Airborne Methyl Jasmonate is Metabolized to Jasmonic Acid and 12-Hydroxyjasmonic Acid, and Induces Jasmonate Biosynthesis in *Marchantia polymorpha*

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Abstract: Methyl jasmonate (MeJA) is a volatile jasmonate compound commonly used to induce defense responses in spermatophytes. This study reports that airborne MeJA-d_3, deuterated MeJA, increases the levels of (dinor-)12-oxo-phytodienoic acids [(dn-)OPDAs] and jasmonic acid (JA) as well as JA-d_3 and 12-hydroxyjasmonic acid-d_2 (12-OH-JA-d_2), MeJA-d_3 metabolites, in the model bryophyte *Marchantia polymorpha*. Enhancement of JA biosynthesis was substantiated by the expression of JA biosynthetic genes induced by airborne MeJA. Additionally, each of enantiomers, (+)-MeJA and (−)-MeJA, was observed to induce the accumulation of JA and (dn-)OPDAs in *M. polymorpha*. This study demonstrates that airborne MeJA is metabolized to JA and 12-OH-JA, and induces JA biosynthesis in *M. polymorpha*. Moreover, the transient increase in endogenous JA level after airborne MeJA treatment provides concrete evidence that *M. polymorpha* biosynthesizes JA.

Introduction

Jasmonate is a lipid-derived plant hormone that regulates development and defense responses in plants. The biosynthetic pathway toward (+)-7-*iso*-jasmonic acid (7-*iso*-JA,**1a**) in Arabidopsis (*Arabidopsis thaliana*) has been largely clarified (Figure 1). [1,2] The biosynthesis starts with the release of α-linolenic acid (**2**) from the chloroplast membranes. Oxygenation of α-linolenic acid (**2**) by lipoxygenase (LOX) and subsequent dehydration-cyclization by allene oxide synthase (AOS) and allene oxide cyclase (AOC) produce *cis*-12-oxo-phytodienoic acid (*cis*-OPDA, **3**), which is a key intermediate in jasmonate biosynthesis. Reduction by OPDA-reductase 3 (OPR3) and three β-oxidation cycles produce (+)-7-*iso*-JA (**1a**) from *cis*-OPDA (**3**). (+)-7-*iso*-JA (**1a**) is also biosynthesized from hexadecatrienoic acid (**4**) via dinor-*cis*-OPDA (dn-*cis*-OPDA, **5**) in a pathway parallel to that from α-linolenic acid (**2**) via *cis*-OPDA (**3**). The conjugation of (+)-7-*iso*-JA (**1a**) with L-isoleucine by Jasmonate-amido synthase (JASMONATE RESISTANT1: JAR1) produces (+)-7-*iso*-jasmonoyl-L-isoleucine [(+)-7-*iso*-JA-Ile, **6a**]. (+)-7-*iso*-JA-Ile (**6a**), but not (+)-7-*iso*-JA (**1a**), triggers the interaction of JASMONATE ZIM-domain proteins (JAZ) and F-box protein CORONATINE INSENSITIVE1 (COI1) in Arabidopsis, which is crucial for jasmonate responses such as the induction of genes related to defense response.[1] (+)-7-*iso*-JA (**1a**) and (+)-7-*iso*-JA-Ile (**6a**) were isomerized to (−)-JA (**1b**) and (−)-JA-Ile (**6b**), respectively, via keto-enol isomerization (Figure 2). (−)-JA-Ile (**6b**) has less ability to induce the interaction of COI1-JAZ than (+)-7-*iso*-JA-Ile (**6a**).[3]

