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Biosynthesis of jasmonic acid in a plant pathogenic fungus, *Lasiodiplodia theobromae*

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ABSTRACT

Jasmonic acid (JA) is a plant hormone that plays an important role in a wide variety of plant physiological processes. The plant pathogenic fungus, *Lasiodiplodia theobromae* also produces JA; however, its biosynthesis in this fungus has yet to be explored. Administration of [1-^{13}C] and [2-^{13}C] NaOAc into *L. theobromae* established that JA in this fungus originates from a fatty acid synthetic pathway. The methyl ester of 12-oxo-phytodienoic acid (OPDA) was detected in the culture extracts of *L. theobromae* by GC–MS analysis. This finding indicates the presence of OPDA (a known intermediate of JA biosynthesis in plants) in *L. theobromae*. ^{2}H NMR spectroscopic data of JA produced by *L. theobromae* with the incorporation of [9,10,12,13,15,16-$^{2}H_6$] linolenic acid showed that five deuterium atoms remained intact. In plants, this is speculated to arise from JA being produced by the octadecanoid pathway. However, the observed stereoselectivity of the cyclopentenone olefin reduction in *L. theobromae* was opposite to that observed in plants. These data suggest that JA biosynthesis in *L. theobromae* is similar to that in plants, but differing in the facial selectivity of the enone reduction.

1. Introduction

Lasiodiplodia theobromae is a pathogenic fungus that infects plants in tropical and subtropical regions of the world, causing considerable damage to crops during storage. This fungus is known to produce a variety of bioactive compounds (Aldridge et al., 1971). Previous studies in our laboratory have demonstrated the presence of various potato-tuber-inducing substances in a culture of *L. theobromae*: (−)-JA (**7**) (see Fig. 1), theobroxide (and related compounds), and lasiodiplodins (Nakamori et al., 1994; Matsuura et al., 1998a,b; Yang et al., 2000; Li et al., 2006; Takei et al., 2006). Among these compounds, theobroxide exhibits the most interesting biological activity, which effects not only potato-tuber-inducing activity in potato (*Solanum tuberosum*), but also induces flower bud formation in morning glory (*Pharbitis nil*) (Yoshihara et al., 2000). Encouraged by the unique biological activities that these compounds display, the biosynthesis of theobroxide (Li et al., 2006) and lasiodiplodins (Kashima et al., 2009a,b) were elucidated. Among the compounds described above, (−)-JA (**7**) was first isolated from the culture of *L. theobromae* as a substance used to accelerate plant senescence in the 1970s (Aldridge et al., 1971). It has since been established that JA (**7**) is a plant hormone that controls responses to environmental stresses and developmental events in flowering plants. It plays an important role in coordinating plant defense responses with physiological stresses associated with herbivores and microbial pathogens. JA (**7**) biosynthesis and its regulatory mechanism, has been thoroughly investigated (Wasternack, 2007). Recently, the presence of JA (**7**) and the activity of allene oxide synthase (AOS), with respect to JA (**7**) biosynthesis, was demonstrated in a model moss *Physcomitrella patens* (Oliver et al., 2009; Bandara et al., 2009).

In plants, JA (**7**) is biosynthetically produced by the octadecanoid pathway as shown in Fig. 1 (Schaller and Stinzi, 2009). The first step in the octadecanoid pathway is the lipoxygenase (LOX)-catalyzed oxygenation of α-linolenic acid (**1**). In the specific case of JA (**7**) biosynthesis, hydroperoxidation to intermediated (**2**) takes place at C-13 of α-linolenic acid (**1**), and is effected by 13-LOX. The resulting hydroperoxide [13(*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT)] (**2**) can be metabolized by AOS into an unstable allene oxide [12,13(*S*)-epoxyoctadecatrienoic acid (12,13-EOT) **3**], in which cyclization is facilitated by allene oxide cyclase (AOC) to provide 12-oxo-phytodienoic acid (OPDA) (**4**). The later is reduced by OPDA reductase 3 (OPR3) to yield 3-oxo-2-[(*Z*)-pent-2-enyl]-cyclopentane-1-octanoic acid (OPC-8:0) (**5**). Three subsequent β-oxidation steps afford (+)-*iso*-JA (**6**) [(3*R*,7*R*)-configuration], which is further epimerized at C-7 to provide (−)-JA (**7**) [(3*R*,7*S*)-configuration]. Due to keto-enol interconversion, the *cis*-isomer, (+)-*iso*-JA (**6**), is readily converted into the more stable *trans*-isomer, (−)-JA (**7**), for steric reasons.

