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Title

Biosynthesis and *in vitro* enzymatic synthesis of the isoleucine conjugate of 12-oxo-phytodienoic acid from the isoleucine conjugate of α-linolenic acid.

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Abbreviations

AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; 12,13-EOT, allene oxide; GC-MS, gas chromatography-mass spectrometry; 13-HPOT, 13-hydroperoxy octadecatrienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; JAR1, jasmonic acid-resistant 1; JAZ, jasmonate-zim domain; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LA-Ile, isoleucine conjugate of α-linolenic acid; OPC-8:0, 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid; OPDA-Ile, isoleucine conjugate of OPDA; OPR, 12-oxo-phytodienoic acid reductase; SCF, skp-cullin-F box.

29 Abstract

30 The isoleucine conjugate of 12-oxo-phytodienoic acid (OPDA-Ile), a new member of the
31 jasmonate family, was recently identified in *Arabidopsis thaliana* and might be a signaling molecule
32 in plants. However, the biosynthesis and function of OPDA-Ile remains elusive. This study reports an
33 *in vitro* enzymatic method for synthesizing OPDA-Ile, which is catalyzed by reactions of lipoxygenase
34 (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) using isoleucine conjugates of
35 α-linolenic acid (LA-Ile) as the substrate. *A. thaliana* fed LA-Ile exhibited a marked increase in the
36 OPDA-Ile concentration. LA-Ile was also detected in *A. thaliana.* Furthermore, stable isotope labelled
37 LA-Ile was incorporated into OPDA-Ile. Thus, OPDA-Ile is biosynthesized via the cyclization of LA-
38 Ile in *A. thaliana.*

39

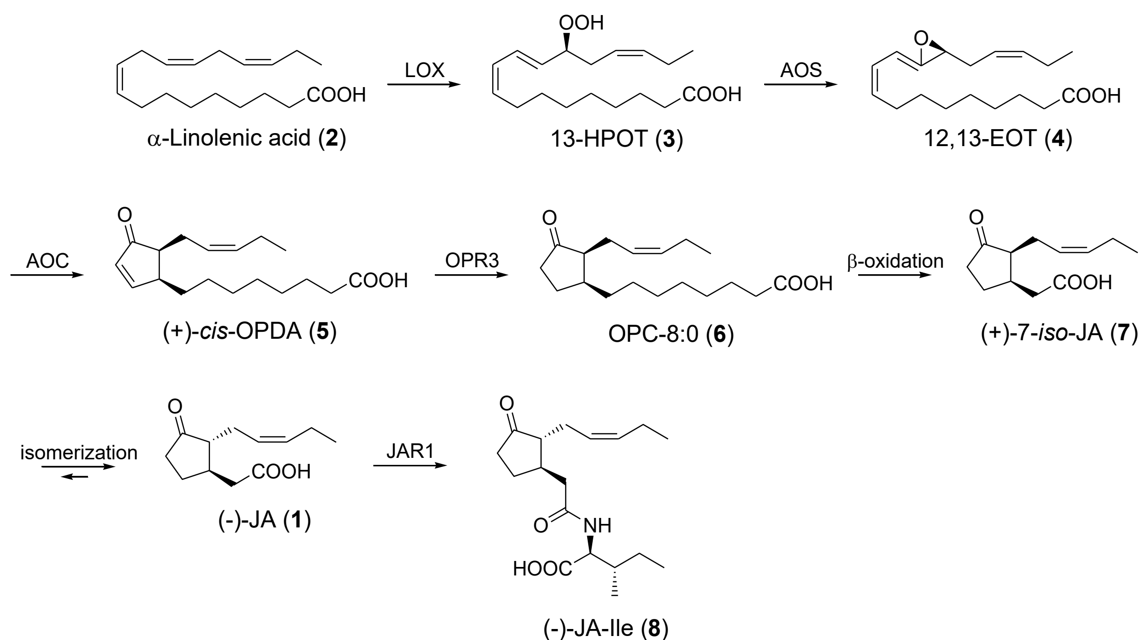
40 Keywords
41 *Arabidopsis thaliana*, jasmonates, LA-Ile, OPDA-Ile, 12-oxo-phytodienoic acid.

42

43 Plants have a wide variety of physiological responses that allow them to adapt to adverse
44 environmental conditions that negatively affect their growth and development. Jasmonic acid (JA, **1**)
45 plays important roles in stress responses and development in plants. JA (**1**) functions as a signaling
46 molecule in numerous plant physiological processes related to development and defense responses.[1]
47 Most enzymes that participate in JA (**1**) biosynthesis have been successfully characterized. JA (**1**) has
48 been shown to be a signaling molecule in both flowering plants and a model lycophyte, *Selaginella*
49 *moellendorffii*.[2] JA (**1**) is a ubiquitous phytohormone detected in vascular plant species.

