/z* 164 and 171 were used to determine the endogenous amounts of CJ.

Feeding experiments of airborne deuterium-labeled compounds: Three *M. suaveolens* plants grown for 6 weeks

under long-day conditions (14 h light /10 h dark) and a filter paper loading 2.6 mg of the tested compound were left in semi-closed translucent container (2.6 L), referring to the reports by Tamogami *et al.*^[21], Oki *et al.*^[22] and Rishni *et al.*^[23]. After 1 week, the plants were frozen, crushed, and extracted with EtOAc. The extract was filtered and concentrated under approximately 20 °C using a rotary evaporator to yield an oil. The resulting oil was redissolved with EtOAc and successfully treated with CH₂N₂ to convert the carboxylic acid moiety to methyl ester. A portion of the solution was subjected to GC–MS/MS analysis.

Synthesis of deuterium-labeled compounds: Details are provided in Supporting Information.

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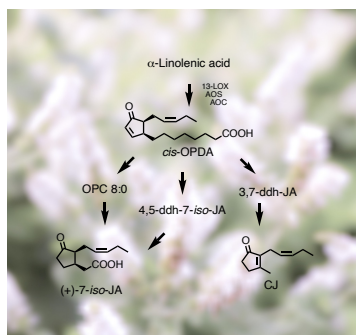
Keywords: *cis*-Jasmone, Jasmonic acid, GC–MS, *Mentha suaveolens*, Lamiaceae

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GC MS/MS experiments using deuterium labeled methyl jasmonate, methyl 4,5-didehydro-jasmonate, methyl 3,7-didehydro-jasmonate, in which the compounds were provided by air propagation, identified that biosynthetic pathway to give jasmonic acid and *cis*-jasmone were completely separate and independent in *Mentha suaveolens*.