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Identification of JA in *Selaginella moellendorffii*

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Identification of jasmonic acid and jasmonoyl-isoleucine and the characterization of AOS, AOC, OPR and JAR1 in the model lycophyte Selaginella moellendorfii

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AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; 12,13-EOT, (9Z,15Z)-(13S)-12,13-epoxyoctadeca-9,11,15-trienoic acid; GC-MS, gas chromatography-mass spectrometry; 13-HPOT, 13-hydroperoxyoctadecatrienoic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; JAR1, jasmonic acid resistant 1; JAZ, jasmonate-zim domain; LOX, lipoxygenase; Ni-NTA, nickel-nitrilotriacetic acid; OPC-8:0, 3-oxo-2-(cis-2’-pentenyl)cyclopentane-1-octanoic acid; OPDA, 12-oxo-phytodienoic acid; OPR, 12-oxo-phytodienoic acid reductase; SCF, skp-cullin-F box; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry
Abstract

Jasmonic acid (JA) is involved in a variety of physiological responses in seed plants. However, the detection and role of JA in lycophytes, a group of seedless vascular plants, have remained elusive until recently. This study provides the first evidence of 12-oxo-phytodienoic acid (OPDA), JA, and jasmonoyl-isoleucine (JA-Ile) in the model lycophyte Selaginella moellendorfii. Mechanical wounding stimulated the accumulation of OPDA, JA, and JA-Ile. These data were corroborated by the detection of enzymatically active allene oxide synthase (AOS), allene oxide cyclase (AOC), 12-oxo-phytodienoic acid reductase 3 (OPR3), and JA-Ile synthase (JAR1) in S. moellendorfii. SmAOS2 is involved in the first committed step of JA biosynthesis. SmAOC1 is a crucial enzyme for generating the basic structure of jasmonates and is actively involved in the formation of OPDA. SmOPR5, a functionally active OPR3-like enzyme, is also vital for the reduction of (+)-cis-OPDA, the only isomer of the JA precursor. The conjugation of JA to Ile by SmJAR1 demonstrates that S. moellendorfii produces JA-Ile. Thus, the four active enzymes have characteristics similar to those in seed plants. Wounding and JA treatment induced the expression of SmAOC1 and SmOPR5. Furthermore, JA inhibited the growth of shoots in S. moellendorfii, which suggests that JA functions as a signaling molecule in S. moellendorfii. This study proposes that JA evolved as a plant hormone for stress adaptation, beginning with the emergence of vascular plants.

Keywords

AOS; AOC; JAR1; Jasmonic acid; OPR3; Selaginella moellendorfii
**Introduction**

As sessile organisms, plants must adapt to many environmental stresses that can negatively affect their development, growth, productivity, and fertility by altering their physiological, morphological, and/or developmental processes. Many studies have found that JA modulates numerous plant physiological processes that are related to development and defense responses (Wasternack and Parthier 1997, Ziegler et al. 2000, Ishiguro et al. 2001, Wasternack 2007).

Indeed, JA is a naturally occurring phytohormone that is ubiquitous in seed plant species (Creelman and Mullet 1997).

The JA biosynthetic pathway (Fig. 1) starts with the lipase-mediated release of α-linolenic acid from the membrane lipids of chloroplasts (Wasternack and Hause 2013), which is triggered by abiotic and biotic stresses (Farmer and Ryan 1992, Mueller et al. 1993, Conconi et al. 1996, Penninckx et al. 1996, Narváez-Vásquez et al. 1999). In chloroplasts, α-linolenic acid is converted into 13(S)-hydroperoxyoctadecatrienoic acid [13(S)-HPOT]; this process is mediated by 13-lipoxygenase (13-LOX). The 13(S)-HPOT intermediate can be dehydrated through the action of allene oxide synthase (AOS), which leads to JA biosynthesis (Yan et al. 2013). In the presence of AOS, the dehydration of the resultant fatty acid hydroperoxide forms an allene oxide (12,13-EOT), which is then cyclized by allene oxide cyclase (AOC) into cis-(+)-12-oxo-phytodienoic acid [(9S,13S)-OPDA]. The specificity of AOC determines the configuration of the side chains in the naturally occurring structure of jasmonates. The resulting OPDA is then transferred into peroxisomes, where OPDA reductase 3 (OPR3) reduces cis-(+)-OPDA to 3-oxo-2-(cis-2’-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0), which is then converted into (+)-7-iso-JA through three β-oxidation steps. Subsequently, (+)-7-iso-JA is isomerized to (−)-JA and released into the cytoplasm via an unknown mechanism (Ziegler et al. 2000, Hyun et al. 2008,
Khan et al. 2012). JA can be enzymatically converted into many derivatives, such as methyl jasmonate and JA-amino acid conjugates. The jasmonic acid resistant 1 (JAR1) gene encodes the enzyme that is responsible for the conjugation of JA to amino acids, i.e., isoleucine and valine. Among these conjugates, JA-Ile is considered to be an important compound in the JA signaling pathway (Stenzel et al. 2012). Under stress conditions, the JA-Ile level increases, and JA-Ile binds to its receptor, COI1, a component of the SCF complex. Subsequently, JA-Ile mediates the initial binding of the JAZ protein to the COI1-JA-Ile unit of the SCF complex, which results in degradation by the 26S proteasome and the release of MYC2 (Fonseca et al. 2009a, 2009b, Sheard et al. 2010, Santino et al. 2013).

Vascular plants have survived on earth for the past 420 million years and have diverged into several descendants. Only two descendants have survived: the euphyllophytes and lycophytes (Kenrick and Crane 1997, Banks et al. 2011). Fossil evidence shows that lycophytes diverged shortly after land plants evolved vascular tissues, which generated all other vascular plants. There are currently only three extant lycophyte groups: the Lycopodiales (club mosses), Isoetales (quillworts), and Selaginellales (spike mosses) (Weng et al. 2005, Banks 2009, Hecht et al. 2011). Recently, the approximately 100 Mbp genome of Selaginella moellendorffii was fully sequenced (Banks et al. 2011). This model lycophyte, as a representative of the earliest and still surviving vascular plant lineage, can potentially provide information for deciphering the evolution of the physiological, biochemical, and developmental processes that are unique to land plants.

The model plants that transitioned from water to land, such as the moss Physcomitrella patens and the liverwort Marchantia polymorpha, produce OPDA but not JA (Stumpe et al. 2010, Yamamoto et al. 2015). These findings strongly suggest that the first half of the octadecanoid
pathway in chloroplasts remains in bryophytes. Thereafter, the difference in JA production between seed plants and bryophytes is one of the significant observations in the study of plant evolution. The lycophytes are intermediate plants between bryophytes and seed plants, and this ancient lineage diverged shortly after land plants evolved vascular tissues. Thus, the origin of JA is of interest for studying plant evolution. In this study, we analyzed the lycophyte model S. moellendorfii to determine the presence of OPDA, JA, and JA-Ile, as well as the enzymatic activities of AOS, AOC, OPR3, and JAR1, which are involved in the biosynthesis of jasmonates. Moreover, we showed the growth inhibitory activity of OPDA, JA, and JA-Ile in S. moellendorfii. These data indicate a possible role for JA as a signaling molecule that regulates growth and response to wounding in S. moellendorfii.