50 The JA (**1**) biosynthetic pathway begins with the lipase-mediated release of α-linolenic acid (**2**)
51 from the membrane lipids of chloroplasts (Fig. 1).[1] In chloroplasts, lipoxygenase (LOX) oxidizes α-
52 linolenic acid (**2**) into 13(*S*)-hydroperoxy octadecatrienoic acid (13-HPOT, **3**). 13-HPOT (**3**) is
53 metabolized by allene oxide synthase (AOS) into an unstable allene oxide (12,13-EOT, **4**), which is
54 cyclized by allene oxide cyclase (AOC) into *cis*-(+)-12-oxo-phytodienoic acid (OPDA, **5**). The AOC
55 reaction provides two side chain configurations in the naturally occurring jasmonate structure.
56 Reduction of the 10,11-double bond in OPDA (**5**) by OPDA reductase 3 (OPR3) then yields 3-oxo-2-

57 (2-*cis*-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0, **6**). Three β-oxidation steps convert OPC-8:0
58 (**6**) into (+)-7-*iso*-JA (**7**), which is naturally isomerized to (−)-JA (**1**). JA (**1**) is converted to the
59 isoleucine conjugate of JA (JA-Ile, **8**) by JAR1. JA-Ile (**8**) is considered a versatile signaling compound
60 in the JA signaling pathway.[1,3] JA-Ile (**8**) binds to its receptor, coronatine insensitive 1 (COI1), and
61 then mediates the binding of the JAZ protein to the COI1-JA-Ile unit of the skp-cullin-F box (SCF)
62 complex, resulting in degradation by the 26S proteasome and the subsequent induction of COI1-
63 dependent JA responses.[4-6] OPDA (**5**) is not only an intermediate in the JA biosynthetic pathway but
64 also exerts individual JA (**1**)-independent biological functions.[7-9] OPDA (**5**) binds cyclophilin 20-3,
65 leading to enhanced redox capability in *Arabidopsis thaliana*.[10] In contrast, OPDA (**5**), but not JA (**1**),
66 is present in the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens,* with functions
67 in defense and development.[11-13] However, the detailed mechanism of the OPDA signaling system
68 remains unknown.



69

70 Fig. 1. Octadecanoid pathway.

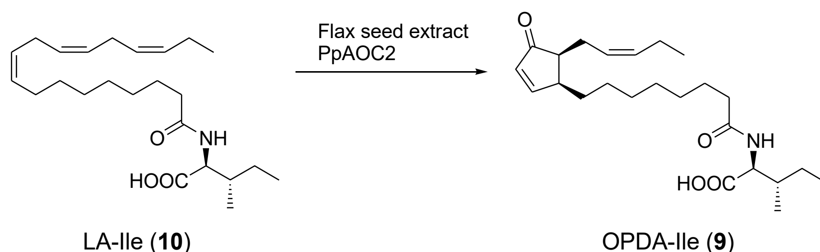
71

72

73 OPDA-Ile (**9**), a new member of the jasmonate family, was recently identified in *A. thaliana*.[14]
74 Moreover, OPDA-Ile (**9**) induces the expression of the *ZAT10* gene, which encodes a salt tolerance

75 zinc finger protein, and the *GRX480* gene, which encodes a GLUTAREDOXIN.[15,16] Based on these
76 findings, OPDA-Ile (**9**) may function as a signaling molecule in plants. The OPDA-Ile (**9**) biosynthetic
77 mechanism has not yet been determined, whereas the *A. thaliana jar1* mutant, which lacks the *jar1*
78 gene encoding a protein that catalyzes the conjugation of JA (**1**) with Ile, produces OPDA-Ile (**9**).[16]
79 Thus, the OPDA-Ile (**9**) biosynthetic pathway, which is independent of JAR1, is proposed to be present
80 in *A. thaliana.*

81 The biological functions of OPDA-Ile (**9**) remain elusive. An efficient method for synthesizing
82 OPDA-Ile (**9**) should be developed to investigate the detailed biological activities of this compound.
83 OPDA-Ile (**9**) was previously produced via the chemical conjugation of Ile and OPDA (**5**) under
84 alkaline conditions.[16,17] The stereochemistry of the two side chains of OPDA (**5**) is easily converted
85 from the *cis*-form to *trans*-form under alkaline conditions; therefore, the previously reported method
86 for synthesizing OPDA-Ile (**9**) is not necessarily optimal. For OPDA (**5**) biosynthesis, reactions with
87 LOX, AOS and AOC occur on the unsaturated alkyl chains of α-linolenic acid (**2**), 13-HPOT (**3**), and
88 12,13-EOT (**4**), respectively.[18-20] Analysis of the crystal structures of AOS and AOC suggests that
89 unsaturated alkyl chains of 13-HPOT (**3**) and 12,13-EOT (**4**) are present in the active sites of the
90 corresponding enzymes.[18,19]



91 LA-Ile (**10**) OPDA-Ile (**9**)

92 Fig. 2. *In vitro* enzymatic synthesis of OPDA-Ile (**9**). LA-Ile (**10**) was incubated in the reaction mixture
93 [50 mM Tris-HCl (pH 8.0), flax seed extract, PpAOC2] at 25 °C for 1 hours.