Phylogenetic analysis indicates that most of the genes responsible for JA biosynthesis and signaling were conserved in the model bryophyte *Marchantia polymorpha*, but two biosynthetic genes, *JAR1* and *OPR3*, were absent in the bryophyte.[4,5] In accordance with deletion of the genes, JA-Ile (**6**) was below the detection limit in *M. polymorpha*. The presence of JA (**1**) is open to argument; Yamamoto et al. reported that JA (**1**) was not detected,[4] but Monte et al. reported that only near detection level of JA (**1**) was detected in *M. polymorpha*.[5] The key components of the COI1-dependent signaling pathway in *M. polymorpha* are functionally conserved, but the notable difference between Arabidopsis and *M. polymorpha* is the ligands of the each signaling; (+)-7-*iso*-JA-Ile (**6a**) is a ligand in *Arabidopsis* AtCOI1, but dn-*cis*-OPDA (**5**) and dn-*iso*-OPDA (**7**) are ligands in *Marchantia* MpCOI1.[5]

Methyl 7-*iso*-jasmonate (7-*iso*-MeJA, **8a**), the methyl ester of 7-*iso*-JA (**1a**), is presumed to play a role as an interplant communication compound. The metabolism of airborne MeJA (**8**) was traced using deuterated compounds in *Achyranthes bidentata* and tomato (*Solanum lycoperisicum*).[6,7] Deuterated JA-Ile (**6'**) and deuterated jasmonoyl-L-leucine (**9'**) were identified after treatment with deuterated MeJA (**8'**) in *A. bidentata*, and deuterated JA-Ile (**6'**), deuterated jasmonoyl-L-valine (JA-Val,**10'**), deuterated 12-hydroxy-JA (12-OH-JA, **11'**), and deuterated 12-glucosyloxy-JA (**12'**) were identified after treatment with deuterated MeJA (**8'**) in tomato.

MeJA (**8**) has been identified as a candidate for volatile interplant signaling in angiosperms. However, the mechanism underlying airborne MeJA (**8**) metabolization and whether airborne MeJA (**8**) is perceived in bryophytes is unknown. In this study, we observed the accumulation of JA, its biosynthetic precursors, *cis*-OPDA (**3**), dn-*iso*-OPDA (**7**), and dn-*cis*-OPDA (**5**), and deuterated JA (**1'**) and deuterated 12-OH-JA (**11'**) in *M. polymorpha* after airborne treatment with deuterated MeJA (**8**). It was also confirmed that airborne MeJA (**8**) induced the transcription of JA biosynthetic genes *MpAOC* and *MpAOSs*.

Results and Discussion

Authentic standards that are indispensable for the analysis of endogenous JA and (dn-)OPDAs. dn-*iso*-OPDA (**7**) was synthesized as shown in Scheme S1 and Supplemental Material and Methods. The synthesized dn-*iso*-OPDA (**7**) and the plant extracts were analyzed using UPLC-MS/MS. The authentic standards of dn-*cis*-OPDA (**5**) and dn-*iso*-OPDA (**7**) were completely separated using UPLC, and both compounds were observed in the *M. polymorpha* plant extract with retention times identical to those of authentic standards (Figure S1).