Results

Identification of OPDA, JA, and JA-Ile in S. moellendorfii

Seed plants can biosynthesize JA and JA-Ile; however, these compounds are absent in the model bryophytes P. patens and M. polymorpha (Stumpe et al. 2010, Yamamoto et al. 2015). In this study, we evaluated S. moellendorfii, which belongs to a lycophyte group that is taxonomically positioned between bryophytes and euphyllophytes, to identify JA and its related compounds. We detected OPDA, JA, and JA-Ile in S. moellendorfii using ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) analysis (Fig. 2). The OPDA analytical data revealed a predominant peak in the chromatogram of an S. moellendorfii extract (m/z 164.99 derived from the peak at m/z 291.37 [M–H]−; Figs. 2A, 2B, and 2C). This peak overlapped with the cis-OPDA standard peak, which indicates that OPDA is present in S. moellendorfii. The minor peak that eluted before cis-OPDA was speculated to be either an
unidentified OPDA-related compound or a *trans*-isomer of OPDA (Fig. 2B). The analytical JA data clearly demonstrated that the standard JA peak overlapped with the peak derived from an extract of *S. moellendorffii* (*m/z* 58.71 derived from the peak at *m/z* 209.09 [M–H]−; Figs. 2D, 2E, and 2F). This result indicated the presence of JA in *S. moellendorffii*. Furthermore, an analysis of JA-Ile showed that the retention time of standard JA-Ile was the same as that of the peak derived from an extract of *S. moellendorffii* (*m/z* 129.68 derived from the peak at *m/z* 323.03 [M–H]−; Figs. 2G, 2H, and 2I), suggesting that *S. moellendorffii* also biosynthesizes JA-Ile. The minor peak that was detected in the extract is potentially an unidentified contaminant that was not further analyzed (Fig. 2H). These data are the first evidence of OPDA, JA, and JA-Ile in *S. moellendorffii*.

**Accumulation of OPDA, JA, and JA-Ile upon wounding stress**

JA plays important roles in stress adaptation. Therefore, the accumulation of JA is considered a response to adverse environmental conditions involving both biotic and abiotic stresses, especially in seed plants (Wasternack and Hause 2013). Wounding activates the octadecanoid pathway, which results in the accumulation of OPDA, JA, and JA-Ile in seed plants. To illustrate the response of *S. moellendorffii* to wounding stress, the photosynthesizing organs (stems and microphylls) were mechanically wounded and then analyzed at different time points. The endogenous levels of OPDA, JA, and JA-Ile were analyzed using UPLC-MS/MS (Fig. 3). *S. moellendorffii* transiently produced a high level of OPDA within the first 10 min after wounding. OPDA was approximately 200-fold more abundant than JA at 10 min after wounding, whereas the JA level peaked at 30 min, following the initial increase at 10 min. In contrast to the rapid increase in the OPDA and JA concentrations, JA-Ile accumulation was delayed and started at 180
min after wounding. The increased levels of JA and JA-Ile were accompanied by a rapid decrease in the OPDA concentration. These data revealed that wounding increased the endogenous levels of these jasmonates in *S. moellendorffii*, similar to the response in seed plants. Accordingly, environmental stress probably activates the octadecanoid pathway. OPDA, JA, and JA-Ile also seem to play a role in regulating the response to wounding in *S. moellendorffii*.

**Putative AOS, AOC, OPR3 and JAR1 genes in *S. moellendorffii***

To further confirm the presence of OPDA, JA, and JA-Ile in *S. moellendorffii*, we investigated the characteristics of important JA biosynthetic enzymes in *S. moellendorffii*. A genomic database analysis using Phytozome v 11.0 (https://phytozome.jgi.doe.gov/pz/portal.html) was performed to screen AOS, AOC, OPR3, and JAR1 homologous genes in *S. moellendorffii* using Arabidopsis amino acid sequences corresponding to these proteins as queries. As a result, the presence of putative AOS (*SmAOS1*, *Sm_271334; SmAOS2*, Sm_177201; *SmAOS3*, Sm_228572), AOC (*SmAOC1*, Sm_91887), OPR3 (*SmOPR1*, Sm_270843; *SmOPR5*, Sm_111662), and JAR1 (*SmJAR1*, Sm_110439) genes was predicted in *S. moellendorffii* (Supplementary Table S1).

**Functional analysis of SmAOS2**

The first committed step of JA biosynthesis is catalyzed by AOS, which converts 13-HPOT into unstable 12,13-EOT. A genomic database analysis of Phytozome v 11.0 revealed the presence of nine putative AOS genes in *S. moellendorffii*. Amino acid sequence analysis of the SmAOS candidates showed that SmAOS1, SmAOS2, and SmAOS3 were promising candidates containing the important sequence motifs: the I-helix GXXX (F/L), EXLR motif, and heme-
binding PXVXNKQCPG, which are characteristic of members of the CYP74 family (Supplementary Fig. S1) (Koeduka et al. 2015). Additionally, the amino acid sequences of SmAOS1, SmAOS2, and SmAOS3 are quite similar (Supplementary Fig. S1). A phylogenetic tree of the AOSs showed that the SmAOSs were separated from the seed plant AOSs (Supplementary Fig. S2). Computational analysis using ChloroP v 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and iPSORT (http://ipsort.hgc.jp/) for subcellular localization predicted the absence of a transit peptide in SmAOS1, SmAOS2, and SmAOS3, whereas most known AOSs are localized in chloroplasts (Schaller and Stintzi 2009).