94

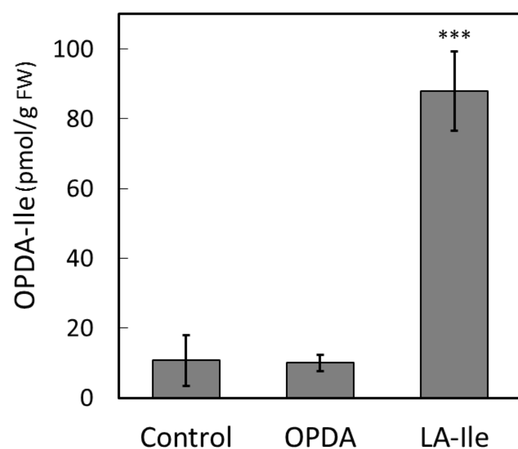
95

96 We attempted the *in vitro* cyclization of LA-Ile (**10**) to produce OPDA-Ile (**9**) by performing
97 continuous reactions with LOX, AOS, and AOC according to the method for *in vitro* stereoselective
98 OPDA (**5**) synthesis (Fig. 2).[21] The mixture used for the *in vitro* synthesis of OPDA-Ile (**10**) contained
99 flaxseed extract, recombinant PpAOC2 derived from the model moss *Physcomitrella patens*, and LA-

100 Ile (**10**) and was incubated at 25 °C for 1 hour. As a result, 11 mg of OPDA-Ile (**9**) was successfully
101 synthesized from 30 mg of LA-Ile (**10**) with a 35% yield (Supplemental data). Analysis of the AOS
102 crystal structure suggests that a lysine residue of AOS near the substrate interacts with the carboxyl
103 group of 13-HPOT (**3**), thereby playing an important role in its binding.[18] While the carboxyl group in
104 linolenic acid (**2**) is replaced by an amide bond in LA-Ile (**10**), a lysine residue near the substrate of
105 AOS may interact with the oxygen of the amide bond in a possible LOX product of LA-Ile (**10**). The
106 alkyl chain of Ile moiety derived from LA-Ile (**10**) must not interfere with binding to LOX, AOS, or
107 AOC. Therefore, the cyclization of LA-Ile (**10**) into OPDA-Ile (**9**) is found to have occurred.
108 Additionally, the *in vitro* enzymatic synthesis of OPDA-Ile (**9**) was conducted under mild conditions
109 and efficiently yielded OPDA-Ile (**9**). Considering the mechanisms of the LOX, AOS, and AOC
110 reactions, the method reported in this study could be applied to the synthesis of other amino acid
111 conjugates of OPDA.

112 The biosynthetic mechanism of OPDA-Ile (**9**) was not revealed until recently. Two possibilities
113 exist for the OPDA-Ile (**9**) biosynthetic pathway. One possibility is that OPDA-Ile (**9**) is synthesized
114 by a protein that conjugates OPDA (**5**) and Ile. In the case of JA-Ile (**8**), a GH3 protein, JAR1
115 conjugates JA (**1**) and Ile.[1] A protein from the GH3 protein family is predicted to catalyze the
116 conjugation of OPDA (**5**) and Ile. The other possibility is that OPDA-Ile (**9**) is biosynthesized by three
117 continuous reactions with LOX, AOS, and AOC with LA-Ile (**10**) as the substrate, similar to the *in*
118 *vitro* enzymatic synthesis of OPDA-Ile (**9**). In a previous study, the marginal conversion of OPDA into
119 OPDA-Ile was observed in WS.[15] The *in vitro* enzymatic synthesis of OPDA-Ile (**9**) in this study
120 supports the hypothesis that OPDA-Ile (**9**) is biosynthesized from LA-Ile (**10**) via LOX-, AOS-, and
121 AOC-mediated reactions in plants. *A. thaliana* plants grown for 30 days under short-day conditions
122 were treated with 100 μM LA-Ile (**10**) or OPDA (**5**), and the accumulation of OPDA-Ile (**9**) was
123 analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).
124 Based on the analytical data, the application of LA-Ile (**10**) significantly increased the OPDA-Ile (**9**)
125 concentration in *A. thaliana*. The amount of OPDA-Ile (**9**) in plants treated with LA-Ile (**10**) was
126 greater than that in untreated plants (Fig. 3). In contrast, the OPDA (**5**) treatment did not increase the
127 OPDA-Ile (**9**) concentration (Fig. 3). These results suggested that OPDA-Ile (**9**) was biosynthesized

128 from LA-Ile (**10**) but not OPDA (**5**).



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130 Fig. 3. UPLC-MS/MS analysis of OPDA-Ile (**9**) in *A. thaliana* treated with OPDA (**5**) or LA-Ile (**10**).
131 Plants were treated with either 100 μM LA-Ile (**10**) or OPDA (**5**). OPDA-Ile (**9**) was analyzed by
132 UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak at *m/z* 130.00 $[M-H]^-$
133 derived from the peak at *m/z* 404.28 $[M-H]^-$. Each value is represented by the mean ± SD of five
134 independent biological replicates. Student's *t*-test, ***$p < 0.001$.

135

136

137 As described above, OPDA-Ile (**9**) was postulated to be converted from LA-Ile (**10**); however, LA-
138 Ile (**10**) has not been reported as a natural product. *A. thaliana* was analyzed by liquid chromatography-
139 tandem mass spectrometry (LC-MS/MS) to determine the presence of LA-Ile (**10**). The analytical data
140 revealed a predominant peak derived from LA-Ile (**10**) in the chromatogram of an *A. thaliana* extract
141 (Fig. 4). The retention time of the peak was the same as the peak for the LA-Ile (**10**) standard. Thus,
142 LA-Ile (10 pmol/g FW, **10**) is present in *A. thaliana*. To our knowledge, this report represents the first
143 evidence identifying LA-Ile (**10**) as a natural product.

