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Verification of the Versatility of the *In Vitro* Enzymatic Reaction Giving (+)-*cis*-12-Oxo-phytodienoic Acid

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ABSTRACT

Jasmonic acid (JA) is a plant hormone involved in the defense response against insects and fungi. JA is synthesized from α-linolenic acid (LA) by the octadecanoid pathway in plants. 12-oxo-Phytodienoic acid (OPDA) is one of the biosynthetic intermediates in this pathway. The reported stereo selective total synthesis of *cis*-(+)-OPDA is not very efficient due to the many steps involved in the reaction as well as the use of water sensitive reactions. Therefore, we developed an enzymatic method for the synthesis of OPDA using acetone powder of flax seed and allene oxide cyclase (PpAOC2) from *Physcomitrella patens*. From this method, natural *cis*-(+)-OPDA can be synthesized in the high yield of approximately 40%. In this study, we investigated the substrate specificity of the enzymatic synthesis of other OPDA analogs with successions to afford OPDA amino acid conjugates, dinor-OPDA (dn-OPDA), and OPDA monoglyceride, and it was suggested that the biosynthetic pathway of arabidopsides could occur via MGDG.

Varieties of JA-amino acid conjugates, such as JA-L-Ile, JA-L-Gly, JA-L-Ala, JA-L-Leu, JA-L-Val, JA-L-Phe, JA-L-Tyr, and JA-L-Trp, have been reported [1]. We speculated that there would also be different types of OPDA analogs in nature; in fact, OPDA-L-Ile was isolated from *Arabidopsis thaliana* [2]. In investigating the other conjugates, it would be helpful to use the authentic compound as an indicator for purification and identification. In a previous paper, an efficient enzymatic synthetic method yielding (9*S*,13*S*)-12-oxo-phytodienoic acid (OPDA) was reported by Kajiwara et al. [3], and the reported method was characterized by the reaction solution containing acetone powder prepared from flax seeds [4] and a recombinant AOC derived from *Physcomitrella patens* [5].

The OPDA yield from the reaction using this system was almost 7-fold higher than that obtained from the conventional reaction with flaxseed extract [4] and gave a compound with an absolute configuration that is consistent with that of natural OPDA. Moreover, OPDA-L-Ile was shown to be biosynthesized from an isoleucine conjugate of α-linolenic acid in *Arabidopsis thaliana*.[6] It was therefore hypothesized that this method could be applied to synthesize OPDA-amino acid conjugates and OPDA analogs such as dinor-OPDA ((7*R*,11*S*)-dn-*cis*-OPDA) and 2,3-dihydroxypropyl 12-oxo-phytodienoate (OPDA monoglyceride).

As a first step, we synthesized 10 types of α-linolenic acid (LA)-amino acid conjugates (LA conjugated with L-Gly, L-Ala, L-Val, L-Leu, L-Ile, L-Phe, L-Tyr, L-Trp, L-Glu, and L-Gln, Figure 1), whose spectroscopic data are given in the Supplementary data. Then, all synthesized compounds were subjected to the method described in the previously reported paper [3] to give OPDA conjugated with L-Gly, L-Ala, L-Val, L-Leu, L-Ile, L-Phe, L-Tyr, L-Trp, L-Glu, and L-Gln. The reactions gave the expected compounds (Figure 1), whose spectroscopic data are given in the Supplementary data, although the conversion of LA-L-Trp into OPDA-L-Trp either did not proceed at all or did not proceed to produce an amount of compound that could withstand instrumental analysis. The synthetic yields of OPDA-L-Glu and OPDA-L-Gln were 38% and 48%, respectively, and the yields of OPDA-L-Gly (26%), OPDA-L-Ala (24%), OPDA-L-Val (25%), OPDA-L-Leu (15%), OPDA-L-Ile (14%), and OPDA-L-Phe (16%), and OPDA-L-Tyr (4%) were lower than those of OPDA-L-Glu and OPDA-L-Gln (Figure 2). The reason why the reaction yield of OPDA-L-Ile in this paper (14%) is lower than the one in the reference paper (35%) [6] is probably due to the fact that the conversion capacity of the enzyme obtained from flax