Four thalli of *M. polymorpha* were grown on an agar medium. A piece of filter paper containing deuterium-labeled (±)-MeJA, (±)-MeJA-d_3 (**8'**), was placed on the center of the plates, and the plates were placed in long shank petri dishes without direct contact with the medium (Figure S2). (±)-MeJA-d_3 (**8'**) was used to determine whether airborne (±)-MeJA (**8**) treatment induced JA biosynthesis. The compounds were extracted from the treated plants after 24, 48, and 72 h, and the endogenous JA (**1**), *cis*-OPDA (**3**), dn-*cis*-OPDA (**5**), dn-*iso*-OPDA (**7**), JA-d_3 (**1'**), and 12-OH-JA-d_2 (**11'**) levels were analyzed using UPLC-MS/MS (Figure 3). The maximum JA-d_3 level was 140 nmol/gFW 48 h after treatment (Figure 3B). 12-OH-JA-d_2 (**11'**) steadily increased up to 72 h, and the endogenous level at 72 h after treatment was 0.73 nmol/gFW (Figure 3C), although non-labeled 12-OH-JA (**11**) was below the detection limit. These results indicate that airborne (±)-MeJA-d_3 was hydrolyzed to JA-d_3 (**1'**) and subsequently hydroxylated to 12-OH-JA-d_2 (**11'**). Additionally, the increase in non-labeled JA (**1**) after (±)-MeJA-d_3 (**8'**) treatment suggested that (±)-MeJA-d_3 (**8'**) induced endogenous JA biosynthesis (Figure 3D). As explained in the Introduction, whether *M. polymorpha* produces JA is unclear (**1**).[4,5] The increase in JA (**1**) after MeJA-d_3 (**8'**) treatment in this study is indicative of the presence of JA (**1**) in *M. polymorpha*. Chini et al. reported an OPR3-independent pathway as a minor JA biosynthetic pathway in Arabidopsis, and that OPR2, which catalyzes the reduction of 4,5-didehydro-JA, is a key enzyme in this pathway.[8] Conservation of the *OPR2* gene in *M. polymorpha* genome suggests that JA (**1**) might be biosynthesized via 4,5-didehydro-JA in *M. polymorpha.* The maximum JA (**1**) content was 0.037 nmol/g FW, which was much lower than that of JA-d_3 (**1'**) (140 nmol/g FW), indicating that most of the accumulated JA (**1**) after the treatment was derived from the hydrolysis of the airborne (±)-MeJA (**8**). *cis*-OPDA (**3**), dn-*cis*-OPDA (**3**), and dn-*iso*-OPDA (**7**) accumulated after (±)-MeJA-d_3 (**8'**) treatment (Figures 3E–G). The maximum *cis*-OPDA (**3**) and dn-*iso*-OPDA (**7**) levels were 1.3 and 11 nmol/gFW, respectively, at 48 h after treatment. dn-*cis*-OPDA (**5**) steadily increased to 0.95 nmol/gFW for 72 h after treatment.

To substantiate the enhancement of JA biosynthesis by airborne (±)-MeJA (**8**), the transcriptional levels of JA biosynthetic genes were analyzed using RT-qPCR. The *M. polymorpha* plants were treated with airborne (±)-MeJA (**8**) as described earlier, and the total RNA was extracted after 48 h. Following reverse transcription, the transcriptional levels of *MpAOC*, *MpAOS1*, and *MpAOS2* were analyzed using quantitative PCR. Expression of *MpAOC*, *MpAOS1*, and *MpAOS2* genes were significantly induced by airborne (±)-MeJA (**8**) (Figure 4). The result demonstrated the enhancement of endogenous JA biosynthetic pathway by airborne (±)-MeJA (**8**). The induction of JA biosynthetic genes by airborne (±)-MeJA (**8**) indicates that (±)-MeJA (**8**) or its metabolites are recognized by unknown receptor other than MpCOI1, whose specific ligands are dn-*iso*-OPDA (**7**) and dn-*cis*-OPDA (**5**).

To confirm the stereochemical requirements for a biological response in *M. polymorpha*, (−)-MeJA (**8b**) and (+)-MeJA (**8c**) were treated as described earlier. Both (−)-MeJA (**8b**) and (+)-MeJA (**8c**) induced accumulation of JA (**1**), *cis*-OPDA (**4**), dn-*cis*-OPDA (**5**), and dn-*iso*-OPDA (**7**) (Figure 5). The JA (**1**) level after treatment with (−)-MeJA (**8b**) was significantly higher than that after treatment with (+)-MeJA (**8c**). It is possible that (−)-MeJA (**8b**), which possesses natural stereochemistry, was more efficiently absorbed and/or hydrolyzed than (+)-MeJA (**8c**). In addition, the dn-*iso*-OPDA (**7**) level after treatment with (−)-MeJA (**8b**) was significantly higher than that after treatment with (+)-MeJA (**8c**).

Conclusion

This study indicates that airborne MeJA (**8**) is hydrolyzed to JA (**1**) and subsequently hydroxylated to 12-OH-JA (**11**) after absorption in *M. polymorpha*. We also elucidated that MeJA (**8**) and/or its metabolite(s) are recognized in *M. polymorpha*, and these compounds induce the endogenous JA biosynthesis. The significant transient increase in endogenous JA (**1**) is clearly indicative of the existence of JA (**1**) in *M. polymorpha*. Further experiments such as the identification of receptor for MeJA (**8**) and/or its metabolite(s) are required, but our results indicate the physiological role of MeJA (**8**) as volatile interplant signals.