To confirm AOS activity in this plant, the production of these recombinant SmAOSs was attempted. However, only recombinant SmAOS2 fused with a His-tag was successfully synthesized in E. coli. Purification of recombinant SmAOS2 by Ni-NTA affinity column chromatography and SDS-PAGE analysis revealed a clear band of SmAOS2 with an expected molecular weight of approximately 50 kDa (Supplementary Fig. S3). The AOS reaction converts 13(S)-HPOT to unstable 12,13-EOT, which is non-enzymatically changed to racemic OPDA. Therefore, AOS enzymatic activity can be determined by analyzing the presence of racemic OPDA. The recombinant SmAOS2 was incubated with 13(S)-HPOT, and then the products were extracted with ethyl acetate, followed by isomerization by alkaline treatment and methylation using ethereal diazomethane. Laudert et al. (1997) demonstrated that chiral GC-MS analysis of methylated OPDA can clearly separate the trans-isomers of methylated OPDA; therefore, isomerization was performed to convert cis-OPDA to trans-OPDA. Peaks of methylated (+)-trans-OPDA and methylated (−)-trans-OPDA were both identified in the SmAOS2 reaction products on the chiral GC-MS chromatogram (Fig. 4). Furthermore, an ion peak at m/z 306, corresponding to the molecular ion peak [M]+ of OPDA methyl ester, was observed together with
unique fragment ion peaks at \( m/z \) 275 ([M–OCH₃]+), \( m/z \) 238 ([M–C₅H₉]+), and \( m/z \) 149 ([M–C₆H₁₀O₂]+) in the GC-MS spectral data of both methylated (+)-trans-OPDA and methylated (-)-trans-OPDA (Supplementary Figs. S4S6, Laudert et al. 1997). These results showed that SmAOS2 had AOS activity similar to that of previously characterized AOSs.

Functional analysis of SmAOC1

AOC has been successfully cloned from seed plants, a moss, and a liverwort (Ziegler et al. 2000, Agrawal et al. 2003, Stenzel et al. 2003, Maucher et al. 2004, Farmaki et al. 2007, Pi et al. 2008, Stumpe et al. 2010, Hashimoto et al. 2011, Yamamoto et al. 2015). The 5’-UTR of SmAOC1 was examined by 5’-RACE to obtain the full-length sequence. A computational analysis using ChloroP v 1.1 and iPSORT predicted that SmAOC1 has a chloroplast transit peptide at its N-terminus. The amino acid sequence alignment of SmAOC1 and AOCs of other plants showed that SmAOC1 had high similarity to other AOCs (Supplementary Fig. S7). The phylogenetic tree of AOCs demonstrated that SmAOC1 was related to MpAOC from the liverwort *M. polymorpha* and to AOCs in seed plants, while it was separated from PpAOC1 and PpAOC2 of the moss *P. patens* (Supplementary Fig. S8).

To determine whether SmAOC1 is involved in the production of OPDA, *SmAOC1* was cloned and overexpressed in *E. coli*. The recombinant SmAOC1 was fused with a His-tag at the N-terminus to replace the chloroplast signal peptide. After purifying SmAOC1 using Ni-NTA affinity column chromatography, a protein with the expected size of 22 kDa was clearly detected as a single band by SDS-PAGE analysis (Supplementary Fig. S3). The purified recombinant SmAOC1 was used for enzymatic analysis.
Due to the instability of 12,13-EOT, which is the substrate for the AOC reaction, the enzymatic activity of SmAOC1 was tested in a reaction mixture containing SmAOC1, PpAOS1, and 13(S)-HPOT as substrates. For a negative control, only PpAOS1 and 13(S)-HPOT were used in a reaction mixture. After terminating the reaction, the resulting cis-OPDA product was converted to trans-OPDA by alkaline treatment, which was followed by methylation using ethereal diazomethane. Trans-OPDA was finally analyzed by chiral GC-MS. The molecular ion peak of the OPDA methyl ester at m/z 306 [M]⁺ was monitored to evaluate SmAOC1 activity (Laudert et al. 1997) (Fig. 5). The GC-MS analysis showed a predominant molecular ion peak (ca. 97%) in the reaction mixture containing SmAOC1. The retention time of this peak coincided with the standard trans-(+)-OPDA methyl ester, which represents cis-(+)-OPDA. In contrast, two molecular ion peaks of the racemic trans-OPDA methyl esters were detected in the product of the PpAOS1 reaction. This result demonstrated that SmAOC1 converts 12,13-EOT into cis-(+)-OPDA, similar to previously reported AOCs.

The first of three enzymes involved in JA biosynthesis (LOX, AOS, and AOC) have been reported to reside in chloroplasts (Schaller and Stintzi, 2009). To examine whether SmAOC1 is localized in chloroplasts, a 35S::SmAOC1-GFP plasmid was constructed and introduced into P. patens protoplasts and was then finally observed by confocal laser scanning microscopy. The green fluorescent signal of SmAOC1 fused with GFP was clearly identified by the red auto-fluorescence of chlorophyll. The overlay data also strongly supported that SmAOC1 is located in chloroplasts, which is similar to the other characterized AOCs (Supplementary Fig. S9). Accordingly, chloroplast could be the functional organelle in the biosynthesis of OPDA in S. moellendorffii.
Functional analysis of SmOPRs

OPRs are classified into two groups based on their ability to reduce OPDA: OPR3-like enzymes (e.g., Arabidopsis AtOPR3, tomato SIOPR3, and rice OsOPR7) and OPR1-like enzymes (e.g., AtOPR1 and SIOPR1) (Breithaupt et al. 2009). OPR3-like enzymes catalyze the reduction of cis-(+)-OPDA, a natural JA precursor, and of cis-(−)-OPDA, which is not involved in JA biosynthesis. The Arabidopsis opr3 mutant shows male sterility and is desensitized to responses involving JA signaling. In contrast, OPR1-like enzymes preferentially reduce cis-(−)-OPDA rather than cis-(+)‐OPDA; therefore, OPR1-like enzymes might function in pathways other than JA biosynthesis.

A genomic database search using Phytozome v 11.0 predicted the presence of six putative OPR genes in S. moellendorffii. All of the amino acid sequences of candidate SmOPRs were aligned with those of other known OPRs (Supplementary Fig. S10). Based on a comparison of two critical amino acids for substrate binding and a phylogenetic analysis of SmOPRs (Supplementary Fig. S10), two genes designated SmOPR1 and SmOPR5 were highly promising candidate genes that encoded OPR3-like enzymes involved in JA biosynthesis. SmOPR1 harbors two important active-site residues, Phe and His, as an OPR3 motif in the active site, which is considered to be necessary to reduce the JA precursor cis-OPDA. However, SmOPR1 was grouped with the other SmOPRs in an independent OPR cluster (Supplementary Fig. S11). In contrast, SmOPR5 was a member of the cluster of OPR3-like enzymes, such as AtOPR3, OsOPR7, SIOPR3, ZmOPR7, and ZmOPR8, which are active in JA biosynthesis (Supplementary Fig. S11). Moreover, SmOPR5 shared 53% amino acid sequence identity with AtOPR3. Nonetheless, instead of Phe and His, which are considered unique to the OPR3 substrate filter,
the active site residues of SmOPR5 were Trp and His. Therefore, these two genes were selected for further analysis to verify their enzymatic activity.

