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An advanced method for the determination of carboxyl methyl esterase activity using gas chromatography–chemical ionization–mass spectrometry

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Abstract

We developed a quantitative method for the determination of methyl esterase activity, analyzing substrate specificity against three major signal molecules, jasmonic acid methyl ester (MeJA), salicylic acid methyl ester (MeSA), and indole-3-acetic acid methyl ester (MeIAA). We used a silylation reagent for chemical derivatization and used gas chromatography (GC)–mass spectroscopy in analyses, for high precision. To test this method, an Arabidopsis esterase gene, *AtME8*, was expressed in *Escherichia coli*, and then the kinetic parameters of the recombinant enzyme were determined for three substrates. Finally, this method was also applied to the direct quantification of phytohormones in petals from lilies and roses.

Keywords: Jasmonic acid methyl ester; Salicylic acid methyl ester; Indole 3-acetic acid methyl ester; Carboxyl methyl esterase; Gas chromatography–mass spectroscopy

1. Introduction

Salicylic acid (SA), jasmonic acid (JA), and indole-3-acetic acid (IAA) are major phytohormones in plants. They have important roles in plant growth, development, and defenses against insect attack and pathogen infections [1–6].

It has been discovered that the methyl esters of phytohormones also have distinct functions in plants. Salicylic acid methyl ester (MeSA) is not an active molecule in plant pathogen resistance, but it is a transfer molecule for intra- or inter-plant signal transduction and a detoxifying sink molecule that consumes SA after levels have been elevated by pathogen infections [7,8]. Jasmonic acid methyl ester (MeJA) is a secondary metabolite in systemic wound signal transduction and biotic or abiotic stress signal transduction [9,10]. Indole-3-acetic acid methyl ester (MeIAA) has been implicated in leaf development in *Arabidopsis* [11].

The role of these methyl esters has been examined by identifying each methyltransferase, such as SA methyltransferase (SAMT) [12], JA methyltransferase (JMT) [9], and IAA methyltransferase (IAMT) [11]. Esterases that hydrolyze the carboxyl methyl group from the methyl esters have also been identified. Two methyl esterases, methyl salicylate esterase (SABP2) [7] and methyl jasmonate esterase (MJE), [13] have been extracted from tobacco and tomato, respectively. In particular, SABP2 has a role in transferring systemic acquired resistance (SAR) signals by hydrolyzing MeSA to SA, which is an active form in plant defense mechanisms [7]. To determine the activity of carboxyl methyltransferase, radiolabeled

Abbreviations: JA, jasmonic acid; SA, salicylic acid; IAA, indole-3-acetic acid; OAA, *o*-anisic acid; MeJA, jasmonic acid methyl ester; MeSA, salicylic acid methyl ester; MeIAA, indole-3-acetic acid methyl ester; MeOAA, *o*-anisic acid methyl ester; JA-TMS, jasmonic acid trimethylsilyl ester; SA-TMS_2, *O*-trimethylsilyl benzoic acid trimethylsilyl ester; IAA-TMS_2, *N*-trimethylsilyl indole 3-acetic acid trimethylsilyl ester; MeSA-TMS, *O*-trimethylsilyl benzoic acid methyl ester; MeIAA-TMS, *N*-trimethylsilyl-indole-3-acetic acid methyl ester; GC, gas chromatography; GC–MS, gas chromatography–mass spectroscopy; SIM, selected ion monitoring; TLC, thin-layer chromatography.

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S-adenosylmethionine (AdoMet) has been used as a methyl donor, and the enzyme reaction products are analyzed with a scintillation counter.

The methylated products are readily determined by gas chromatography–mass spectroscopy (GC–MS) because of their volatility. The phytohormones in plants were successfully determined by methylation and GC–MS analysis [14–17]. Furthermore, a lot of volatile organic compounds as well as phytohormones were separated and quantified simultaneously through GC–MS after methylation [17]. However, this method is not applicable to the determination of esterase activity. Because the substrates are methylated compounds, derivatization through other than chemical methylation have been reported [7,18]. For instance, to determine the specific activity of SABP2, the produced acids are re-methylated, using methyltransferases and ^{14}C-AdoMet [7]. The additional enzyme reaction step makes the experiments difficult to perform. For the analysis of tomato MJE activity, the enzyme reaction mixture was extracted with diethyl ether, ethylated with diazoethane, and analyzed by GC–MS. This was a good approach for the measurement, but stronger signals on GC–MS were obtained by tert-butyldimethylsilylation or trimethylsilylation of the phytohormones, rather than ethylation using diazoethane on IAA, SA, or some acids [19].

In the present study, we showed that silylation and GC–MS analysis is a good quantitative method for detection of enzymatic activity of esterases. In a direct quantitative analysis of phytohormones and their methyl esters in plant extracts, we were able to separate the acid phytohormone derivatives from the phytohormone methyl esters. For easy handling and simplicity, we introduced *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) as a silylation reagent [20]. This reagent effectively silylates the various functional groups of the target compounds.