As referred to above, the detailed mechanism of JA (**7**) signaling and biosynthesis in plants has been elucidated. However, there is a little information about the biosynthetic pathway and functions of JA (**7**) in *L. theobromae*. Thakkar et al. (2004) reported that development of systemic acquired resistance (SAR) was restricted in the plants infected with *L. theobormae* due to deficiency of salicylic

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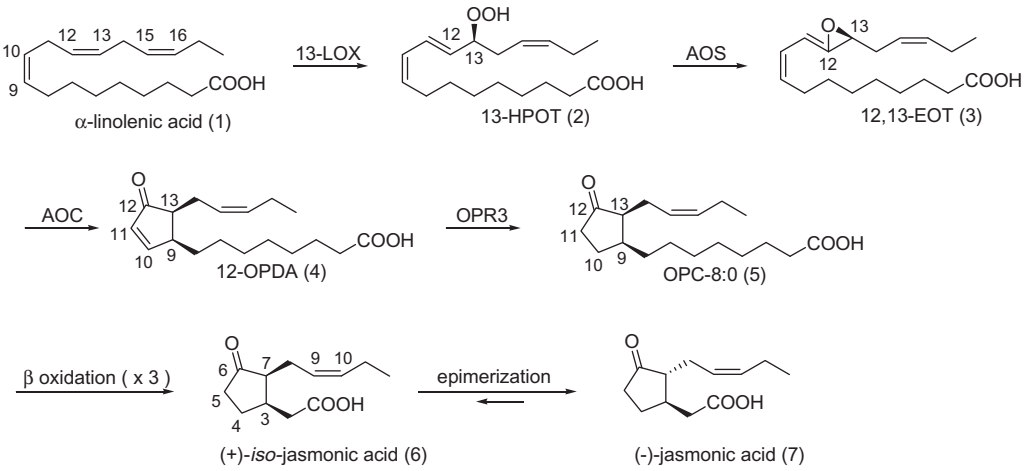

Fig. 1. The octadecanoid pathway.

acid (SA). Inhibition of SA biosynthesis in the infected plants, which was caused by JA (**7**) released from *L. theobromae*, might contribute to the infection of this fungus to plants. In the case of *Pseudomonas syringae*, coronatine, a phytotoxin functioning like JA (**7**), was shown to induce suppression of SA-mediated defense system, and disease development in tomato (Uppalapati et al., 2007).

In this work, it is demonstrated that JA (**7**) is synthesized *via* a fatty acid synthetic pathway in *L. theobromae*, which is supported by ^{13}C labeling experiments. The incorporation of a synthetic ^{2}H-labeled linolenic acid (**1**) into *iso*-JA indicates that JA (**7**) biosynthesis in *L. theobromae* is similar to that of plants, differing only in the facial selectivity of the cyclopentenone reduction (i.e., α- vs. β-hydride attack); the facial selectivity observed in plants is posited on the basis of the X-ray crystal structure data of tomato OPR3 (Breithaupt et al., 2006).

2. Results and discussion

L. theobromae was statically incubated in 1% potato-glucose medium at 29 °C for 7 days. ^{13}C-labeled sodium acetate ([1-^{13}C] and [2-^{13}C]) was administered to the culture at a concentration of 10 mM. The culture was filtered to separate the mycelia and supernatant after an additional 10 days of incubation. The aqueous layer was extracted with ethyl acetate (150 ml) and subsequently purified by chromatography to provide 3.9 mg/150 ml of [1-^{13}C]acetate-derived JA, and 2.8 mg/150 ml of [2-^{13}C]acetate-derived JA, respectively.