To confirm the enzymatic activities, recombinant SmOPR1 and SmOPR5 proteins fused with a His-tag were produced in E. coli. SDS-PAGE analysis showed that the recombinant SmOPR1 and SmOPR5 appeared as single bands with an expected molecular weight of approximately 40 kDa (Supplementary Fig. S3). The enzymatic reactions were conducted according to the method of Schaller et al. (1998) with some modifications. Recombinant SmOPR1 and SmOPR5 were incubated in 50 mM potassium phosphate buffer (pH 7.4) containing NADPH and cis-(±)-OPDA. After terminating the OPR reactions, the cis-isomers of OPDA and OPC-8:0 in the OPR reaction solutions were converted into trans-isomers of OPDA and OPC-8:0 by alkaline treatment. The reaction products were finally analyzed by chiral GC-MS after methylation (Vick and Zimmerman 1983, Laudert et al. 1997). In the GC-MS chromatogram of SmOPR1 reaction products, only the peak of methylated trans-(−)-OPC-8:0, which was derived from the non-JA precursor cis-(−)-OPC-8:0, appeared; there was no peak for methylated trans-(+) -OPC-8:0, which was derived from cis-(+) -OPDA, a natural JA biosynthetic intermediate (Fig. 6). Therefore, SmOPR1 did not participate in JA biosynthesis. In contrast, SmOPR5 catalyzed the reduction of both cis-(±)-OPDA enantiomers to both (±)-OPC-8:0 enantiomers (Fig. 6). The fragmentation of methylated trans-(+) -OPC-8:0 (Peak 1) derived from cis-(+) -OPDA was similar to that of standard methylated OPC-8:0 (Supplementary Figs. S12 and S13). These data indicated that SmOPR5 encodes a functional OPR3 that participates in JA biosynthesis in S. moellendorffii.

Enzymatic production of JA-Ile by SmJAR1
JA-Ile is a versatile signaling molecule for JA-mediated defense and developmental events and is produced by the conjugation of JA and Ile. JAR1s are GH3 proteins that have the ability to synthesize JA-Ile (Staswick and Tiryaki 2004). An Arabidopsis jar1 mutant, which is unable to produce JA-Ile, fails to trigger JA-mediated responses (Suza and Staswick 2008). Thus, JAR1 activity is necessary for JA signal transduction in seed plants. Here, we found 14 JAR1 homologous genes in the Phytozome database using AtJAR1 as a query (Supplementary Table S1). Multiple sequence alignment and phylogenetic analysis using Clustal Omega software were performed to show the relationship between these JAR1 homologous proteins in S. moellendorffii and the other existing JAR1 proteins (Supplementary Figs. S14 and S15). As a result, the candidate Sm_110439, which was designated SmJAR1, was most closely related to AtJAR1, OsJAR1 and OsJAR2, which can synthesize JA-Ile (Staswick and Tiryaki 2004, Wakuta et al. 2011). Moreover, SmJAR1 also has three short conserved motifs involved in ATP/AMP binding (Chang et al. 1997): SSGTSQGRPK (motif 1), YGSSE (motif 2), and YRLGD (motif 3) (Supplementary Fig. S14). To determine whether SmJAR1 encodes functional JA-Ile synthase in S. moellendorffii, SmJAR1 was isolated to check its enzymatic activity.

To evaluate JAR1 activity, E. coli expressing SmJAR1 were incubated with (-)-JA as a substrate. JA-Ile in the culture supernatant of E. coli was analyzed by UPLC-MS/MS. The analytical data clearly showed that SmJAR1 catalyzes the conjugation of JA to Ile using endogenous ATP and Ile in E. coli (Fig. 7). To investigate the substrate specificity of SmJAR1, the conjugation of JA to other amino acids was also analyzed. No peak derived from another conjugate of JA-Trp, JA-Phe, or JA-Val appeared in the culture supernatant of E. coli expressing SmJAR1 (Supplementary Fig. S16). Therefore, SmJAR1 was hypothesized to prefer Ile for conjugation with JA.
Expression of genes encoding key enzymes in JA-Ile biosynthesis under wounding stress and JA treatment

Wounding and JA induce the expression of a wide variety of genes in seed plants, such as defense-related genes, and genes encoding enzymes involved in JA biosynthesis. To determine if wounding and JA treatment affect gene expression in *S. moellendorffii*, quantitative RT-PCR analysis was performed to analyze the expression of *SmAOCl*, *SmOPR5*, and *SmJAR1*. Wounding increased the transcription levels of *SmAOCl*, *SmOPR5*, and *SmJAR1* (Fig. 8). The expression of *SmAOCl* was transiently induced at 10 min after wounding and then decreased until 180 min. The accumulation of *SmOPR5* mRNA reached a maximum 20 min after wounding and decreased thereafter. By contrast, only weak expression was observed for *SmJAR1* until 60 min, followed by an increase in *SmJAR1* expression at 180 min. These results indicate that the expression kinetics of *SmAOCl*, *SmOPR5*, and *SmJAR1* coincided with the accumulation profiles of OPDA, JA, and JA-Ile in wounded *S. moellendorffii*.

The analytical data on *SmAOCl* and *SmOPR5* expression in JA-treated *S. moellendorffii* revealed an initial increase in transcription levels in the first 30 and 10 min, respectively, after JA treatment (Fig. 9). The accumulation of *SmAOCl* continued to increase until 60 min after JA application and decreased thereafter, whereas the transcription level of *SmOPR5* decreased 30 min after treatment. The expression profiles of *SmAOCl* and *SmOPR5* in the plants treated with JA were similar to those in the wounded plants. In contrast to *SmAOCl* and *SmOPR5*, the expression of *SmJAR1* was suppressed within 10 min after JA treatment and remained at a low level thereafter.
Effect of exogenous OPDA, JA, and JA-Ile on the growth of S. moellendorffii shoots

Growth inhibition caused by JA is one of the most significant physiological responses in seed plants. To examine the ability of OPDA, JA, and JA-Ile to inhibit the growth of S. moellendorffii, bulbils of S. moellendorffii (Supplementary Fig. S17) were sprinkled onto soil, and the germinated plants were grown with or without treatment with OPDA, JA, or JA-Ile at concentrations of 25, 50, and 100 µM. After four weeks, the lengths of the shoots of S. moellendorffii were measured. All jasmonates tested in this study were shown to have growth inhibitory effects on shoots of S. moellendorffii (Fig. 10, supplementary Fig. 18). Both OPDA and JA inhibited growth in a dose-dependent manner, with a stronger effect with OPDA than with JA. JA-Ile also retarded the growth of the shoots of this plant, but the growth inhibitory effect did not increase with increasing concentrations of JA-Ile. This is the first report that OPDA, JA, and JA-Ile inhibit the growth of seedless vascular plants, such as lycophytes.

