Running title: Biosynthesis of *cis*-jasmone in *Lasiodiplodia theobromae*.

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Note

Feeding experiment using uniformly ^{13}C-labeled α-linolenic acid supports the involvement of the decarboxylation mechanism to produce *cis*-jasmone in *Lasiodiplodia theobromae*

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In our previous report, it was found that *Lasiodiplodia theobromae* produced *cis*-jasmone via partially utilizing the biosynthetic pathway of JA. A feeding experiment using uniformly ^{13}C-labeled α-linolenic acid, which was added to the culture media of the fungus, strongly supported that the fungus produced CJ via the decarboxylation step of the biosynthetic pathway.

cis-Jasmone (CJ, Figure 1A) is an important industrial material used to produce many commodities, such as perfume and cosmetics. It is also well known that plants utilize the compound as a type of signal mediator. Loughrin et al. [1] reported that CJ was detected as a volatile compound collected from leaves of cotton subjected to beet armyworm feeding, which implied that the plant volatiles might play in herbivore/ predator relationships, and Birkett et al. [2] reported that CJ was a semiochemical used to attract insects to plants. Koch et al. [3] reported that CJ is one of the deactivated form of jasmonic acid (JA).

The pathway for the biosynthesis of CJ using methyl jasmonate (MeJA) via 3,7-didehydroJA (Figure 1A) was postulated in the report by Koch et al. (1997) [3], which described a feeding experiment using [$^2H_1$-7, $^2H_2$-5, $^2H_2$-2] MeJA and resulted in the formation of [$^2H_2$-4, $^2H_2$-1] CJ (Figure 1B). The conversion of 3,7-didehydroJA into CJ was also postulated to occur by a decarboxylation step. The accumulation of JA was enhanced by wound stress, and similarly, CJ also accumulated in response to wound stress [1], which suggested that the synthesis of CJ may be tied to the biosynthetic pathway of JA [4]. Another type of biosynthetic pathway was reported by Dabrowska et al. [5] in which it was found that other types of biosynthetic pathways via *iso*-12-oxo-phytodienoic acid (*iso*-OPDA, Figure 1A) were used in plants, although 3,7-didehydroJA was used as a common intermediate. However, it was recently found that a pathogenic fungus, *Lasiodiplodia theobromae*, produced CJ, which was derived from α-linolenic acid (LA), OPDA, and *iso*-OPDA but not from JA [6]. The proposed biosynthetic pathway to produce CJ is shown in Figure S1 in the supplemental material.

Interestingly, [$^2H_2$-4, $^2H_2$-3] CJ (Figure 1C) was detected in the case of feeding [$^2H_2$-11,$^2H_2$-10,$^2H_2$-8,$^2H_2$-2] methyl-*iso*-OPDA to the culture media. Assuming that CJ was synthesized in *L. theobromae* using the same pathway as it was synthesized in plants, the product should be [$^2H_2$-4, $^2H_2$-3, $^2H_2$-1] CJ. Thus, further study should be done to ensure the biosynthesis of CJ in *L. theobromae*. In this study, we showed that CJ was produced by *L. theobromae* via a decarboxylation step, which seemed to be preceded by the replacement of deuterium.

It was hypothesized that *L. theobromae* might synthesize CJ by the decarboxylation step of 3,7-didehydroJA, which is preceded by loss of the deuterium labeling form C-2 methylene. To get more detailed information of the fragmentation patterns of CJ, unlabeled and [$^2H_2$-4, $^2H_2$-3 $^2H_3$-1] CJ was analyzed by GC-MS, as shown in Figure 2A. Unlabeled CJ had a molecular ion with *m/z* 164. The fragment ions corresponded to the signals at *m/z* 149, 135, and 122. It was assumed that the fragment with *m/z* 149 derived from the release of methyl carbon groups from the C1 or C11 positions, and the fragments with *m/z* 135 and 122 were from the release of $–CH_2CH_3$ from the C11-C10 partial structure and $=CHCH_2CH_3$ from the C11-C19 partial structure, respectively (Figure 2A-I). These considerations were supported by the results of the EI-MS analysis of [$^2H_2$-4, $^2H_2$-3 $^2H_3$-1] CJ (Figure 2A-II) due to signals observed at *m/z* 171 (molecular ion), 156 (M-CH_3 of C11 carbon), 153 (M-CD_3 of C1 carbon), 142 (M- CH_3CH_2 of C11-C10 partial structure) and 129 (M- $CH_3CH_2CH=$ of C11-C19 partial structure).

A culture of *L. theobromae* was added to potato dextrose media, and after 7 days, [$^{2}H_{2}$-4, $^{2}H_{2}$-3 $^{2}H_{3}$-1] CJ was added to the culture and inoculated for 7 more days. The culture medium was extracted according to a previously reported method [6] with a modification: the extract was not treated with CH_2N_2, and a portion of the extract was analyzed by GC-MS. Fungus derived CJ was analyzed by GC-MS using selected ion monitoring (SIM) mode set at *m/z* 164, which resulted in an ion peak with a retention time of 15.8 min (Figure 2B-I). However, the GC-MS chromatograph for the compound assumed to be [$^{2}H_{2}$-4, $^{2}H_{2}$-3] CJ showed no peak using SIM mode set at *m/z* 168 (Figure 2B-II), which suggested that the replacements of deuterium at the C1 position of [$^{2}H_{2}$-4, $^{2}H_{2}$-3 $^{2}H_{3}$-1] CJ did not occur after the production of CJ. To prove that decarboxylation was involved in the production of fungus derived CJ, uniformly labeled ^{13}C [U-^{13}C] α-LA was used as an additive. The GC-MS chromatograph for the compound assumed to be [U-^{13}C] CJ showed an ion peak with a retention time of 15.8 min using SIM mode set at *m/z* 175 (Figure 2B-III), while non feeding experiment of ^{3}C [U-^{13}C] α-LA resulted in no peak (Figure 2B-IV) for *m/z* 175. In order to substantiate the structure detected using SIM mode set at *m/z* 175 as shown in Figure 2B-III, the GC-MS chromatograph was examined, in which the measurement was performed for ions from *m/z* 30 to 200. The GC-MS chromatograph showing the ion patterns derived from the peak eluted at a retention time of 15.8 min is shown in Figure 2C. In the chromatograph, the fragment pattern of the major component coincided well with that of CJ as shown in Figure 2A-I, which proved that the major component of the peak was CJ. On the other hand, the minor peaks were observed for ions with *m/z* 175, 159, and