seeds used in the reaction cannot be kept constant each time. One of the most likely reasons for the inconsistency may be the quality of the flax seeds. Notably, the reaction using LA-L-Trp as astarting material did not give the expected compound. In general, it was thought that starting materials with high hydrophilicity, such as LA-L-Glu and LA-L-Gln, gave better yields and that compounds conjugated with larger amino acid molecules with lower hydrophilicity, such as L-Trp, were unsuitable for the reaction.

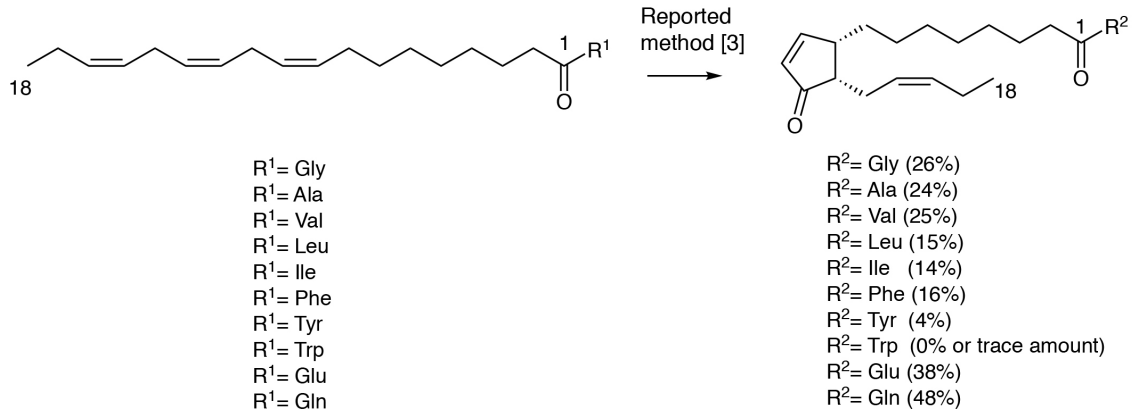

Figure 1. Chemical structures of LA and OPDA amino-monoglyceride.

In 2018, Monte et al. [7] reported that (7*R*,11*S*)-dn-*cis*-OPDA [8] was a crucial component that regulates defense, growth, and developmental responses in *Marchantia polymorpha*, and it was assumed that the reported method to obtain OPDA from LA should be applicable to give (7*R*,11*S*)-dn-*cis*-OPDA from (7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoic acid. (7*Z*,10*Z*,13*Z*)-Hexadeca-7,10,13-trienoic acid was isolated from radish leaves (*Raphanus sativus*) (2 kg) according to the procedure described in the Supplementary data, and the isolated compound was subjected to the reaction, yielding the expected compound (7*R*,11*S*)-dn-*cis*-OPDA (Figure 2) in a conversion rate of 23%, whose spectroscopic data given in the Supplementary data

coincided well with the reported those of synthetic (7*R*,11*S*)-dn-*cis*-OPDA [9]. However, the optical rotation ($[\alpha]_D^{23}$ + 110.5) of (7*R*,11*S*)-dn-*cis*-OPDA synthesized in this study was lower than that of reported one ($[\alpha]_D^{23}$ + 135.5) [9]. The reason for the smaller optical rotation was considered to be the isomerization of the *cis* isomer in the process of isolating the compound, although the contamination of *trans* isomer was not judged from ^{1}H-NMR spectrum. Wang et al. reported that a typical resonance for *cis* isomer is δ_H 7.72 (dd, J = 5.8, 2.7 Hz, 1H), whereas that for trans isomer is δ_H 7.59 (dd, J = 5.8, 2.6 Hz, 1 H).