Discussion

Identification and accumulation of OPDA, JA, and JA-Ile

In this study, the presence of OPDA, JA, and JA-Ile in S. moellendorffii was successfully demonstrated (Fig. 2). Moreover, wounding transiently increased the endogenous concentrations of OPDA and JA in S. moellendorffii within 10 min (Fig. 3), which is probably due to the role of jasmonates in regulating responses to environmental stress in S. moellendorffii. Given that wounding also stimulated OPDA and JA in the fern Pteridium aquilinum (Radhika et al. 2012), the accumulation of JA in response to wounding is likely a common physiological response among all vascular plant species (Maucher et al. 2004). However, JA-Ile accumulated more slowly than OPDA and JA after wounding. It is possible that the early rise in JA levels within
min represents an early response, whereas the slow JA-Ile accumulation may be attributable to a late stress response in this plant.

The transient increases in JA and OPDA after wounding observed in S. moellendorfii are similar to those observed in seed plants. It has been suggested that another metabolic pathway is required to inactivate JA responses in S. moellendorfii. In Arabidopsis, the oxidation of JA-Ile, which decreases the JA-Ile concentration, is important for regulating JA activity (Kitaoka et al. 2011, Koo et al. 2011). A phylogenetic analysis of cytochrome P450s in A. thaliana, P. patens, and S. moellendorfii revealed that SmCYP94Js (SmCYP94J1, SmCYP94J2, SmCYP94J3, SmCYP94J5Pv1, and SmCYP94J5Pv2) are members of a cluster that includes AtCYP94B3 and AtCYP94C1 as enzymes responsible for the oxidation of JA-Ile and 12OH-JA-Ile (Banks et al. 2011). SmCYP94Js are likely related to the oxidation of JA-Ile, leading to the inactivation of JA activity.

In studies of P. patens and M. polymorpha, only OPDA has been detected in both plants (Stumpe et al. 2010, Yamamoto et al. 2015). In contrast to vascular plants, JA is not important for the physiology of these model bryophytes. Hence, our data suggest that JA and JA-Ile biosynthesis first appeared after bryophytes in plant evolution (Figs. 2 and 3). Additionally, there is a significant difference between bryophytes and lycophytes. Lycophytes have a vascular system for the efficient transport of water, nutrients, and molecules, as well as for enhancing plant height and size. It is likely that the emergence of the JA biosynthetic pathway after OPDA is related to the acquisition by plants of a vascular system.

Enzymatic activity
There have been no previous studies on enzymes in the JA biosynthetic pathway of non-seed vascular plants. To support the analytical data showing the presence of OPDA, JA, and JA-Ile, the enzymatic activities of AOS, AOC, OPR3, and JAR1 were investigated. Here, genes encoding functional AOS, AOC, OPR3, and JAR1 proteins (SmAOS2, SmAOC1, SmOPR1 and SmOPR5, and SmJAR1, respectively) were identified in S. moellendorffii.

**SmAOS2.** The AOS reaction is the first committed step of JA biosynthesis in plants. AOS is classified as a member of the CYP74 family and requires an oxygenated fatty acid hydroperoxide substrate instead of oxygen or a redox partner (Howe and Schilmiller 2002, Werck-Reichhart et al. 2002). This study revealed three AOS candidate genes (SmAOS1, SmAOS2, and SmAOS3) in S. moellendorffii (Supplementary Figs. S1 and S2). Among them, recombinant SmAOS2, which was successfully produced in E. coli, exhibited AOS activity similar to that of previously reported AOSs (Fig. 4, Supplementary Figs. S4–S6). Computer programs analyzing protein subcellular localization did not predict a transit peptide in the N-terminus of SmAOS2. Because most known AOSs are localized in chloroplasts, SmAOS2 is likely located in chloroplasts for JA biosynthesis. Since the amino acid sequences of SmAOS1, SmAOS2, and SmAOS3 are similar, further investigation is needed to clarify the physiological functions of each of these SmAOSs.

**SmAOC1.** AOC is a critical enzyme that establishes the enantiomeric structure of OPDA, which contributes to the basic structure of jasmonates. The enzymatic activity of SmAOC1 is required for the synthesis of naturally occurring OPDA (Fig. 5). The stereochemistry of OPDA in S. moellendorffii is the same as that in other studied plants. Our microscopic observations using SmAOC1 fused to GFP showed that SmAOC1 localized to chloroplasts (Supplementary Fig. S9).
These results suggest that the first half of the octadecanoid pathway for synthesizing OPDA is present in the chloroplasts of *S. moellendorfii*. The presence of LOX, AOS, and AOC in chloroplasts may have been conserved during the process of land plant evolution.

*SmOPR1 and SmOPR5*. The enzymatic analysis of SmOPR1 and SmOPR5 revealed that *S. moellendorfii* possesses two types of OPRs that are classified by substrate preference: OPR1-like enzymes and OPR3-like enzymes. SmOPR1 and SmOPR5 are similar in their molecular mass and isoelectric point (pI) (Table 1) but differ in their substrate specificity for *cis*-OPDA isomers. Of these two SmOPRs, only SmOPR5 reduced *cis*-($\pm$)-OPDA, including the endogenous substrate *cis*-(+)-OPDA, which is the natural JA precursor (Fig. 6, Supplementary Figs. S12 and S13). In contrast to SmOPR5, SmOPR1 reduced only *cis*-(−)-OPDA, which is the unnatural type (Fig. 6). SmOPR1 might instead be required for the oxylipin metabolic pathway.

A structural comparison of tomato SlOPR1 and SlOPR3 indicated that two active site residues, Tyr78 and Tyr246 in SlOPR1 and Phe74 and His244 in SlOPR3, are critical for substrate specificity (Breithaupt et al. 2009). SlOPR3 is less enantioselective due to its two relatively smaller amino acid residues that form a larger substrate binding pocket. In contrast, the relatively larger amino acids (two Tyr residues) in SlOPR1 permit access to only *cis*-(−)-OPDA, the unnatural type, at the substrate binding site. This study showed that SmOPR5, but not SmOPR1, actively converts the endogenous substrate *cis*-(+) OPDA to *cis*-(+) OPC-8:0. This result contrasts with the prediction from the substrate preference analysis of their active site residues but agrees with the phylogenetic analysis of OPRs (Supplementary Figs. S10 and S11). Mutation of the important amino acids Phe and His into Tyr in SlOPR3 did not completely change the substrate specificity (Breithaupt et al. 2009). There is an unidentified factor in the
structure of OPRs that is important for substrate specificity, and this factor must be identified by further crystal structure analysis.

Li et al. (2009) showed that OPRs from the lower land plants *P. patens* (PpOPR1, PpOPR2, PpOPR4, and PpOPR5) and *S. moellendorffii* (SmOPR1, SmOPR2, SmOPR3, SmOPR4, and SmOPR6) clustered together in subgroup VI. Because SmOPR1 showed OPR1 activity, the other OPRs in subgroup VI may be unrelated to JA biosynthesis. In contrast, the other known OPR3-like enzymes are positioned in the same cluster in subgroup II. It is most likely that OPR3-like enzymes independently evolved to expand the substrate binding pocket to accept cis- (+)-OPDA.