Experimental Section

Chemicals and general procedures. (±)-MeJA (**8**) was provided by ZEON Corporation (Tokyo, Japan). (−)-MeJA (**8b**) and (+)-MeJA (**8c**) were prepared using the procedure described by Miyamoto et al.[9]

Plant material and growth condition. The male accession of *M. polymorpha*, Takaragaike-1 (Tak-1), was asexually maintained.[10] *M. polymorpha* plants were grown on 1/2 Gamborg's B5 medium containing 1.4% agar in a growth chamber (day cycle, 16 h light and 8 h dark; temperature, 22 °C).

Analysis of the internal contents of oxylipins after airborne MeJA treatment. The agar plates on which the four Tak-1 plants were grown were transferred to a long shank petri dish (ϕ 11 cm x 9 cm). MeJA-d_3 was dissolved

in MeOH (1 mg/mL, 336 μL) and transferred to a paper disk (ϕ 21 mm). After MeOH was evaporation from the paper disk, it was placed on vial caps (ϕ 15 cm x 9 mm) in the center of the agar plate. The treated thalli (ca. 300 mgFW) were frozen using liquid nitrogen, crushed, and subjected to extraction with EtOH (10 mL). Internal standards (JA-d_6, 100 ng; TA-d_5, 100 ng; OPDA-$U^{13}C$, 200 ng) were added to the filtered extract. The volatile components of the extract were removed under reduced pressure, and the residue was dissolved in 80% MeOH (2 mL). The solution was applied to a Bond Elut C18 cartridge column (Agilent Technologies, Santa Clara, CA, USA), which was successively eluted with a solution of 80% MeOH (2 mL × 2). After evaporation of the volatile components of the eluate (6 mL), the remaining residue was dissolved in 80% MeOH (300 μL), and a portion of the mixture (5 μL) was subjected to UPLC MS/MS. The dn-*cis*-OPDA (**5**) and dn-*iso*-OPDA (**7**) levels were quantified using calibration curves with OPDA-$U^{13}C$ as an internal standard. The UPLC and mass spectrometry conditions are listed in Tables S2 and S3, respectively.

Expression analysis of the genes of JA biosynthetic genes. Tak-1 plants were treated with airborne MeJA (**8**) for 72 h using the method described in the previous section. Total RNA was extracted and purified from the thali using ISOSPIN Plant RNA (Nippon Gene, Tokyo, Japan). The first strand of cDNA was synthesized using M-MLV Reverse Transcriptase (Nippon Gene) with oligo (dT) primers. The Thermal Cycler Dice Real Time System (Takara Bio, Shiga, Japan) was used to perform RT-qPCR using the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan). The *elongation factor 1α* (*EF1α*, Mp3g23400) gene was used as an internal control to normalize the data. The thermal cycling conditions were 95 °C for 5 s, followed by 40 cycles of 95 °C for 5 s and annealing for 30 s at the temperatures shown in Table S1. The primer sequences are listed in Table S1.

Supporting Information Summary

Supporting information includes the analytical condition and chromatogram of UPLC MS/MS (Supplemental Material and Methods, Tables S2-3, Figure S1), the synthetic methods for dn-*iso*-OPDA (**7**) (Supplemental Material and Methods, Scheme S1), the primer sequences (Table S1), the picture showing airborne MeJA treatment (Figure S2), and the MS and NMR spectra of dn-*iso*-OPDA (**7**) (Figures S3-8).