The $^{13}C\{^{1}H\}$-NMR spectrum of [1-^{13}C]acetate-derived JA (**7**) showed enhanced signals at C-1, -3, -5, -7, -9, and -11. Meanwhile, the $^{13}C\{^{1}H\}$ NMR spectrum of JA (**7**) derived from [2-^{13}C]acetate had intensified signals at C-2, -4, -6, -8, -10, and -12 (Table 1 and Fig. 2). Biosynthetically ^{13}C-labeled JA (**7**) was converted into the methyl ester with ethereal diazomethane, and then its specific incorporation ratio was calculated by the relative intensity of the methyl ester signal normalized to 1.11% (natural abundance). The specific incorporation of ^{13}C generally observed was 1.3–5.7 atom% for [1-^{13}C]acetate-derived carbons and 4.5–7.8 atom% for [2-^{13}C]acetate-derived carbons. These data provide clear evidence that JA (**7**) is produced through a fatty acid biosynthetic pathway in *L. theobromae*.

In plants, α-linolenic acid (**1**) is transformed into JA (**7**) through the octadecanoid pathway. We have been interested in whether OPDA (**4**)—a key intermediate of the octadecanoid pathway—is present in the culture of *L. theobromae*; however, the presence of OPDA (**4**) in this fungus has not been reported. In an attempt to test this hypothesis, the acetone extract of mycelia of *L. theobromae* was partially purified by preparative TLC, and subsequently subjected to GC–MS analysis. In conclusion, OPDA (**4**) was not detected in free form; however, the OPDA methyl ester was observed in the selected ion mode monitored at *m*/*z* 306 ($[M]^+$), 275 ($[M-OCH_3]^+$), and 238 (Fig. 3) (Laudert et al., 1996). The retention time associated with all of the selected ion peaks in this analysis was 87.2 min, which was the same as that of the molecular ion peaks of an authentic (+)-*trans*-OPDA methyl ester prepared (i.e., CH_2N_2, Et_2O) from natural OPDA (**4**). These data strongly suggested that *L. theobromae* produces natural occurring OPDA (**4**). JA (**7**) production by way of a fatty acid synthetic pathway, and the presence of OPDA (**4**) in *L. theobromae*, suggests that this fungus produces JA (**7**) *via* OPDA (**4**). As previously stated, OPDA (**4**) is an intermediate

Table 1
Incorporation of ^{13}C-labeled sodium acetate ([1-^{13}C] and [2-^{13}C]) into jasmonic acid (7).

Position	δ_c (ppm)	^{13}C-atom%	
		[1-^{13}C]acetate	[2-^{13}C]acetate
1	172.4	2.41	1.80
2	38.8	0.69	5.49
3	38.0	2.63	1.73
4	25.5	0.86	4.62
5	37.7	3.00	1.19
6	219.0	0.65	7.81
7	54.0	1.30	1.17
8	27.2	1.06	5.89
9	124.9	2.16	0.73
10	134.1	0.63	4.45
11	20.6	5.68	1.79
12	14.1	1.27	5.70
OMe	51.6	1.11	1.11

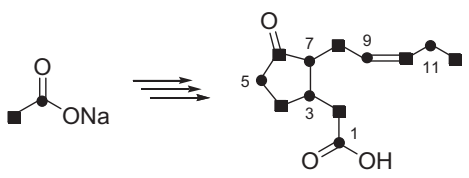

Fig. 2. Labeling patterns of jasmonic acid (**7**) following incorporation of ^{13}C-labeled sodium acetate into *L. theobromae*. Filled square and circle symbols represent [1-^{13}C] and [2-^{13}C] in ^{13}C-labeled sodium acetate, respectively.