*SmJAR1*. JAR1 is responsible for conjugating JA to amino acids. Because JA-Ile is a versatile molecule in JA signaling, JAR1 is necessary for JA-mediated physiological events in seed plants (Wasternack and Hause 2013). JAR1 is a member of the GH3 proteins, which are classified into three groups based on their enzymatic activity. Group I proteins, such as *Arabidopsis* AtJAR1, can synthesize the JA-amino acid conjugates JA-Ile, JA-Leu and JA-Val (Staswick and Tiryaki 2004). Group II proteins conjugate IAA and SA to various amino acids (Staswick et al. 2005). Group III proteins accept benzoates as substrates for amino acid conjugation (Okrent et al. 2009). A group I GH3 protein from seed plants showed JA-Ile synthase activity, which functions in defense (Kang et al. 2006, Wang et al. 2007, Suza et al. 2010).

*S. moellendorffii* produces JA-Ile based on evidence of a functionally active SmJAR1 (Fig. 7). The identification of JA-Ile and an active SmJAR1 strongly suggests that JA-Ile-mediated signaling is functional in *S. moellendorffii*. Moreover, SmJAR1 was hypothesized to prefer Ile as a substrate for conjugation with JA (Fig. 7 and Supplementary Fig. S16). The same characteristic was also found in AtJAR1 (Staswick and Tiryaki 2004, Suza and Staswick 2008). Considering
the substrate specificity of SmJAR1, JA-Ile seems to be more important than the other JA-amino acid conjugates. Additionally, endogenous conjugates of JA with another amino acid, such as JA-Trp, JA-Phe, JA-Gly, JA-Leu, and JA-Val, have not been found in *S. moellendorffii* (Supplementary Fig. 19).

In the case of rice (*Oryza sativa*), OsJAR2 in the cluster of group II GH3 proteins can conjugate JA to Ile, similar to OsJAR1 in the cluster of group I GH3 proteins (Wakuta et al. 2011). The other thirteen of JAR1 homologs found in *S. moellendorffii* might also show JA-Ile synthetic activity. Given that GH3 proteins synthesize conjugates of JA and amino acids in *P. patens* (Ludwig-Müller et al. 2009), land plants are suggested to have enzymes with ubiquitous JAR1 activity. JA synthesis and JAR1 activity might have evolved independently in land plants.

**Expression of SmAOC1, SmOPR5, and SmJAR1**

Wounding and JA induce the expression of various genes involved in defense responses in seed plants, i.e., genes encoding enzymes in JA biosynthesis. In *S. moellendorffii*, the expression of *SmAOC1* and *SmOPR5* under wounding treatment was increased, respectively (Fig. 8). These results were consistent with the transient increases in OPDA and JA in *S. moellendorffii* (Fig. 3). Based on the *SmOPR5* expression level, the accumulation of *SmOPR5* appears to precede the production of OPDA. Additionally, the late accumulation of JA-Ile coincided with the late expression level of *SmJAR1* after wounding (Fig. 8). The expression profiles of *SmAOC1*, *SmOPR5*, and *SmJAR1* support the accumulation patterns of OPDA, JA, and JA-Ile, probably due to the role of jasmonates in regulating responses to environmental stress in *S. moellendorffii*.

The present study also demonstrated that JA induced the expression of *SmAOC1* and *SmOPR5* in *S. moellendorffii*. JA treatment led to the accumulation of genes encoding enzymes in
the octadecanoid pathway, indicating positive feedback regulation of JA biosynthesis. The expression of SmAOC1 and SmOPR5 was enhanced upon JA application, and these expression patterns were similar to those observed under wounding treatment (Fig. 9). The JA-induced expression of SmAOC1 and SmOPR5 allows us to infer the presence of positive feedback regulation of JA biosynthesis in S. moellendorffii. In contrast to the up-regulation of SmJAR1 by wounding, JA inhibited SmJAR1 expression at the transcriptional level (Fig. 9). Although the detailed JA signaling pathway has not been elucidated in S. moellendorffii, the suppression of SmJAR1 expression by JA appears to function as negative feedback regulation of JA-Ile-mediated signaling and/or an increase in JA signaling that is independent from JA-Ile. Wounding probably activates various signaling pathways that are JA-dependent and JA-independent. In the case of SmJAR1 expression, integrated signaling caused by wounding might induce SmJAR1 expression against stresses.

OPDA, JA, and JA-Ile inhibit the growth of S. moellendorffii

P. patens and M. polymorpha cannot produce JA; instead, OPDA inhibits their growth, and JA does not induce any significant physiological responses (Stumpe et al. 2010, Yamamoto et al. 2015). However, P. patens responds to methyl jasmonate, which has not been detected in P. patens, by reducing its colony size (Ponce de León et al. 2012). Many studies have reported the growth inhibitory activity of JA in seed plants. OPDA, JA, and JA-Ile treatment retarded the growth of S. moellendorffii in a manner similar to those of seed plants (Fig. 10, Supplementary Fig. 18). This report is the first evidence that OPDA, JA and JA-Ile inhibit the growth of non-seed vascular plants. The growth inhibitory activity of JA and JA-Ile is probably a characteristic physiological response that is conserved in vascular plants. During plant evolution, JA
biosynthesis and signaling in vascular plants might have developed due to the need to adapt to life on land and/or to organize more complicated physiological processes.

It has recently been reported that the genes encoding proteins related to JA signaling perception, such as COI1 and JAZ, are present in bryophytes and lycophytes (Wang et al. 2015). The JA responsiveness of *S. moellendorffii* shown in this study indicates that JA might function through a signal transduction mechanism similar to that observed in seed plants: the COI1-JAZ system. Given that JA does not have any significant effect in bryophytes, vascular plants are suggested to have developed the COI1-JAZ system, which is suitable for JA signaling. Investigating the functional difference in the COI1-JAZ system between bryophytes and lycophytes is important for understanding the functional evolution of JA signal transduction in plants. *S. moellendorffii* will be a key plant for understanding the function of JA in vascular plants.

In conclusion, we provide the first evidence of the presence of OPDA, JA, and JA-Ile in the model lycophyte *S. moellendorffii*, which is taxonomically positioned between bryophytes and euphyllophytes. Moreover, active homologous enzymes involved in the octadecanoid pathway, including an AOS, an AOC, an OPR3-like enzyme, and a JAR1, were also identified in this plant. The application of exogenous OPDA, JA, and JA-Ile significantly inhibited the growth of *S. moellendorffii*. Thus, OPDA, JA, and JA-Ile are probably important signaling molecules in stress responses and growth regulators in *S. moellendorffii*.

**Materials and Methods**

*Plant material*
**S. moellendorffii** was grown at 25°C under white fluorescent light with a photoperiod of 14 h light/10 h dark.