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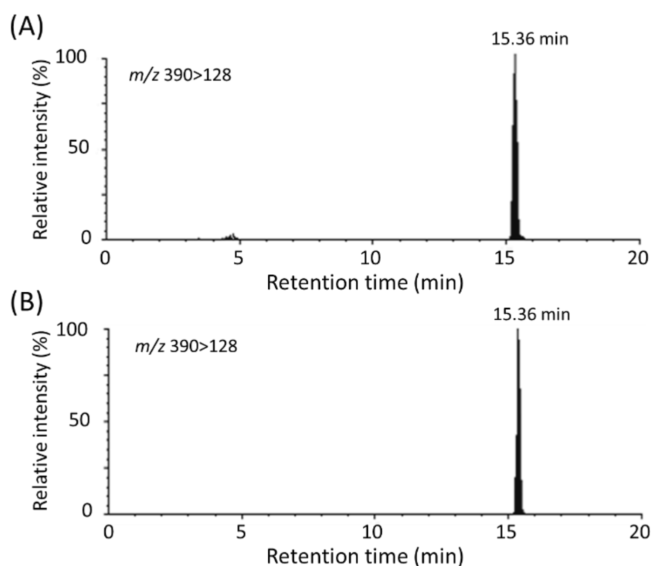

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146 Fig. 4. Analysis of LA-Ile (**10**) in *A. thaliana.* LA-Ile (**10**) was analyzed by LC-MS/MS. The MRM
147 mode was used to analyze a specific fragment peak at *m/z* 128.0 $[M-H]^-$ derived from the peak at *m/z*
148 390.3 $[M-H]^-$. (A): standard; (B): plant extract.

149

150

151 To examine whether OPDA-Ile (**9**) was synthesized by an *A. thaliana* protein extract, a reaction
152 mixture comprising LA-Ile (**10**) and an *A. thaliana* protein extract was incubated for 1 hour, and then
153 the reaction mixture was analyzed for the presence of OPDA-Ile (**9**) by UPLC-MS/MS. The peak
154 derived from OPDA-Ile (**9**) appeared clearly in the reaction mixture of LA-Ile (**10**) and the protein
155 extract (Fig. 5). In contrast, no clear peak derived from OPDA-Ile (**10**) was detected in the protein
156 extract lacking LA-Ile (**10**) or in the buffer used to generate the protein extract supplemented with LA-
157 Ile (**10**) (Fig. 5). *Arabidopsis* protein extract was shown to exhibit sequential LOX, AOS, and AOC
158 enzymatic activities to convert LA-Ile (**10**) into OPDA-Ile (**9**).

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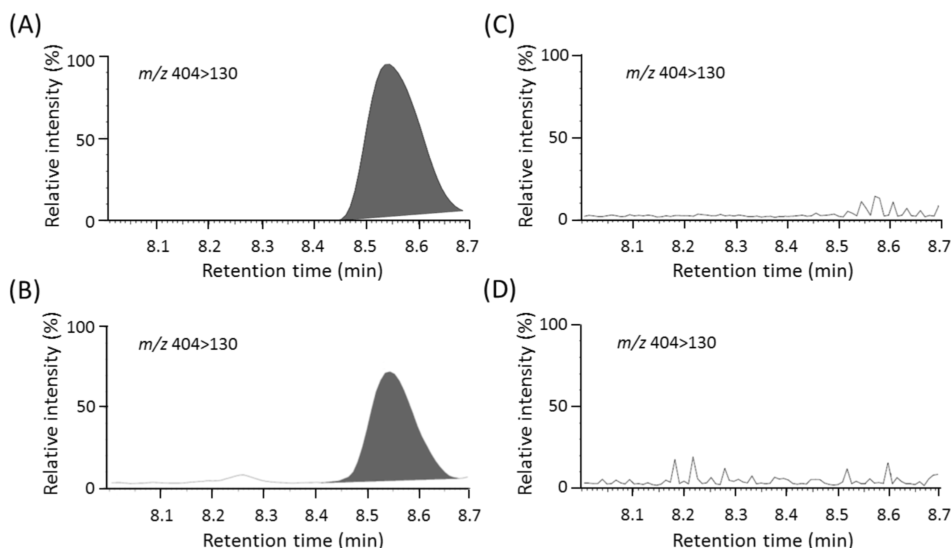

160

161 Fig. 5. *In vitro* synthesis of OPDA-Ile (**9**) by protein extracts from *A. thaliana.* LA-Ile (**10**) was
162 incubated with a protein extract prepared from *A. thaliana* at 25 °C for 1 hour, and the mixture was
163 then analyzed for OPDA-Ile (**9**) by UPLC-MS/MS. The MRM mode was used to analyze a specific
164 fragment peak at *m/z* 130.00 $[M-H]^-$ derived from the peak at *m/z* 404.28 $[M-H]^-$. (A): standard
165 OPDA-Ile (**9**); (B): LA-Ile (**10**) in protein extract; (C): protein extract without added LA-Ile (**10**); (D):
166 LA-Ile (**10**) in the buffer used for protein extraction.