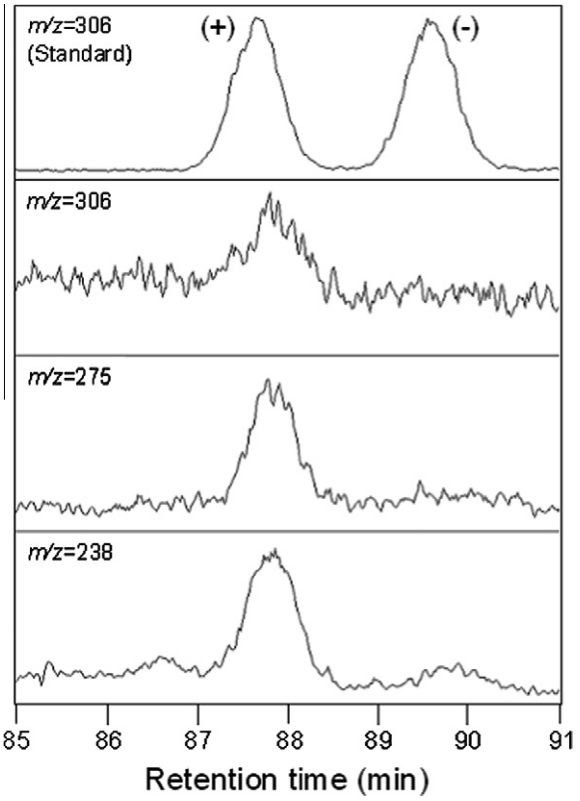

Fig. 3. Segment of selected ion monitored chromatograms of GC–MS spectral data of OPDA methyl ester produced by *L. theobromae*. Column; a chiral β-DEX fused silica capillary column (0.25 mm i.d. × 30 m).

derived from α-linolenic acid (**1**) by way of the known plant pathway.

Next, it was investigated as to whether α-linolenic acid (**1**) is also a precursor of JA (**7**) in *L. theobromae*; therefore, [9,10,12,13,15,16-$^2H_6$]linolenic acid ([$^2H_6$]-LA) (**1a**) was synthesized according to Hungerford and Kitching (1998). [$^2H_6$]-LA (**1a**) was supplemented to a 7-day-old culture of *L. theobromae*, reaching a final concentration of 1 mM, and the fungus was incubated for an additional 10 days. Chromatographic separation of a residue from the culture filtrate of *L. theobromae* gave *iso*-JA (**6a**) incorporating [$^2H_6$]-LA (**1a**) (1.5 mg). After methyl ester formation, the ^2H NMR spectrum of the resulting *iso*-JA methyl ester was immediately taken. The ^2H NMR spectroscopic data established the presence of five ^2H signals corresponding to H-3, -4α, -7, -9, and -10 (Fig. 4) (Seto et al., 1999). Analysis of the EI-MS data by comparison of $[M+4]^+$ ion peak (m/z = 228, [$^2H_4$]-JA (**7a**)) and $[M+5]^+$ ion peak (m/z = 229, [$^2H_5$]-*iso*-JA (**6a**)) with the methyl ester of biosynthetically labeled and natural JA (**7**) showed that 6.98% and 2.96% of the peak intensities were enhanced, respectively. Considering the isomerization, 9.94% of ^2H was enriched in the *iso*-JA (**6a**) derived from [$^2H_6$]-LA (**1a**). This incorporation demonstrates that α-linolenic acid (**1**) is a precursor in JA (**7**) biosynthesis of *L. theobromae* (Fig. 5). The presence of *iso*-JA (**6**) suggests that it is produced first and subsequently isomerized to JA (**7**) in *L. theobromae*. Moreover, it is interesting that the positions of ^2H incorporation in [$^2H_6$]-LA (**1a**) derived *iso*-JA (**6a**) are in accordance with the positions speculated by the cyclization mechanism involved in reactions mediated by AOS and AOC in plants (Schaller and Stinzi, 2009). Accordingly, OPDA (**4**) is most likely metabolized to JA (**7**) through reduction of the cyclopentenone moiety and subsequent β-oxidation of the side-chain. While the reduction occurs prior to the β-oxidation in plants, the order of events (i.e., reduction or β-oxidation) are unknown in *L. theobromae*.