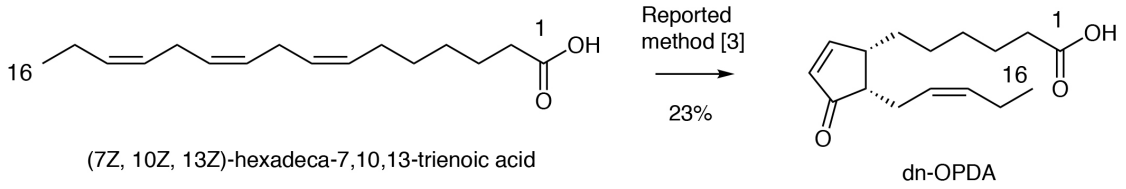

Figure 2. Chemical structures of LA and dn-OPDA.

Arabidosides are well known characteristic secondary metabolites of *A. thaliana*. Arabidosides are presumed to be a metabolite derived from monogalactosyldiacylglycerol (MGDG), but it was also conceivable that the compounds might be biosynthesized in the series of reaction, in which OPDA might bind to propane-1,2,3-triol. The detailed biosynthetic pathway that affords arabidosides has not yet been clarified, although it has been suggested that fatty acids remain attached to galactolipids during the enzymatic conversion to give OPDA [10]. The synthesis of LA monoglycerides was accomplished according to a reported method [11], whose spectroscopic data are given in the Supplementary data, and the obtained LA monoglyceride was then subjected to the

reaction described in the report of Kajiwara et al. [3] We could detect the expected compounds by TLC analysis in the case of former reactions, however no expected compounds were detected in the case of the reaction using LA monoglyceride. (Figure 3A). This unexpected result was thought to be due to hydrolysis caused by the activity of lipase in the enzyme solution containing the acetone powder originating from flax seeds. Therefore, an attempt was made to detect the compound using UPLC MS/MS, which is able to detect trace amount of compounds. After synthesizing authentic OPDA monoglyceride, whose synthetic procedure is given in supplementary data and optimizing the parameters to detect the monoglyceride, a portion of the reaction mixture using LA monoglyceride as a substrate was subjected to UPLC MS/MS. The features of the UPLC MS/MS chromatographs are given in Figure 3B, and it was found that the reaction using LA monoglyceride gave OPDA monoglyceride based on the compound from the reaction mixture and the authentically synthesized OPDA monoglyceride having the same retention time. This result revealed that the system using acetone powder of flax seed and allene oxide cyclase was able to carry out the series of reactions of LOX, AOS, and AOS and accept LA monoglyceride as a substrate. In order to suggest the hypothesis that the giving trace amount of OPDA may be due to the lipase activity in the reaction mixture, we tried to detect OPDA in the reaction solution after attempting the conversion of an LA monoglyceride. The features of the UPLC MS/MS chromatograph are given in Figure 3C, and OPDA was detected as expected. However, it might also be possible that OPDA, which originated from the LA released from LA monoglyceride after lipase treatment, formed an ester bond with the liberated propane-1,2,3-triol to give OPDA monoglyceride. In order to verify this possibility, the method reported by Kajiwara et al. [3] was performed with some modifications, in which OPDA and propane-1,2,3-triol were added as

substrates instead of LA monoglyceride. The UPLC MS/MS data is shown in Figure 3D, indicating that the esterification of OPDA with propane-1,2,3-triol did not proceed, whose result ruled out a reaction mechanism in which OPDA is formed and then combined with propane-1,2,3-triol to produce OPDA-monoglyceride.