**Analysis of OPDA, JA, and JA-Ile**

The microphylls and stems of **S. moellendorffii** (ca. 500 mg) were harvested 10, 20, 30, 60 or 180 min after wounding with tweezers and were homogenized in liquid nitrogen, followed by ethanol extraction for 24 h. UPLC-MS/MS analysis was performed according to Sato et al. (2009) and Yamamoto et al. (2015).

**JA growth inhibitory activity assay in S. moellendorffii**

**S. moellendorffii** bulbils (Supplementary Fig. S17) were sprinkled onto a Jiffy-7 pellet (Sakata Seed Corporation, Japan). The bulbils were grown at 25°C under white fluorescent light with a photoperiod of 14 h light/10 h dark. After germination and one week of growth, the plants were treated with OPDA, JA, or JA-Ile at concentrations of 25 µM, 50 µM, and 100 µM every other day for four weeks. The shoot growth of the plants was evaluated by morphometric analysis.

**Bioinformatics analysis**

All of the putative genes in **S. moellendorffii** were screened in the Phytozome v 11.0 database (https://phytozome.jgi.doe.gov/pz/portal.html) using the *Arabidopsis* amino acid sequence as a query (Supplementary Table S1). Multiple sequence analysis was performed using Clustal Omega and viewed in the GeneDoc software program. A phylogenetic tree was generated using Mega version 5.2 (Neighbor-joining method). Bootstrap analysis was performed by resampling the datasets 1,000 times. The sub-cellular localization of SmAOCl was predicted using ChloroP v

Molecular cloning of SmAOS2, SmAOC1, SmOPR1, SmOPR5, and SmJAR1-1

The SmAOS2, SmAOC1, SmOPR1, SmOPR5, and SmJAR1 genes were cloned according to a conventional method. The details are described in the Supplemental Information.

Synthesis of recombinant SmAOS2, SmAOC1, SmOPR1 and SmOPR5

Recombinant SmAOS2, SmAOC1, SmOPR1 and SmOPR5 were produced according to a conventional method. The details are described in the Supplemental Information.

Enzyme assay of SmAOS2

To measure AOS activity, SmAOS2 was reacted with 13(S)-HPOT according to Bandara et al. (2009) with some modifications. Five milligrams of recombinant SmAOS2 was reacted with 6 µg of 13(S)-HPOT as a substrate in a total volume of 0.1 ml of 50 mM phosphate buffer (pH 7.0). The reaction mixture was incubated at 25°C for 1 h. To terminate the enzymatic reaction, the mixture was treated with 1 M HCl until it reached pH 3. To convert cis-OPDA to trans-OPDA, the reaction mixture was subjected to alkaline treatment by stirring with 1 ml of 1 M NaOH for 30 min. After acidification, the resulting solution was extracted three times with an identical volume of ethyl acetate, evaporated, and methylated using ethereal diazomethane. After incubation for 30 min on ice, the reaction was terminated with the addition of 1 or 2 drops of acetic acid until the color changed from yellow to colorless. The methylated residue was
dissolved in 100 µl of CHCl₃ for chiral GC-MS analysis (1200 L GC-MS/MS system, Varian, USA) equipped with a β-DEX fused silica capillary column (0.25 mm × 30 m × 0.25 µm, Supelco, USA). Helium was used as the carrier gas at a constant flow rate of 1.0 ml/min. The ion source and vaporizing chamber were heated to 200°C and 220°C, respectively, and the ionization voltage was 70 eV. The column was initially heated to 50°C and held isothermally for 1 min, and then the temperature was subsequently increased at a rate of 10°C/min to a final temperature of 190°C, which was held for 80 min.

**Enzyme assay of SmAOC1 activity**

The enzymatic reaction was performed in a mixture of 5 µg of 13-HPOT and 5 µg of PpAOS1 in 1 ml of 10 mM sodium phosphate buffer (pH 7.8) with or without 10 µg of SmAOC1. The reaction mixture was incubated at 25°C for 1 h. The products of the SmAOC1 reaction were analyzed according to the method of OPDA analysis described in the previous section.

**Enzyme assays for OPR activity**

The enzymatic reaction was performed according to the method of Schaller et al. (1998) with some modifications. Five micrograms of affinity-purified recombinant SmOPR1 or SmOPR5 was incubated in 50 mM potassium phosphate buffer (pH 7.4) containing 7.5 µg of (±)-cis-OPDA as a substrate and 1.0 mM NADPH in a total volume of 0.1 ml for 1 h at 25°C. At the end of the incubation, (±)-cis-OPDA and (±)-cis-OPC-8:0 were converted to methylated trans-isomers according to the method described above. OPC-8:0 was evaluated by chiral GC-MS analysis (same conditions as the enzymatic analysis for SmAOC1).
Subcellular localization of SmAOC1

Subcellular localization of SmAOC1 was analyzed according to the method of Yamamoto et al. (2015). The details are described in the Supplemental Information.

Enzyme assays for JAR1 activity

SmJAR1 activity was assayed according to Wakuta et al. (2011) with some modifications. E. coli BL21 (DE3) cells carrying pET23a-SmJAR1 were cultured overnight in 5 ml of LB medium supplemented with 100 µg/ml ampicillin, at 37°C. A portion of the culture was inoculated into 2 ml of fresh selective LB medium, incubated until the OD_{600} reached 0.2, and treated with 1 mM IPTG. The culture was incubated at 37°C for 5 h to induce protein production. Subsequently, 0.2 mM JA was added to the culture. The incubation was continued at 37°C for 3 h with shaking. JA-Ile in the culture was analyzed using the UPLC-MS/MS system as previously described (Sato et al. 2009).

Quantitative RT-PCR for SmAOC1, SmOPR5, and SmJAR in S. moellendorffii exposed to wounding stress and JA treatments

The coding sequences (CDSs) for SmAOC1, SmOPR5, and SmJAR1 were used to design specific primers for quantitative RT-PCR (qRT-PCR). The primers used to amplify these genes are listed in Supplementary Table S1. Total RNA of S. moellendorffii was extracted 0, 10, 20, 30, 60, and 180 min after wounding or 50 µM JA treatment. The first-strand cDNA was used as a template. In the present study, KOD SYBR qPCR Mix (Toyobo, Japan) was used according to the manufacturer’s protocol. Each reaction mixture contained 12.5 µl of KOD SYBR® qPCR Mix, 1 µl of each primer (10 mM), 1 µl of cDNA, and 9.5 µl of MilliQ water qRT-PCR was performed
on a Thermal Cycler Dice Real Time system (Takara TP800, Japan). The qRT-PCR conditions were as follows: pre-incubation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The specificity of each PCR amplicon was assessed with a dissociation curve (95°C for 15 s, 60°C for 30 s, and 95°C at 15 s). Each target gene was calculated and expressed as fold regulation in comparison to the housekeeping genes actin (XM_002976966) for the wounding treatment and ubiquitin EST (DN839032, Kirkbride et al. 2013) for the JA treatment.