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Keywords: Biosynthesis • *Marchantia polymorpha* • Methyl jasmonate • Phytochemistry • Plant hormone

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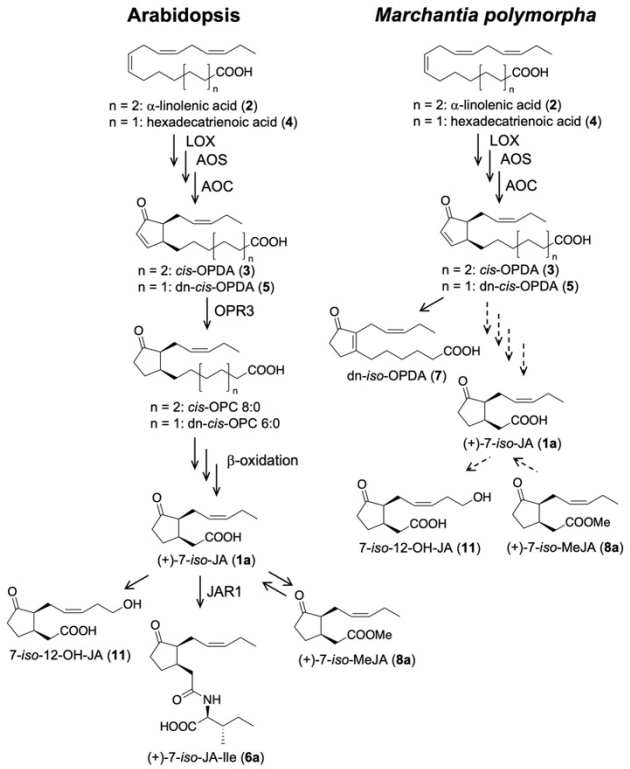

Figure 1. Proposed biosynthetic pathway of jasmonates in Arabidopsis and *M. polymorpha*. Dotted arrows indicate the reaction steps proposed in this study.

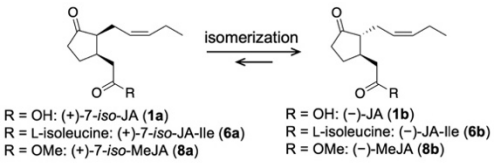

Figure 2. Isomerization of (+)-7-*iso*-JA (**1a**), (+)-7-*iso*-JA-Ile (**6a**), and (+)-7-*iso*-MeJA (**8a**).

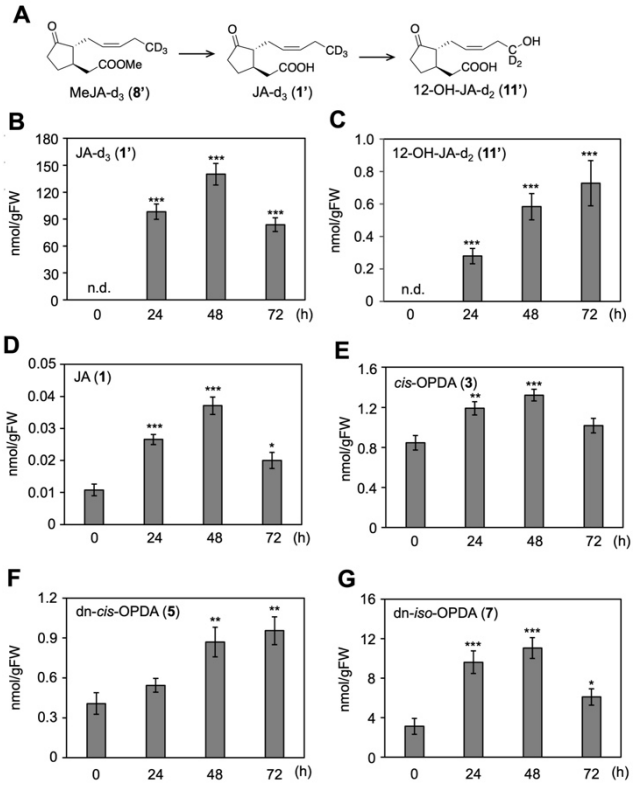

Figure 3. Endogenous amounts of the labeled and non-labeled JA-related compounds in *M. polymorpha* after treatment with airborne (±)-MeJA-d_3 treatment. (A) The chemical structures of the treated (±)-MeJA-d_3 (**8'**) and its metabolites, JA-d_3 (**1'**) and 12-OH-JA-d_2 (**11'**). Endogenous amounts of JA-d_3 (**1'**) (B), 12-OH-JA-d_2 (**11'**) (C), JA (**1**) (D), *cis*-OPDA (**3**) (E), dn-*cis*-OPDA (**5**) (F), and dn-*iso*-OPDA (**7**) (G) in the airborne (±)-MeJA-d_3 (**8'**) treated *M. polymorpha*. Asterisks denote significant difference between 0 h and the time points 24, 48, and 72 h at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***); Student's *t* test. Each data point represents the mean ±S.E. of eight biological replicates. n.d.: not detected.