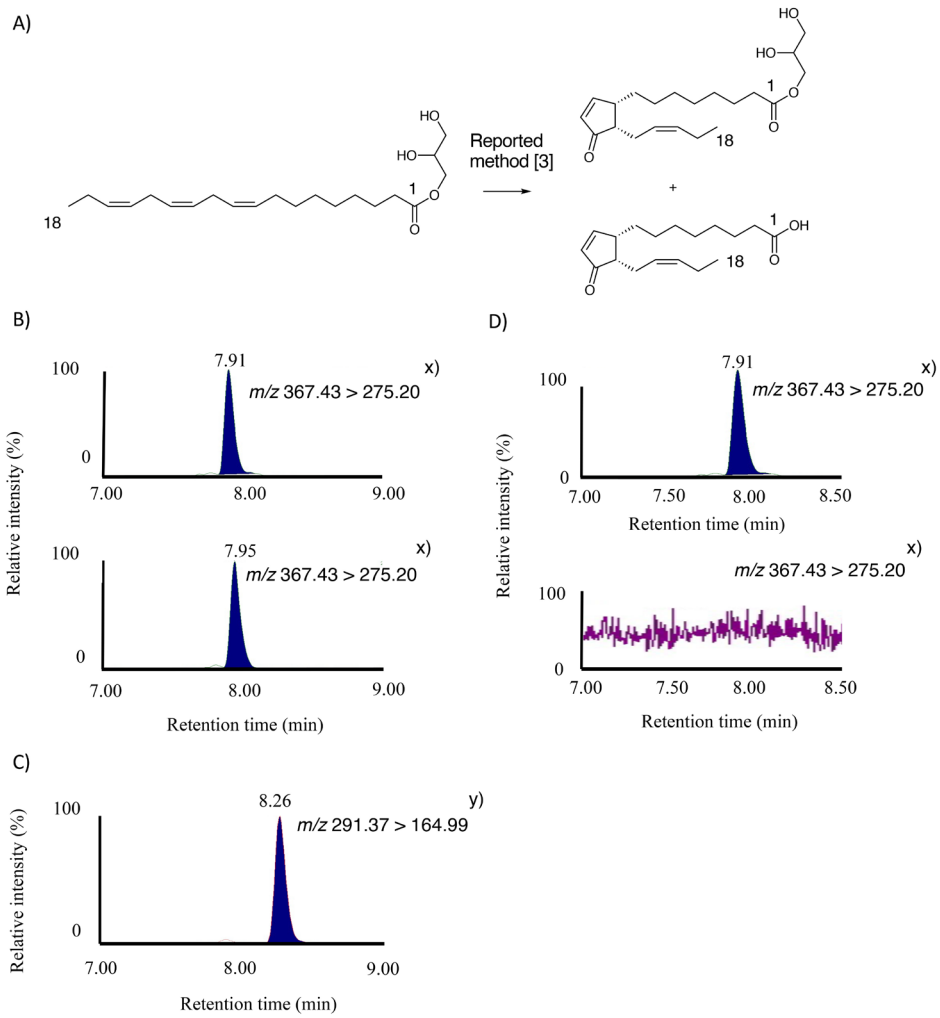

Figure 3. Enzymatic conversion of LA mono-glyceride to OPDA mono-glyceride. A) Enzymatic conversion of LA-mono-glyceride to OPDA mono-glyceride (*m/z* 366) and OPDA (*m/z* 290). B) Detection of OPDA mono-glyceride using UPLC MS/MS in positive mode. Upper column for the reaction mixture. Lower column for authentic sample, C) Detection of OPDA in the reaction mixture using UPLC MS/MS in negative mode, D) Detection of OPDA mono-glyceride using UPLC MS/MS in positive mode. Upper column for the reaction mixture. Lower column for authentic sample. x): indicating pseudo molecular and transition ions to detect OPDA mono-glyceride. y): indicating pseudo molecular and transition ions to detect OPDA.