From a stereochemical perspective, the reductase could facilitate hydride attack from either the α- or β-face, which would determine the configuration at C-4 in [$^2H_6$]-LA derived *iso*-JA (**6a**). In plants, the X-ray crystal structural data of tomato OPR3 suggests that the reduced flavin cofactor is placed on the opposite side of the OPDA (**4**) side-chain (i.e., α-face) (Breithaupt et al., 2006). The attack of a hydride from the α-face would result in the original proton attached at C-10 in OPC-8:0 to be in a β-orientation. On the other hand, the ^2H NMR spectrum of the [$^2H_6$]-LA (**1a**) derived *iso*-JA methyl ester indicated an α-orientation of ^2H-4. This supports the contention that the hydride attack from the reductase occurs on the β-face of the cyclopentenone plane at C-4, which is in contrast to that in plants. These data suggest that the cyclopentenone reduction mechanism in *L. theobromae* is different from that in plants.

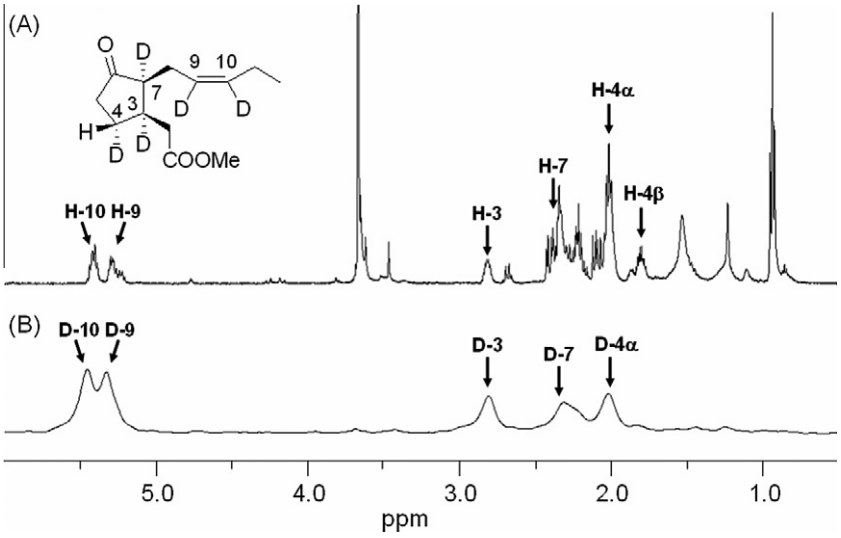

Fig. 4. ^1H NMR spectrum of natural jasmonic acid (**7**) (A) and ^2H NMR spectrum of biosynthetically labeled *iso*-jasmonic acid (**6a**) derived from [9,10,12,13,15,16-$^2H_6$]linolenic acid (**1a**) (B).

