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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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ABSTRACT

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

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

The JA (**1**) biosynthetic pathway begins with the lipase-mediated release of α-linolenic acid (**2**) from the membrane lipids of chloroplasts (Fig. 1).[1] In chloroplasts, lipoxygenase (LOX) oxidizes α-linolenic acid (**2**) into 13(*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT, **3**). 13-HPOT (**3**) is metabolized by allene oxide synthase (AOS) into an unstable allene oxide (12,13-EOT, **4**), which is cyclized by allene oxide cyclase (AOC) into *cis*-(+)-12-oxo-phytodienoic acid (OPDA, **5**). The AOC reaction provides two side chain configurations in the naturally occurring jasmonate structure. Reduction of the 10,11-double bond in OPDA (**5**) by OPDA reductase 3 (OPR3) then yields 3-oxo-2-(2-*cis*-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0, **6**). Three β-oxidation steps convert OPC-8:0 (**6**) into (+)-7-*iso*-JA (**7**), which is naturally isomerized to (−)-JA (**1**). JA (**1**) is converted to the isoleucine conjugate of JA (JA-Ile, **8**) by JAR1. JA-Ile (**8**) is considered a versatile signaling compound in the JA signaling pathway.[1,3] JA-Ile (**8**) binds to its receptor, coronatine insensitive 1 (COI1), and then mediates the binding of the JAZ protein to the COI1-JA-Ile unit of the skp-cullin-F box (SCF) complex, resulting in degradation by the 26S proteasome and the subsequent induction of COI1-dependent JA responses.[4–6] OPDA (**5**) is not only an intermediate in the JA biosynthetic pathway but also exerts individual JA (**1**)-independent biological functions.[7–9] OPDA (**5**) binds cyclophilin 20–3, leading to enhanced redox capability in *Arabidopsis thaliana*.[10] In contrast, OPDA (**5**), but not JA (**1**), is present in the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens,* with functions in defense and development.[11–13] However, the detailed mechanism of the OPDA signaling system remains unknown.

Abbreviations: AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; 12,13-EOT, allene oxide; FW, fresh weight; GC–MS, gas chromatography-mass spectrometry; 13-HPOT, 13(*S*)-hydroperoxyoctadecatrienoic 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.

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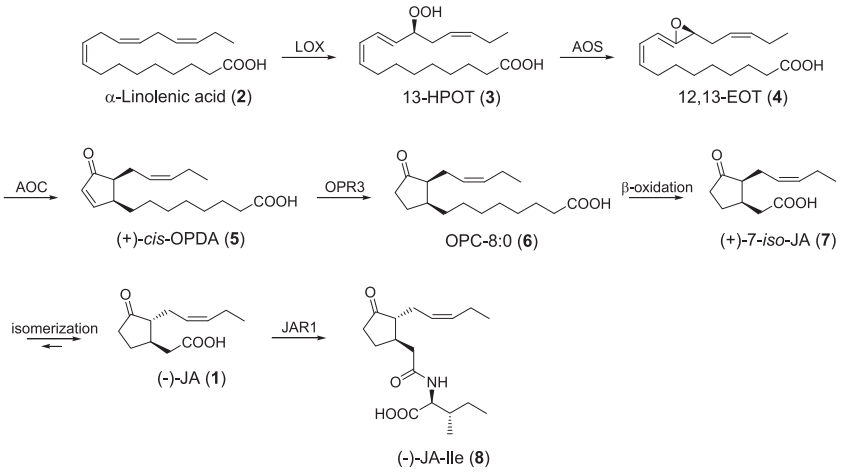

Fig. 1. Octadecanoid pathway.

OPDA-Ile (**9**), a new member of the jasmonate family, was recently identified in *A. thaliana*.[14] Moreover, OPDA-Ile (**9**) induces the expression of the *ZAT10* gene, which encodes a salt tolerance zinc finger protein, and the *GRX480* gene, which encodes a glutaredoxin.[15,16] Based on these findings, OPDA-Ile (**9**) may function as a signaling molecule in plants. The OPDA-Ile (**9**) biosynthetic mechanism has not yet been determined, whereas the *A. thaliana jar1* mutant, which lacks the *jar1* gene encoding a protein that catalyzes the conjugation of JA (**1**) with Ile, produces OPDA-Ile (**9**).[16] Thus, the OPDA-Ile (**9**) biosynthetic pathway, which is independent of JAR1, is proposed to be present in *A. thaliana*.