167

168

169 Next, we investigated whether stable-isotope-labelled LA-Ile (**9**) was incorporated into OPDA-Ile
170 (**9**). LA-$[^{13}C_6,^{15}N]$Ile was fed to *A. thaliana*, and OPDA-$[^{13}C_6,^{15}N]$Ile in *A. thaliana* was then analyzed
171 by UPLC-MS/MS. The analytical data showed that the peak derived from OPDA-$[^{13}C_6,^{15}N]$Ile (*m/z*
172 411>137) clearly appeared, and the retention time of OPDA-$[^{13}C_6,^{15}N]$Ile was in accordance with that
173 of non-labelled OPDA-Ile (*m/z* 414>130) (Fig. 5). Accordingly, OPDA-Ile (**9**) is biosynthesized via
174 cyclization of the LA-Ile (**10**) substrate in *A. thaliana.*

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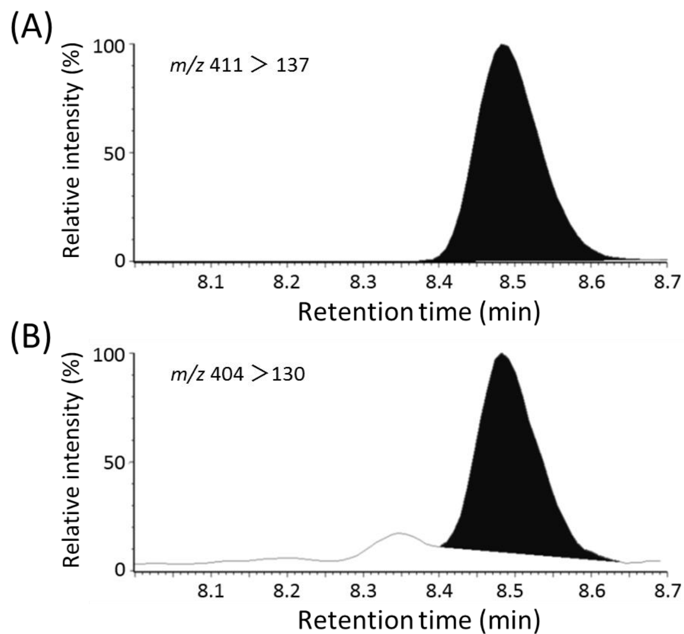

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177 Fig. 5. Incorporation of LA-[$^{13}C_6$,^{15}N]Ile into OPDA-[$^{13}C_6$,^{15}N]Ile into *A. thaliana*. Plants were treated
178 with or without 100 μM LA-[$^{13}C_6$,^{15}N]Ile, and the resulting mixture was analyzed for OPDA-
179 [$^{13}C_6$,^{15}N]Ile by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak of
180 OPDA-[$^{13}C_6$,^{15}N]Ile at *m/z* 137.00 $[M-H]^-$ derived from the peak at *m/z* 411.28 $[M-H]^-$ and a specific
181 fragment peak of OPDA-Ile at *m/z* 130.00 $[M-H]^-$ derived from the peak at *m/z* 404.28 $[M-H]^-$. (A);
182 extract of plant treated with LA-[$^{13}C_6$,^{15}N]Ile: (B); extract of control plant.

183

184

185 Based on the data described above, OPDA-Ile (**9**) is biosynthesized in *A. thaliana* via LOX-, AOS-,
186 and AOC-mediated reactions, which participate in the octadecanoid pathway, using LA-Ile (**10**) as the
187 substrate. This result is supported by previous studies showing that the *jar1* mutant still produces
188 OPDA-Ile (**9**), that the *aos* mutant does not produce OPDA-Ile and that marginal conversion of OPDA
189 into OPDA-Ile occurs in *A. thaliana.*[14,15] Because the three proteins, LOX, AOS and AOC, are
190 localized in chloroplasts, OPDA-Ile (**9**) is predicted to be located in chloroplasts. Additionally,
191 arabidopsides, monogalactosyl glycerol lipids containing OPDA,[20] are likely synthesized by a
192 combination of LOX-, AOS-, and AOC-mediated reactions using monogalactosyldiacylglycerol as the
193 substrate.[22] The previously reported data also support the results obtained in this study. Thus, the

194 present study suggests that an α-linolenic acid-related compound with a modified carboxylic acid can
195 become a substrate in the octadecanoid pathway. It is possible that a variety of OPDA-related
196 compounds are biosynthesized through the octadecanoid pathway in plants.

197 As described above, LA-Ile (**10**) is a synthetic precursor of OPDA-Ile (**9**). LA-Ile (**10**) synthesis is
198 also a crucial step in OPDA-Ile (**9**) biosynthesis; however, the conjugation mechanism of α-linolenic
199 acid (**2**) and Ile in *A. thaliana* remains unclear. As GH3 proteins conjugate amino acids and plant
200 hormones, such as indole-3-acetic acid and JA (**1**),[23] a member of the GH3 protein family likely plays
201 an important role in OPDA-Ile (**10**) biosynthesis. The identification of an enzyme that catalyzes the
202 conjugation of α-linolenic acid (**2**) and Ile is required to elucidate the total biosynthetic pathway for
203 OPDA-Ile (**9**).
204

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208

209 Supplementary data
210 The supplementary data associated with this article can be found in the online version.
211

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Supplemental data

1. General methods

The ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol EX-270 NMR spectrometer (Jeol, Tokyo, Japan). ^{1}H-NMR chemical shifts are referenced to the residual $CDCl_3$ solvent peak at δ7.24 ppm. ^{13}C-NMR chemical shifts are referenced to the residual $CDCl_3$ solvent peak at δ77.0 ppm. Field desorption-high resolution mass spectra (FD-HR-MS) were recorded on a JEOL JMS T100GCV mass spectrometer (Jeol, Tokyo, Japan). Specific rotation values were measured on a JASCO DIP-310 polarimeter (Jasco Corporation, Tokyo, Japan).