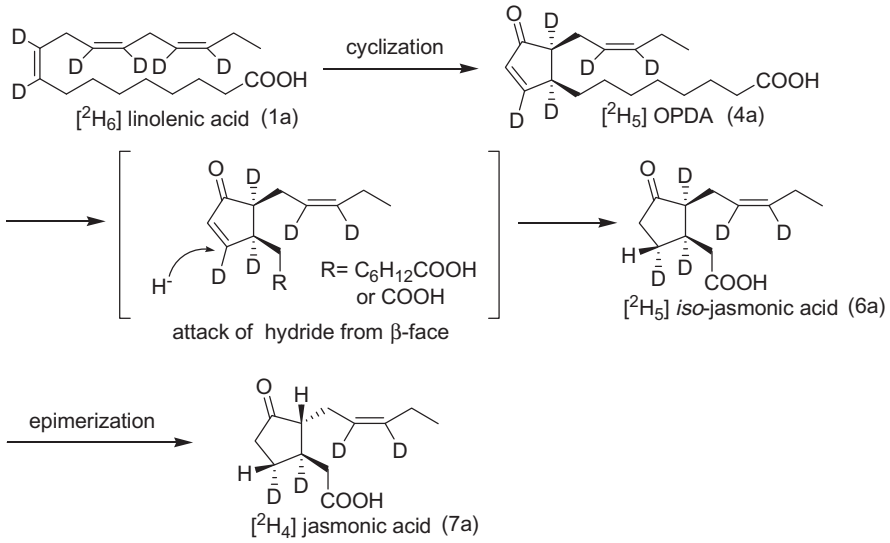

Fig. 5. The presumed jasmonic acid biosynthetic pathway in *L. theobromae*.

3. Conclusions

This study demonstrates that JA (**7**) biosynthesis in *L. theobromae* is similar to that in plants (e.g., cyclization). It is interesting that the cyclization mechanism in *L. theobromae* appears to be identical to that in plants, which was supported by administration experiments of stable isotope-labeled compounds. Therefore, *L. theobromae* potentially has proteins, which have similar enzymatic activities to 13-LOX, AOS, and AOC. In stark contrast, the reduction of the cyclopentenone moiety differed between *L. theobromae* and plants. Highlighted by the case of gibberellin biosynthesis, there are large differences between plant and fungal genes involved in gibberellin biosynthesis. Fungi evolved a gibberellin biosynthetic pathway independently (Bömke and Tudzynski, 2009). Considering the clear difference between the observed facial selectivity of the cyclopentenone reduction that was observed in *L. theobromae* and that in plants, it can be suggested that this fungus has an independently evolved JA (**7**) biosynthesis pathway. The detailed mechanism of JA (**7**) biosynthesis in *L. theobromae* requires further investigation. Additional studies aimed at determining the mechanism and specific enzymes involved for each biosynthetic step are required to fully define the JA (**7**) biosynthetic route in *L. theobromae*. In addition, these studies could lend new insight into plant–microbe interactions on a fundamental level.

4. Experimental

4.1. General

Data were obtained with the following instruments: ^{1}H NMR (270 MHz) and ^{13}C-NMR (67.8 MHz), Jeol JMN-EX270 FT-NMR spectrometer; ^{2}H NMR (76.5 MHz), Bruker AMX500 FT-NMR spectrometer; EI-MS, Jeol JMS-T100GCV mass spectrometer; GC–MS, Varian 1200L GC/MS/MS system. The NMR shift values were referenced to residual solvent signals as follows; ^{1}H NMR, $CDCl_3$ (δ_{1H} = 7.24 ppm); ^{2}H NMR, $CHCl_3$ (δ_{2H} = 7.24 ppm); ^{13}C-NMR, $CDCl_3$ (δ_{13C} = 77.0 ppm).

4.2. Administration of ^{13}C-labeled sodium acetate in L. theobromae

Spores of *L. theobromae* were maintained on 1% potato-glucose agar medium at 30 °C and transferred at intervals of 6 months. A piece (approximately 1 cm^2) of agar bearing the spore-formed culture of *L. theobromae* was inoculated into a Erlenmeyer flask (500-ml) containing 150 ml of a 1% potato-glucose medium. Each medium was statically incubated at 29 °C in the dark. After 7 days of incubation, [1-^{13}C]NaOAc acetate and [2-^{13}C]NaOAc (99 atom%, respectively) were dissolved in H_2O (1 ml) which was supplemented to give a final concentration of 10 mM, and then the incubation was continued for an additional 10 days. The 17-day-old culture (150 ml) for each administration experiment was filtered, made alkaline by addition of 5% aqueous $NaHCO_3$, and extracted with EtOAc (250 ml). The resultant aqueous layer was acidified by addition of 6 M HCl (30 ml), and extracted with EtOAc (300 ml). After the EtOAc extract was concentrated *in vacuo*, the resultant residue was purified by preparative SiO_2 gel TLC (*n*-hexane:EtOAc:AcOH = 60:40:1, Merck, USA), and subsequently isolated by HPLC (TSK gel ODS80Ts,TOSOH, 20 mm i.d. × 250 mm, MeOH–H_2O (4:1%, v/v) solution, 5.0 ml/min, retention time = 16.2 - min). For the calculation of incorporated ratio, JA (**7**) was converted to the methyl ester with ethereal diazomethane.