In order to substantiate the above mentioned conclusion, the application of (9*Z*,12*Z*,15*Z*)-*N*-(2,3-dihydroxypropyl)octadeca-9,12,15-trienamide, which has an amide bond that is less susceptible to lipase degradation than the ester bond, was carried out (Figure 4). The synthesis of (9*Z*,12*Z*,15*Z*)-*N*-(2,3-dihydroxypropyl)octadeca-9,12,15-trienamide was performed according to the detailed procedure in the Supplementary data, and the synthesized substrate was subjected to the reaction according to the method described in the literature [3], which gave enough amount of *N*-(2,3-dihydroxypropyl)-8-((1*S*,5*S*)-4-oxo-5-((*Z*)-pent-2-en-1-yl)cyclopent-2-en-1-yl)octanamide (24% synthetic yield) (Figure 4A) to withstand detection using TLC and the ^{1}H-NMR measurements, whose feature of ^{1}H-NMR are given in Figure 4B and spectroscopic data given in the Supplementary data.

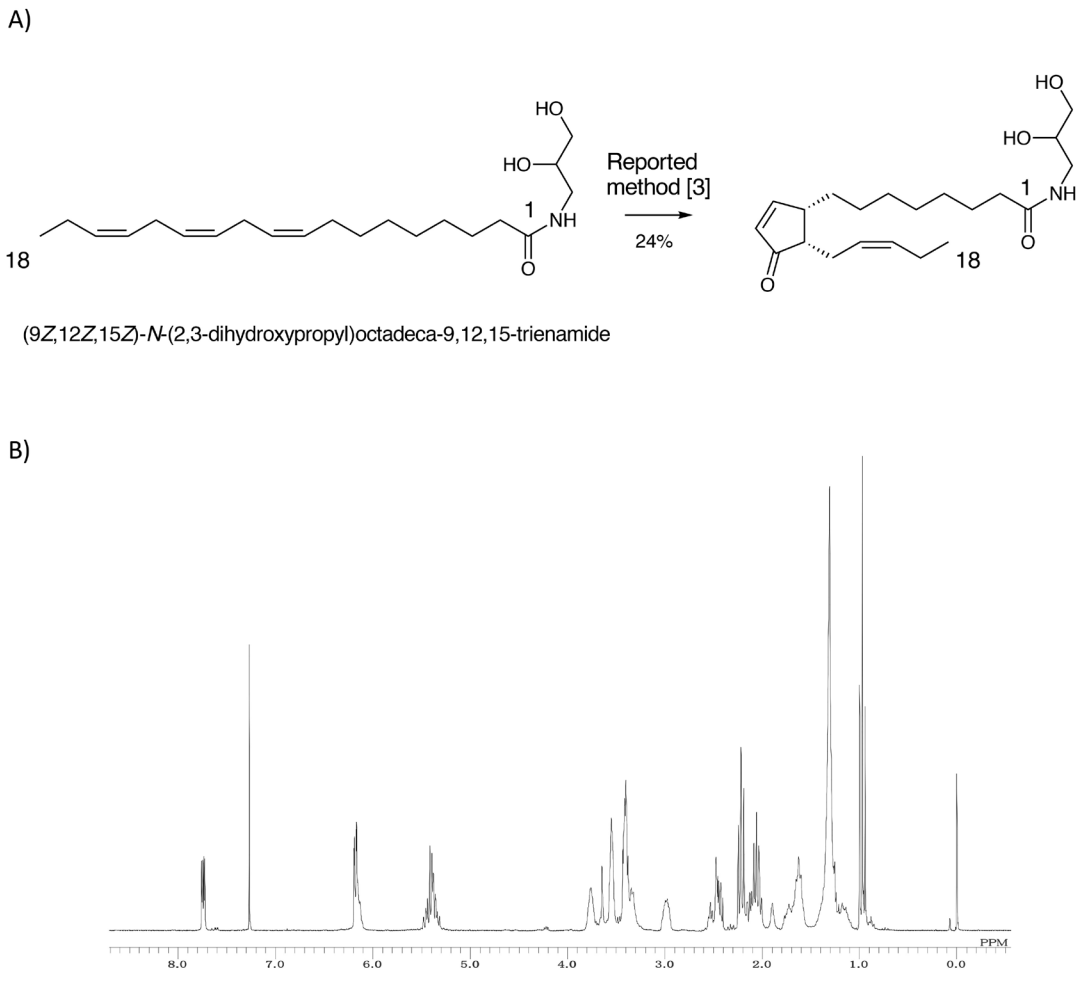

Figure 4. Enzymatic conversion to give *N*-(2,3-dihydroxypropyl)-8-((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl) cyclopentyl)octanamide.