2. LA-Ile (**10**) synthesis

α-Linolenic acid (**2**, 0.88 mmol, 245 mg) was dissolved in tetrahydrofuran (11 ml) with trimethylamine (0.98 mmol, 0.14 ml). Chloroformic acid ethyl ester (0.1 mmol, 0.1 ml) was added while the mixture was stirred at −10 °C. After the resulting solution was stirred for 20 min, a 0.3 M aqueous NaOH solution (6.9 ml) containing Ile (1.77 mmol, 232 mg) was added to the solution and stirred for an additional 25 min at room temperature. After evaporation to remove the solvent, the obtained residue was cooled at 0 °C, and poured into 1 M HCl, extracted with ethyl acetate, and dried over Mg_2SO_4. The extract was evaporated and purified by SiO_2 gel column chromatography (Kanto Chemical, Tokyo, Japan), which was developed with a mixed solvent of acetic acid/ethyl acetate/*n*-hexane (1/30/70, v/v). LA-Ile (**10**) was obtained as a colorless oil (293.2 mg, 85%). FD-HR-MS: found *m/z* 392.3150 $[M+H]^+$; calculated *m/z* 392.3165 for $C_{24}H_{42}NO_3$; $[\alpha]^{25}_{D}$ +19.8 (*c* 0.3, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 11.03 (s, 1H), 6.44 (d, *J* = 8.6 Hz, 1H), 5.38-5.21 (m, 6H), 4.59 (dd, *J* = 4.6, 8.6 Hz, 1H), 2.77-2.73 (m, 4H), 2.22 (t, *J* = 8.1 Hz, 2H), 2.08-1.85 (m, 6H), 1.60-1.38 (m, 3H), 1.22-1.07 (m, 8H), 0.97-0.81 (m, 9H).

3. LA-[$^{13}C_6$,^{15}N]Ile synthesis

Instead of Ile, [$^{13}C_6$,^{15}N]Ile was conjugated to a-linolenic acid. The reaction was carried out according to the method described in the previous section.

306

307 4. OPDA-[$^{13}C_6$,^{15}N]Ile synthesis

308 Instead of LA-Ile (**10**), LA-[$^{13}C_6$,^{15}N]Ile was cyclized to afford OPDA-[$^{13}C_6$,^{15}N]Ile according to

309 the method described in the previous section.

310

311 4. *In vitro* enzymatic synthesis of OPDA-Ile (**9**)

312 OPDA-Ile (**9**) was synthesized *in vitro* using LA-Ile (**10**) as the substrate, according to the method

313 used for the *in vitro* synthesis of OPDA (**5**) (Kajiwara et al., 2012). The reaction mixture used for the

314 *in vitro* synthesis of OPDA-Ile (**9**) contained a flaxseed extract, recombinant PpAOC2 and LA-Ile (**10**).

315 An acetone powder of the flaxseed extract (625 mg) was extracted with 5 ml of 50 mM Tris-HCl buffer

316 (pH 8.0, 20 mM NaCl) containing 500 μg of recombinant PpAOC2 and then centrifuged at 21,500 ×

317 *g* for 30 min at 4 °C. The prepared enzyme solution was incubated with 30 mg of LA-Ile (**10**) and

318 stirred under an oxygen atmosphere for 3 hours at room temperature. The reaction mixture was

319 extracted with ethyl acetate. After the extract was evaporated, the resulting residue was purified by

320 SiO_2 gel column chromatography, which was developed with a mixed solvent of acetic acid/ethyl

321 acetate/*n*-hexane (1/30/70, v/v) to obtain 11 mg of OPDA-Ile (**9**). $[\alpha]^{25}_{D}$ +42.3 (*c* 0.6, $CHCl_3$). FD-

322 HR-MS: found *m/z* 404.2804 [M-H]; calculated *m/z* 404.2801 for $C_{24}H_{38}NO_4$. ^{1}H-NMR ($CDCl_3$, 270

323 MHz) δ: 7.74-7.70 (dd, *J* = 5.8, 2.6 Hz, 1H), 6.20-6.12 (dd, *J* = 3.1, 1.8 Hz, 1H), 6.08-5.98 (d, *J* = 8.3

324 Hz, 1H), 5.48-5.28 (m, 2H), 4.64-4.54 (dd, *J* = 5.6, 4.8 Hz, 1H), 3.03-2.88 (m, 1H), 2.55-2.46 (m, 1H),

325 2.46-2.36 (m, 1H), 2.26-2.17 (t, *J* = 7.4 Hz, 2H), 2.15-2.07 (m, 1H), 2.06-1.96 (m, 2H), 1.96-1.84 (m,

326 1H), 1.77-1.65 (m, 1H), 1.64-1.54 (m, 2H), 1.53-1.40 (m, 1H), 1.34-1.24 (m, 6H),1.23-1.09 (m, 2H),

327 1.05-0.72 (m, 11H). ^{13}C-NMR ($CDCl_3$, 67.5 MHz) δ: 208.8, 172.4, 171.1, 165.0, 130.5, 129.8, 124.3,

328 53.8, 47.4, 41.8, 35.1, 34.0, 28.1, 27.0, 26.6, 26.5, 25.0, 23.1, 22.6, 21.2, 18.2, 12.7, 11.4, 9.0.