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**Disclosures**

The authors have no conflicts of interest to declare.

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References


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Table 1. Properties of *SmAOC1*, *SmOPR1*, *SmOPR5*, and *SmJAR1*.

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<th>Protein length (aa)</th>
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* The numbering of *SmOPRs* was previously described by Li et al. (2009).
Figure legends

Fig. 1. The biosynthesis of JA and JA-Ile.

Fig. 2. UPLC-MS/MS analysis of OPDA, JA, and JA-Ile in S. moellendorffii. (A–C) MRM mode analysis of a specific daughter peak at m/z 164.99 that was derived from the peak at m/z 291.37 [M–H]^−. (D–F) MRM mode analysis of a specific daughter peak at m/z 58.71 that was derived from the peak at m/z 209.09 [M–H]^−. (G–I) MRM mode analysis of a specific daughter peak at m/z 129.68 that was derived from the peak at m/z 322.03 [M–H]^−. (A, D, G) Standard. (B, E, H) S. moellendorffii extract. (C, F, I) Co-injection of the standard and S. moellendorffii extract.

Fig. 3. Accumulation of OPDA, JA, and OPDA in S. moellendorffii after wounding. S. moellendorffii was subjected to mechanical wounding and then collected at the indicated time after wounding. The concentrations of OPDA, JA, and JA-Ile were analyzed by UPLC-MS/MS. The data are presented as the mean ± SD (n = 3). The asterisks represent a significant difference between the treated and control plants (Student’s t-test, *p < 0.05, **p < 0.01).

Fig. 4. Chiral GC-MS analysis of the OPDA methyl ester. Recombinant SmAOS2 was reacted with the substrate 13(S)-HPOT, directly treated with alkaline solution and then methylated by ethereal diazomethane. The methylated OPDA was analyzed by GC-MS using a chiral capillary column. The data are presented as the total ion current chromatogram. The former and latter peaks represent the trans-(+)- and (-)-OPDA methyl esters, respectively (Laudert et al. 1997).
Fig. 5. Chiral GC-MS analysis of the OPDA methyl ester. Recombinant SmAOC1 was reacted with the substrate 13-HPOT and an allene oxide synthase (PpAOS1), directly treated with alkaline solution and then methylated by ethereal diazomethane. The methylated OPDA was analyzed by GC-MS using a chiral capillary column. The molecular ion peak of the OPDA methyl ester at m/z 306 was monitored. The former and latter peaks represent the trans-(+)- and (-)-OPDA methyl ester, respectively (Laudert et al. 1997). (A) Racemic OPDA standard. (B) Product of the SmAOC1 enzymatic reaction.

Fig. 6. Chiral GC-MS analysis of the reaction products catalyzed by SmOPR1 and SmOPR5. Recombinant SmOPR1 and SmOPR5 were each reacted with racemic OPDA, directly treated with alkaline solution and then methylated by ethereal diazomethane. The methylated reaction products were analyzed by GC-MS using a chiral capillary column. The data are presented as the total ion current chromatogram. Peaks 1 to 4 represent trans-isomers of (+)-OPC-8:0 (natural type), (-)-OPC-8:0, (+)-OPDA (natural type) and (-)-OPDA, respectively, that were derived from cis-isomers of these compounds.

Fig. 7. UPLC-MS/MS analysis of JA-Ile in E. coli overexpressing SmJAR1. MRM mode analysis of a specific daughter peak of JA-Ile-d₆ at m/z 129.68 that was derived from the peak at m/z 328.03 [M−H]⁻ and of the peak at m/z 129.68 that was derived from the peak of JA-Ile at m/z 322.03 [M−H]⁻. (A) Internal standard JA-Ile-d₆. (B) Culture supernatant of E. coli overexpressing SmJAR1. (C) Culture supernatant of E. coli containing pET23a as a control. *The value of 100% is equal to 9 × 10⁵ counts, which was converted by the algorithm in Mass Lynx software (Waters Corporation, Milford, MA, USA).
Fig. 8. Quantitative RT-PCR analysis of *SmAOC1*, *SmOPR5*, and *SmJAR1* in wounded *S. moellendorffii*. Plants were subjected to mechanical wounding and then collected at the indicated times after wounding. To evaluate the transcript levels of *SmAOC1*, *SmOPR5*, and *SmJAR1*, qRT-PCR was performed using RNA isolated from control and wounded plants. The data are presented as the mean ± SD (n = 3). The asterisks represent a significant difference between the treated and control plants (Student’s t-test, *p* < 0.05; **p** < 0.01).

Fig. 9. Quantitative RT-PCR analysis of *SmAOC1*, *SmOPR5*, and *SmJAR1* in *S. moellendorffii* treated with JA. Plants were treated with 50 μM JA and then collected at the indicated times after JA treatment. To evaluate the transcript levels of *SmAOC1*, *SmOPR5*, and *SmJAR1*, qRT-PCR was performed using RNA isolated from control and JA-treated plants. The data are presented as the mean ± SD (n = 3). The asterisks represent a significant difference between the treated and control plants (Student’s t-test, *p* < 0.05; **p** < 0.01; ***p** < 0.001).

Fig. 10. Growth inhibitory activities of OPDA, JA, and JA-Ile in *S. moellendorffii*. *S. moellendorffii* was treated with OPDA, JA, and JA-Ile for four weeks, and then the shoots were measured. The data are presented as the mean ± SD (n = 5). The asterisks represent a significant difference between the treated and control plants (Student’s t-test, **p** < 0.01; ***p** < 0.001).
Fig. 1.

α-linolenic acid
-----------
<table>
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<tr>
<th>Lipoxigenase</th>
<th>13-HPOT</th>
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<tbody>
<tr>
<td>(LOX)</td>
<td>Allene oxide synthase (AOS)</td>
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<tr>
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13-HPOT

racemic OPDA α-ketol γ-ketol

Allene oxide synthase (AOS)

non-enzymatically

JAR1

β-oxidation (x3)

(-)-JA

(+)-7-iso-JA

(-)-JA-ile

(+)-7-iso-JA-ile

OPC-8:0
Fig. 2.
Fig. 3.

Concentration (pmol/gFW$^{-1}$)

Time after wounding (min)

**OPDA**

**JA**

**JA-Ile**

* and ** indicate significance levels compared to the control.

Concentration levels increase over time, with significant differences marked by * and **.
Fig. 4.
Fig. 5.
Fig. 6.

Retention time (min)

Relative intensity (%)
Fig. 7.

![Graph showing relative intensity over time for Standard, SmJAR1, and Control samples with peaks labeled JA-Ile and JA-Ile-d6.](image)
Fig. 8.

- **SmAOC1**
- **SmOPR5**
- **SmJAR1**
Fig. 9.
Fig. 10.