144. The ion peak with *m/z* 175 was thought to derive from the molecular ion of [U-^{13}C] CJ, and the fragment ion peaks with *m/z* 159 and 144 were thought to be from [M-$^{13}CH_3$]$^+$ and [M- $^{13}C_2H_5$]$^+$, respectively. The mass spectra also indicated that the extract contained [U-^{13}C] CJ.

By combining the abovementioned experimental results, the following biosynthetic pathways of CJ were proposed (Figure 3). Given that α-LA, OPDA, and *iso*-OPDA (Figure 1C) are intermediates of the biosynthesis of fungus derived CJ, and JA was produced from OPC 8:0 via three β-oxidation steps (Figure S1 in the supplemental material), the feeding experiment using [U-^{13}C] α-LA (Figure 2B, 2C) indicated that decarboxylation was involved in the production of CJ in *L. theobomae*. Because the replacement of deuteriums at the C1 position of [$^2H_2$-4, $^2H_2$-3 $^2H_3$-1] CJ did not occur in the culture media (Figure 2B-II), it was suggested that the replacement occurred before the production of CJ, and it was assumed that the replacement might occur immediately after the production of 3,7-didehydroJA. Since the C2 methylene moiety of 3,7-didehydroJA was sandwiched between α, β-unsaturated carbonyl and C1 carboxyl moieties, it was reasonable to say that its *p*Ka value was relatively lower than those of the C3 and C4 methylene protones, which promoted the replacement of deuteriums. However, the abovementioned biological mechanism could not be applied to the production of CJ in plants (Figure 1B) because the acidity-basicity of plants might be significantly different than that of fungi, and the decarboxylation step had a higher priority than the deprotonation that occurs at the C2 position in 3,7-dehydroJA. Thus, the further study is needed to explain the discrepancy and get real picture for CJ

production.

EXPERIMENTAL SECTION

General experimental procedures

GC-MS analyses were performed using a Varian instrument. CJ and [U-^{13}C] α-LA were purchased from Sigma-Aldrich and Medical Isotope, respectively. The *L. theobromae* (MAFF No. 306027) fungus culture was obtained from the Genetic Resources Center, National Agriculture and Food Research Organization (NARO).

GC-MS conditions

The parameters for GC-MS analysis were set according to previously reported data [6].

Feeding experiments

The experiments were performed according to previously reported data [6].

Author Contributions

RM and KT performed the experiments. RM and HM designed the study and wrote the paper. KM and TK helped to draft the manuscript.

Acknowledgments

A GC-MS system (Waters, USA) at the Research Faculty of Agriculture, Hokkaido University, was used for this study.

Disclosure statement

No potential conflicts of interest were reported by the authors.

Supplemental materials

The supplemental materials for this paper are available at http://

Figure legends

Figure 1. The chemical structures of CJ and related compounds for the biosynthesis of CJ.

A) Chemical structures of CJ, 3,7-dihydroJA, and *iso*-OPDA. B) Production of [$^2H_2$-4,$^2H_2$-1] CJ from [$^2H_1$-7,$^2H_2$-5,$^2H_2$-2] MeJA. C) Production of [$^2H_2$-4,$^2H_2$-3] CJ from [$^2H_2$-11,$^2H_2$-10,$^2H_2$-8,$^2H_2$-2] methyl-*iso*-OPDA.

Figure 2. GC-MS analysis of unlabeled and labeled CJ.

GC-MS fragmentation patterns for unlabeled CJ (I) and [$^2H_2$-4,$^2H_2$-3,$^2H_3$-1] CJ (II). B) GC-MS chromatograph for fungus derived CJ for the experiment in which [$^2H_2$-4,$^2H_2$-3,$^2H_3$-1] CJ was added to the culture media (I), GC-MS chromatograph for analyzing [$^2H_2$-4,$^2H_2$-3] CJ for the experiment in which [$^2H_2$-4,$^2H_2$-3,$^2H_3$-1] CJ was added to the culture media (II), GC-MS chromatograph for analyzing [U-^{13}C] CJ for the experiment, in which [U-^{13}C] α-LA was added to the culture media (III), and GC-MS chromatograph for analyzing [U-^{13}C] CJ for the experiment, in which [U-^{13}C] α-LA was not added to the culture media (IV). C) GC-MS fragmentation patterns for the peak with a retention time of 15.8 min, which was measured using MS range from *m/z* 30 to 200.

Figure 3. Proposed biosynthetic pathway from 3,7-dihydrohJA to CJ in plants and *L. theobromae*.

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