A) Chemical structures of (9*Z*,12*Z*,15*Z*)-*N*-(2,3-dihydroxypropyl) octadeca-9,12,15-trienamide and *N*-(2,3-dihydroxypropyl)-8-((1*S*,5*S*)-4-oxo-5-((*Z*)-pent-2-en-1-yl)cyclopent-2-en-1-yl) octanamide. B) ^{1}H NMR spectrum of *N*-(2,3-dihydroxypropyl)-8-((1*S*,5*S*)-4-oxo-5-((*Z*)-pent-2-en-1-yl) cyclopent-2-en-1-yl)octanamide (270 MHz, $CDCl_3$).

Because OPDA monoglycerides could be enzymatically synthesized from 2,3-dihydroxypropyl linoleate (LA monoglyceride), this result could support the story that LOX, AOS, and AOC act upon MGDG to afford arabidosides in nature, similar to the conclusion reported by Nilsson et al. [10] Proposed biosynthetic pathway to give

arabidopside via Path A are given in Figure 5 together with experimentally concreated biosynthetic pathway to afford (+)-7-iso-JA and (-)-JA.

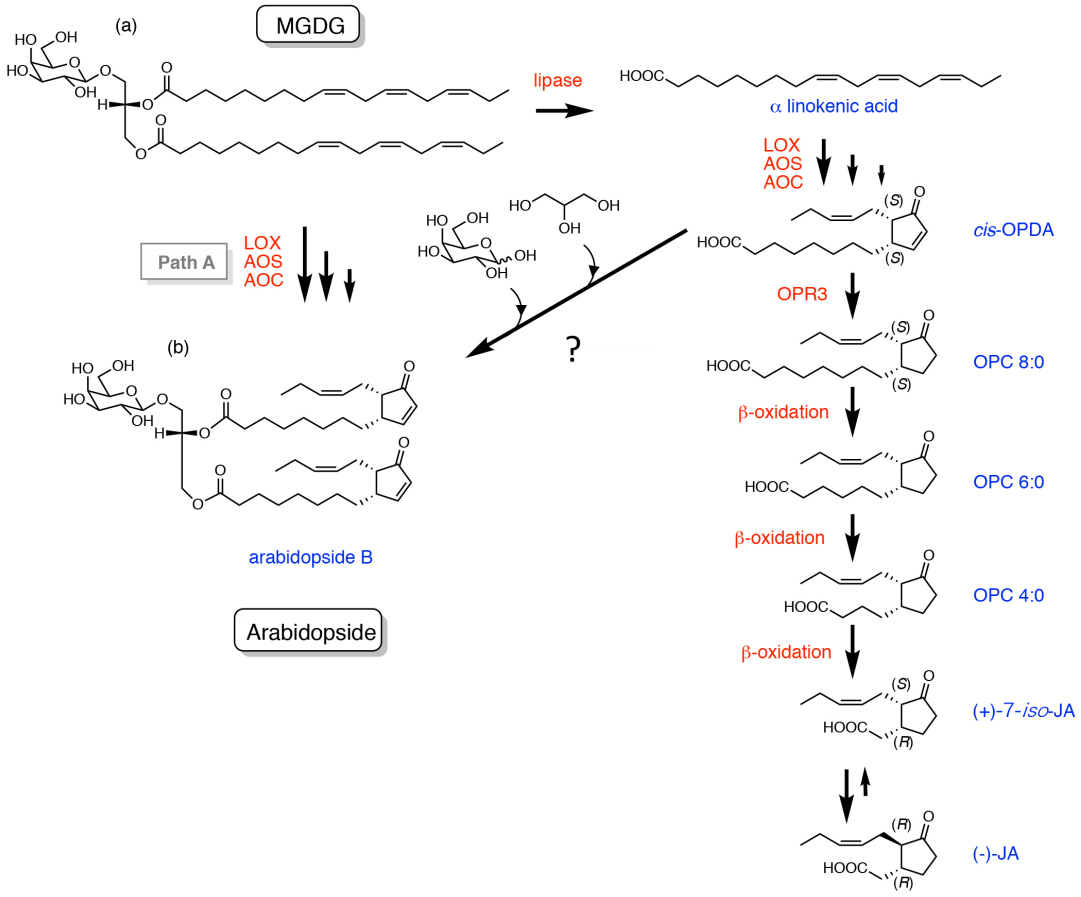

Figure 5. Biosynthetic pathway to afford arabidopside and jasmonic acid.
Chemical structures of a representative MGDG, *bis*-α linolenic acid form (a) and representative arabidoside, arabidoside B (b). *cis*-OPDA: *cis*-12-oxo-phytodienoic acid, OPC 8:0: 3-oxo-2-(2'-[*Z*]-pentenyl)-cyclopentane-1-octanoicacid, OPC 6:0; 3-oxo-2-(2'-[*Z*]-pentenyl)-cyclopentane-1-hexanoic acid, OPC 4:0: 3-oxo-2-(2'-[*Z*]-pentenyl)-cyclopentane-1-butanoicacid.
LOX: lipoxygenase, AOS: alene oxide synthase, AOC: alene oxide cyclase, OPR3: *cis*-12-oxophytodienoate reductase 3, JA: jasmonic acid.

In the present study, the enzymatic method for the synthesis of OPDA developed by Kajiwara et al. [3] was found to be applicable to synthesize OPDA analogs such as