2. Experimental

2.1. Reagents and standards

JA, SA, IAA, MeSA, and MeIAA were purchased from Sigma Chemical (St. Louis, MO). MeJA, *o*-anisic acid (OAA), and hydrochloric acid (HCl) (37%) were purchased from Aldrich Chemical (Milwaukee, WI). Adsorbent Super Q (80/100 mesh) was purchased from Alltech (Deerfield, IL). The silylation reagent, BSTFA + TMCS, was purchased from SUPELCO (Bellefonte, PA). Methyl *o*-anisic acid (MeOAA) was obtained from OAA by a methylation reaction with diazomethane.

2.2. Protein purification and enzyme assay

For over-expression of the AtME8 protein in *Escherichia coli*, the coding region of *AtME8* was amplified from U12118 (a complementary DNA [cDNA] clone obtained from the Arabidopsis Biological Resource Center; http://www.arabidopsis.org) by polymerase chain reaction (PCR), with 5′-TATGGATCCATATGAGTGAGGAGAAGAGGAAGCA-3′ and 5′-TAAGGATCCTTAACAGAAATTGTCAGCGATTG-3′ as primers. PCR products were digested with BamHI, ligated into the expression vector pGEX-5X-1 (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and then introduced into *E. coli* BL21 (DE3) pLysS. Protein purification was conducted as described previously [9]. Esterase activity of purified recombinant enzyme was determined at 25 °C in 50 mM Tris–HCl (pH 7.5) buffer containing 5 mM potassium chloride with 2 mM MeJA, MeSA, or MeIAA [18]. After a 30-min incubation, 10 μL 1 M HCl was added and the reaction mixture was extracted with diethyl ether. The diethyl ether phase was concentrated and loaded on a thin-layer chromatography (TLC) plate or was silylated after evaporating the ether for GC–MS analysis.

2.3. Thin-layer chromatography

Methyl esters and acid forms of JA, IAA, and SA were separated on a TLC plate (Silica gel 60 F254, Merck), using the developing solvent mixture hexane:ethyl acetate:acetic acid (70:30:1).

JA/MeJA and IAA/MeIAA were visualized by "burning" the plate with burning solution or by ultraviolet (UV) irradiation. The burning solution contained 2.5% (v/v) anisaldehyde, 1% (v/v) acetic acid, and 3.5% (v/v) sulfuric acid in 95% ethanol. SA/MeSA was visualized by UV irradiation (254 nm).

2.4. Chromatographic equipment

The GC–MS system used was a GC-17A gas chromatograph and QP-5000 mass spectrometer (SHIMADZU, Japan) equipped with an AOC-20i auto injector. Sample injection was done in splitless mode and separation occurred on a DB-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Inc., Santa Clara, CA). The column stayed at 40 °C for 1 min and was then heated by 15 °C min^{-1} to 250 °C (for 5 min) with helium gas as a carrier, at 1 mL min^{-1}. Measurements were made either through electron impact ionization (GC–EI–MS) with total ion count (TIC) or chemical ionization (GC–CI–MS) with selected ion monitoring (SIM) mode. All substrates and products were identified in GC–EI–MS mode, and deduced mass fragment patterns were detected at time points 9.06 min (MeSA), 10.33 min (MeOAA), 10.95 min (*O*-trimethylsilyl benzoic acid methyl ester [MeSA-TMS]), 11.40 min (*o*-anisic acid trimethylsilyl ester [OAA-TMS]), 11.90 min (*O*-trimethylsilyl benzoic acid trimethylsilyl ester [SA-TMS_2]), 13.3 min (MeJA), 14.07 min (jasmonic acid trimethylsilyl ester [JA-TMS]), 15.24 min (MeIAA), and 15.71 min (*N*-trimethylsilyl indole 3-acetic acid trimethylsilyl ester [IAA-TMS_2]). For the quantitative analysis of reaction products and plant samples, the area of each parent ion was monitored in GC–CI–MS mode as follows: MeSA $[M+H]^+$ (*m*/*z* 153), MeOAA $[M+H]^+$ (*m*/*z* 167), MeSA-TMS $[M+H]^+$ (*m*/*z* 225), OAA-TMS $[M+H]^+$ (*m*/*z* 225), SA-TMS_2 $[M+H]^+$ (*m*/*z* 283), MeJA $[M+H]^+$ (*m*/*z* 225), JA-TMS $[M+H]^+$ (*m*/*z* 283), MeIAA $[M+H]^+$ (*m*/*z* 262), and IAA-TMS_2 $[M+H]^+$ (*m*/*z* 320).