329

330 5. Plants and chemical treatments

331 *A. thaliana* (Col-0) was grown on soil under short day conditions (10 hours of light/14 hours of

332 dark) at 25 °C for 30 days under a white fluorescent light. Plants were sprayed with 100 μM OPDA

333 and LA-Ile once per day for 3 days. For the feeding experiment with LA-[$^{13}C_6$,^{15}N]Ile, *A. thaliana* was

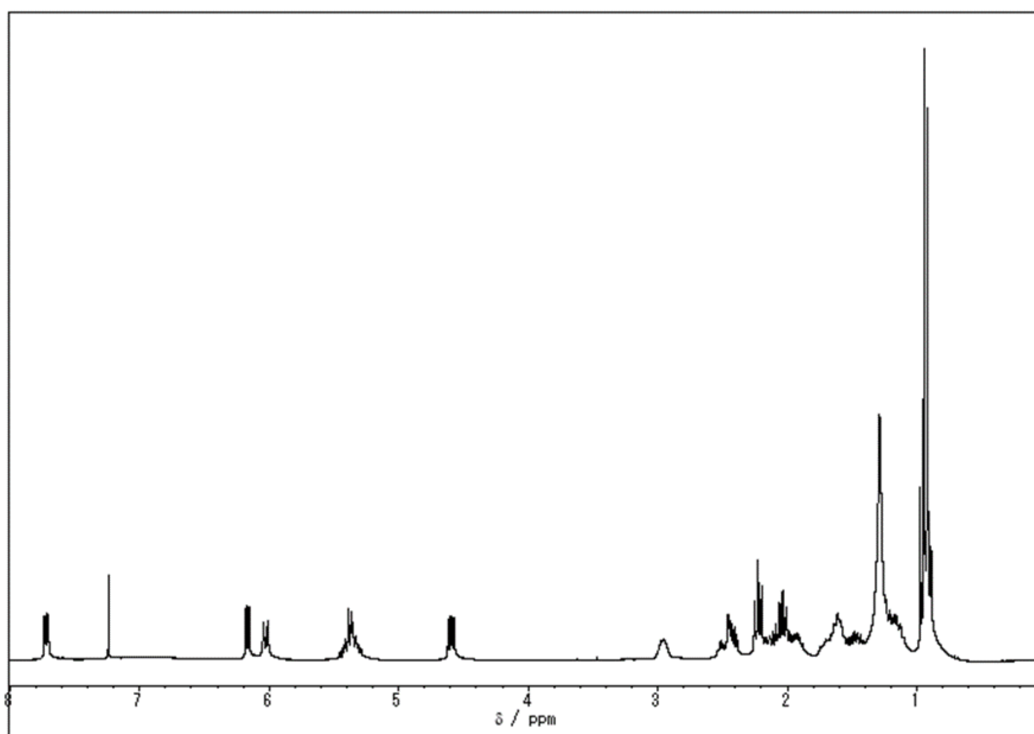
334 harvested at 12 hours after spraying 100 μM LA-[$^{13}C_6$,^{15}N]Ile. OPDA was synthesized according to
335 the method reported by Kajiwara et al. (2012).
336
337 6. Analysis of OPDA-Ile (**9**)
338 *A. thaliana* plants were grown on Jiffy-7 (Sakata Seed Corporation, Yokohama, Japan) for 5 weeks
339 at 22 °C under a white fluorescent light with 10 h/14 h photoperiods (short-day conditions). Samples
340 were prepared according to the method developed by Floková et al. (Phytochemistry. 2016; 122: 230–
341 237). UPLC was performed using an ACQUITY UPLC system (Waters Corporation, Milford, MA,
342 USA) equipped with a binary solvent manager and a sample manager. MS/MS was subsequently
343 performed using a Micromass Quattro Premier tandem quadrupole MS (Waters Corporation, Milford,
344 MA, USA). The UPLC/MS system was controlled by Micromass MassLynx 4.0 (Waters Corporation,
345 Milford, MA, USA). The UPLC conditions were described previously (Sato et al., Plant Cell Physiol.
346 2011; 52: 509-517). The MS parameters for the detection of OPDA-Ile were set according to the
347 method described by Floková et al. (Phytochemistry. 2016; 122: 230–237). OPDA-[$^{13}C_6$,^{15}N]Ile was
348 used as a standard for quantitative analysis.
349
350 7. Analysis of LA-Ile (**10**)
351 *A. thaliana* plants were grown according to the method described in the previous section. Plants
352 (500 mg) were then extracted with 10 ml of an 80% aqueous MeOH solution. The resulting extract
353 was applied onto a C18 solid phase extraction cartridge (Bond Elut, 6 ml, Agilent Technologies, CA,
354 USA) that had been equilibrated with 80% aqueous MeOH. After the cartridge was washed with 6 ml
355 of 80% MeOH, LA-Ile (**10**) was eluted with 6 ml of MeOH. The eluate was evaporated, and the
356 obtained residue was dissolved in 500 μl of 80% aqueous MeOH for analysis. The detection and
357 quantification of LA-Ile (**10**) were performed using a 4000Q TRAP LC-MS/MS system (Sciex,
358 Framingham, MA, USA) equipped with an electrospray ionization (ESI) source (turbo V) and 1290
359 Infinity HPLC system (Agilent, Santa Clara, CA, USA). Chromatographic separation was
360 performed at 40 °C on a TSK-gel ODS-100V column (150 mm × 2 mm inner diameter (i.d.), 5 μm)
361 (Tosoh Corporation, Tokyo, Japan). Eluents were composed of water/formic acid (99.9/0.1, v/v)