OPDA-amino acid conjugates, (7*R*,11*S*)-dn-*cis*-OPDA, and OPDA-monoglyceride. These results indicated that there was loose substrate recognition of the carboxylic acid moiety of fatty acids by the biosynthetic enzymes. As mentioned above, *A. thaliana* synthesizes a series of unique compounds, arabidopsides A-E. Arabidopside A promotes senescence of barley leaves (*Hordeum vulgare*) [12], and arabidopsides A, B, and D inhibit the elongation of roots [13]. Anderson et al. [14] reported that arabidopside E showed an inhibitory effect on the growth of *Pseudomonas syringae in vitro*. The proteins AvrRpm1 and AvrRpt2 are attenuated proteins derived from *P. syringae*, and it has been reported that arabidopside E accumulation occurs when these two proteins are recognized by *Arabidopsis thaliana* [15]. However, the biosynthetic pathway that produces these compounds have not been completely uncovered. Anders et al. [10] reported that fatty acids could remain attached to galactolipids during the enzymatic conversion to (dn) OPDA, and our present report supports this experimental result, in which the conversion of MGDG into arabidopsides proceeds while the fatty acids are bound to the galactolipids. However, in order to give a true picture of the biosynthesis of arabidopsides, further studies are needed.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/**********

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Figure Legends

Figure 1. Chemical structures of the LA and OPDA amino acid conjugates.

Figure 2. Enzymatic conversion to give (7R,11S)-dn-*cis*-OPDA.

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Highlights

1) OPDA amino conjugates were synthesized using an in vitro enzymatic reaction.

2) (7*R*,11*S*)-dinor-*cis*-OPDA was synthesized using an in vitro enzymatic reaction.

3) OPDA monoglyceride was synthesized using an in vitro enzymatic reaction

4) It was found a fatty acid remained attached to the alcohol during the conversion.