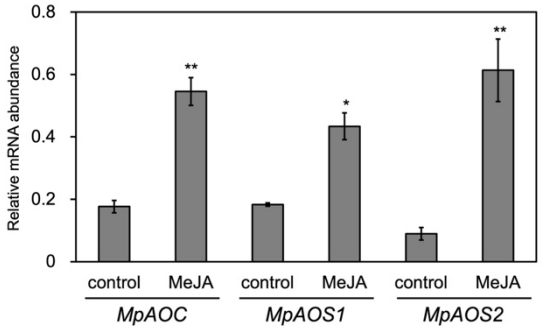

Figure 4. Relative transcript abundance of the JA biosynthetic genes in *M. polymorpha* after treatment with the airborne (±)-MeJA (**8**). Transcriptional levels of *MpAOC* (Mp7g06220), *MpAOS1* (Mp3g21350), and *MpAOS2* (Mp5g16260) after the airborne MeJA treatment were analyzed using RT-qPCR. Asterisks denote significant difference between control and (±)-MeJA (**8**) treatment at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***); Student's *t* test. Each data point represents the mean ±S.E. of six biological replicates.

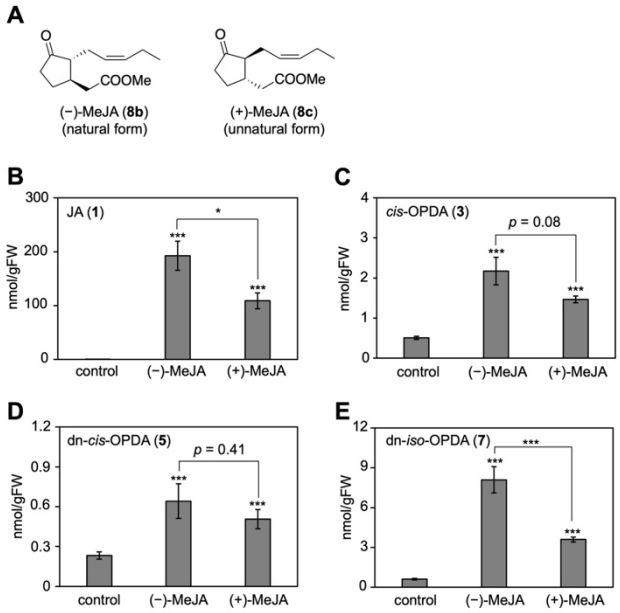

Figure 5. Endogenous amounts of JA (**1**) and its biosynthetic precursors (**3**, **5**, and **7**) in *M. polymorpha* after treatment with (−)-MeJA (**8b**) and (+)-MeJA (**8c**). (A) The chemical structures of the treated (−)-MeJA (**8b**) and (+)-MeJA (**8c**). The endogenous amounts of JA (**1**) (B), *cis*-OPDA (**3**) (C), dn-*cis*-OPDA (**5**) (D), and dn-*iso*-OPDA (**7**) (E) in *M. polymorpha* plants treated with the airborne (−)-MeJA (**8b**) and (+)-MeJA (**8c**) were analyzed using UPLC-MS/MS. Asterisks above graphs denote significant difference between control and each treatment at $p < 0.01$ (*) or $p < 0.001$ (**); Student's *t* test. Asterisks or *p* values above lines between graphs of (−)-MeJA (**8b**) and (+)-MeJA (**8c**) denote significant difference between (−)-MeJA (**8b**) and (+)-MeJA (**8c**) treatment at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***); Student's *t* test. Each data point represents the mean ±S.E. of eight biological replicates.