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

We attempted the *in vitro* cyclization of LA-Ile (**10**) to produce OPDA-Ile (**9**) by performing continuous reactions with LOX, AOS, and AOC according to the method for *in vitro* stereoselective OPDA (**5**) synthesis (Fig. 2).[21] The mixture used for the *in vitro* synthesis of OPDA-Ile (**10**) contained flaxseed extract that has LOX and AOS activities, recombinant PpAOC2 derived from the model moss *Physcomitrella patens*, and LA-Ile (**10**) and was incubated at 25 °C for 1 h. As a result, 11 mg of OPDA-Ile (**9**) was successfully synthesized from 30 mg of LA-Ile (**10**) with a 35% yield (Supplemental data). Analysis of the AOS crystal structure suggests that a lysine residue of AOS near the substrate interacts with the carboxyl group of 13-HPOT (**3**), thereby playing an important role in its binding.[18] While the carboxyl group in linolenic acid (**2**) is replaced by an amide bond in LA-Ile (**10**), a lysine residue near the substrate of AOS may interact with the oxygen of the amide bond in a possible LOX product of LA-Ile (**10**). The alkyl chain of Ile moiety derived from LA-Ile (**10**) must not interfere with binding to LOX, AOS, or AOC. Therefore, the cyclization of LA-Ile (**10**) into OPDA-Ile (**9**) is found to have occurred. Additionally, the *in vitro* enzymatic synthesis of OPDA-Ile (**9**) was conducted under mild conditions and efficiently yielded OPDA-Ile (**9**). Considering the mechanisms of the LOX, AOS, and AOC reactions, the method reported in this study could be applied to the synthesis of other amino acid conjugates of OPDA.

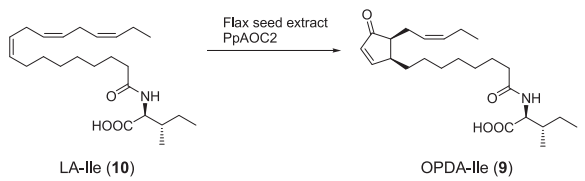

Fig. 2. *In vitro* enzymatic synthesis of OPDA-Ile (**9**). LA-Ile (**10**) was incubated in the reaction mixture [50 mM Tris-HCl (pH 8.0), flax seed extract, PpAOC2] at 25 °C for 1 h.

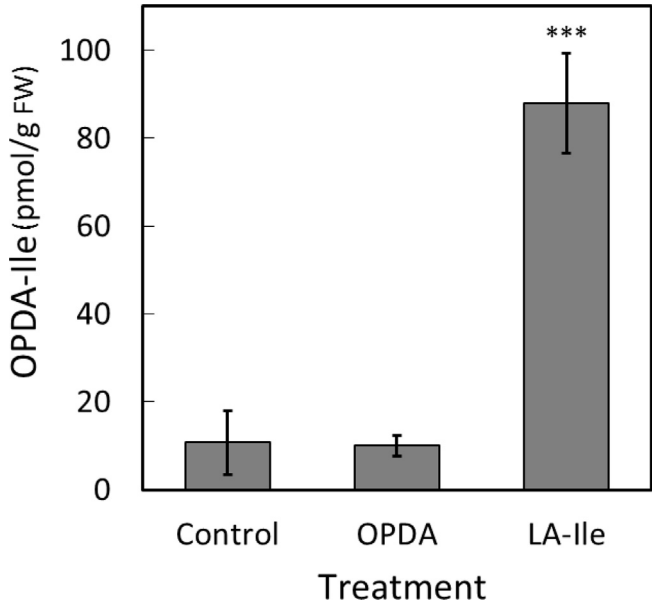

Fig. 3. UPLC-MS/MS analysis of OPDA-Ile (**9**) in *A. thaliana* treated with OPDA (**5**) or LA-Ile (**10**). Plants were treated with either 100 μM LA-Ile (**10**) or OPDA (**5**). OPDA-Ile (**9**) was analyzed by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak at *m/z* 130 $[M-H]^-$ derived from the peak at *m/z* 404 $[M-H]^-$. Each value is represented by the mean ± SD of five independent biological replicates. Student's *t*-test, $^{***}p < 0.001$.