(eluent A) and methanol/formic acid (99.9/0.1, v/v) (eluent B). Elution was conducted at a flow rate of 0.20 ml/min with the following linear gradient: 0–3 min, 50% B; 3–18 min, 50–97% B; 18–22 min, 97% B; 22–22.1 min, 97–50% B; and 22.1–29 min, 50% B. The injection volume was 10 μl. MS data were acquired in multiple reaction monitoring (MRM) mode. The conditions of the interface were as follows: ion spray voltage, −4500 V; source temperature, 450 °C; curtain gas pressure, 10 psi; nebulizing gas pressure, 70 psi; and turbo gas pressure, 80 psi. The parameters used for the mass spectrometry of LA-Ile (**10**) are listed in Supplemental Table S1. Analyst 1.6.2 software was used for data acquisition and processing. LA-[$^{13}C_6$,^{15}N]Ile was used as a standard for quantitative analysis. The values given are the mean ± SD of five independent biological replicates.

8. *In vitro* synthesis of OPDA-Ile (**9**) by an *A. thaliana* protein extract

Plants (1.0 g) were ground in liquid nitrogen and extracted with 10 ml of 100 mM sodium phosphate buffer (pH 7.8). The residue was removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was then used as a protein extract to synthesize OPDA-Ile (**9**). One milliliter of the protein extract supplemented with 1 mM LA-Ile (**10**) was incubated at 25 °C for 1 hour. The pH of the reaction solution was adjusted to approximately 3, and the solution was next extracted with an equal volume of ethyl acetate and then evaporated. The resulting residue was dissolved in 200 μl of 80% aqueous MeOH, and OPDA-Ile (**9**) was analyzed by UPLC-MS/MS according to the method described above. A protein extract without added LA-Ile (**10**) and 100 mM sodium phosphate buffer (pH 7.8) supplemented with LA-Ile (**10**) were used as controls.

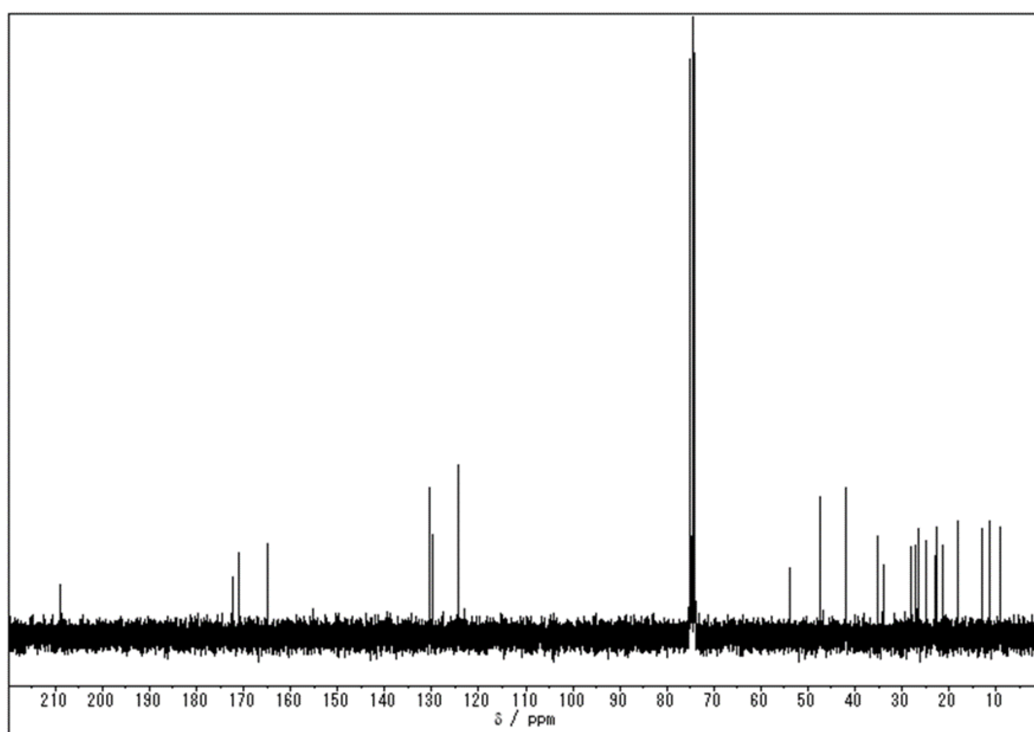
390



391

392 Supplemental Fig. S1. ^{1}H-NMR spectrum of OPDA-Ile (**9**) (270 MHz, $CDCl_3$).

393



394

395 Supplemental Fig. S2. ^{13}C-NMR spectrum of OPDA-Ile (**9**) (67.5 MHz, $CDCl_3$).

396

397 Supplemental Table S1. Optimized MS parameters for the analysis of LA-Ile.

Compound	Scan mode	MRM transition	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision exit potential (V)
LA-Ile	–	390.3/128.0	-80	-10	-34	-19

398