4.3. Identification of OPDA methyl ester in L. theobromae

L. theobromae was statically cultured in a Erlenmeyer flask (500 ml) containing 1% potato-glucose medium (150 ml) at 29 °C for 17 days in the dark. The mycelia were soaked in acetone, and the extract was concentrated *in vacuo*. The resulting aqueous solution was extracted with EtOAc (20 ml). After concentration, the EtOAc extract (23 mg) was loaded onto silica gel TLC plate (Merck, USA), which was developed with $CHCl_3$. A band (Rf ≈ 0.3–0.4) was scraped, extracted with a mixed solution of MeOH and $CHCl_3$ (3:7), and concentrated *in vacuo*. The residue (1 mg) was dissolved in $CHCl_3$ (100 μl), and 1 μl of the solution was analyzed by GC–MS spectrometry.

The GC–MS spectrometer was a 1200L GC/MS/MS system (Varian, USA) equipped with a β-DEX fused silica capillary column (0.25 mm × 30 m, 0.2 mm film thickness, Supelco, USA). He was used as carrier gas at a constant flow rate of 1.0 ml/min. The temperature of the ion source and vaporizing chamber were heated to 200 °C, with an ionization voltage of 70 eV. The temperature gradient was initiated at 50 °C, was held isothermal for 1 min, and subsequently raised at a 10 °C/min to a final temperature of 190 °C, which was held for 80 min. The retention times of (+) and (−)-*trans*-OPDA methyl esters were 87.6 min and 89.7 min,

respectively. Standard racemic *trans*-OPDA methyl ester was prepared according to the method of Bandara et al. (2009).

4.4. Administration of ^{2}H-labeled linolenic acid (**1a**) in L. theobromae

[9,10,12,13,15,16-$^2H_6$]linolenic acid (**1a**) was synthesized according to the method of Hungerford and Kitching (1998). A 1% potato-glucose medium (100 ml) was prepared in an Erlenmeyer (300 ml) flask. Incubation of *L. theobromae* was carried out as described above. After 7 days of incubation, [9,10,12,13,15,16-$^2H_6$]linolenic acid (**1a**) was dissolved in 2 mM NH_4OH aqueous solution (500 μl), which was supplemented to give a final concentration of 1 mM. The incubation was continued for an additional 10 days. The filtrate of the culture, in which an equal volume of 2 M HCl was added, was extracted with EtOAc (300 ml). After concentration, the extract (120 mg) was subjected to preparative TLC (SiO_2 gel, *n*-hexane:EtOAc:AcOH = 60:40:1, Merck, USA). The resultant residue (8.9 mg) was purified by HPLC (TSK gel ODS80Ts,TOS-OH, 20 mm i.d. × 250 mm, MeOH–H_2O (4:1, v/v), 5.0 ml/min, retention time = 16.2 min) to give pure *iso*-JA (**6**) (1.5 mg). After methyl ester formation with ethereal diazomethane, the ^{2}H NMR of the methyl ester of *iso*-JA was taken immediately. The intensities of $[M+4]^+$ ion peak (m/z = 228, [$^2H_4$]-JA (**7a**)) and $[M+5]^+$ ion peak (m/z = 229, [$^2H_5$]*iso*-JA (**6a**)) with the methyl ester of biosynthetically labeled JA were given as the percent relative to the intensity of the molecular ion peak of natural JA (m/z = 224) as 100% in EI-MS data.

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