2.5. Preparation of plant samples

The petals of roses and lilies were extracted following a procedure reported previously [16] with H_2O/acetone (30/70, v/v) containing 50 mM citric acid. Internal standards (MeOAA and OAA, 50 ng each, dissolved in the extraction solution) were added to each extract and samples were sonicated at room temperature. Acetone in the extraction solution was evaporated, and

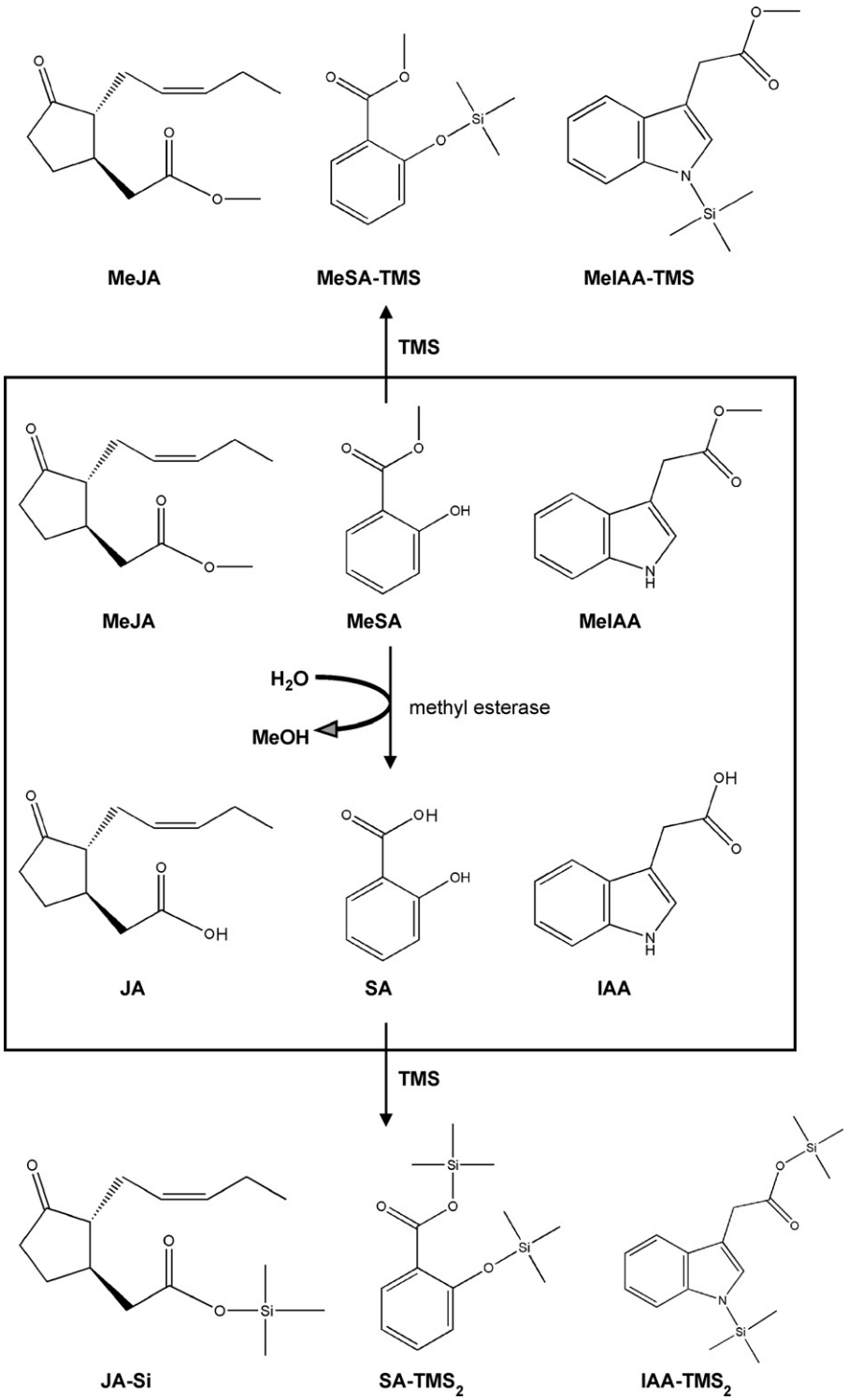


Fig. 1. Reaction of methyl esterase with carboxyl methyl JA, SA, and IAA. The silylated products from TMS reactions are also depicted. Silylated products were analyzed by GC–MS. "TMS" and "TMS_2" indicate silylation on single and double sites of each chemical, respectively.

the remaining water phase was extracted with diethyl ether. The extracted ether phase was completely dried at room temperature and silylated with 100 μL BSTFA + TMCS solution at 80 °C for 30 min. This solution was then completely evaporated at 80 °C through a Super Q filter trap, which was filled with about 50 mg of adsorbent and inserted into the reaction vessel. Adsorbed compounds were eluted with about 500 μL dichloromethane and analyzed by GC–MS.

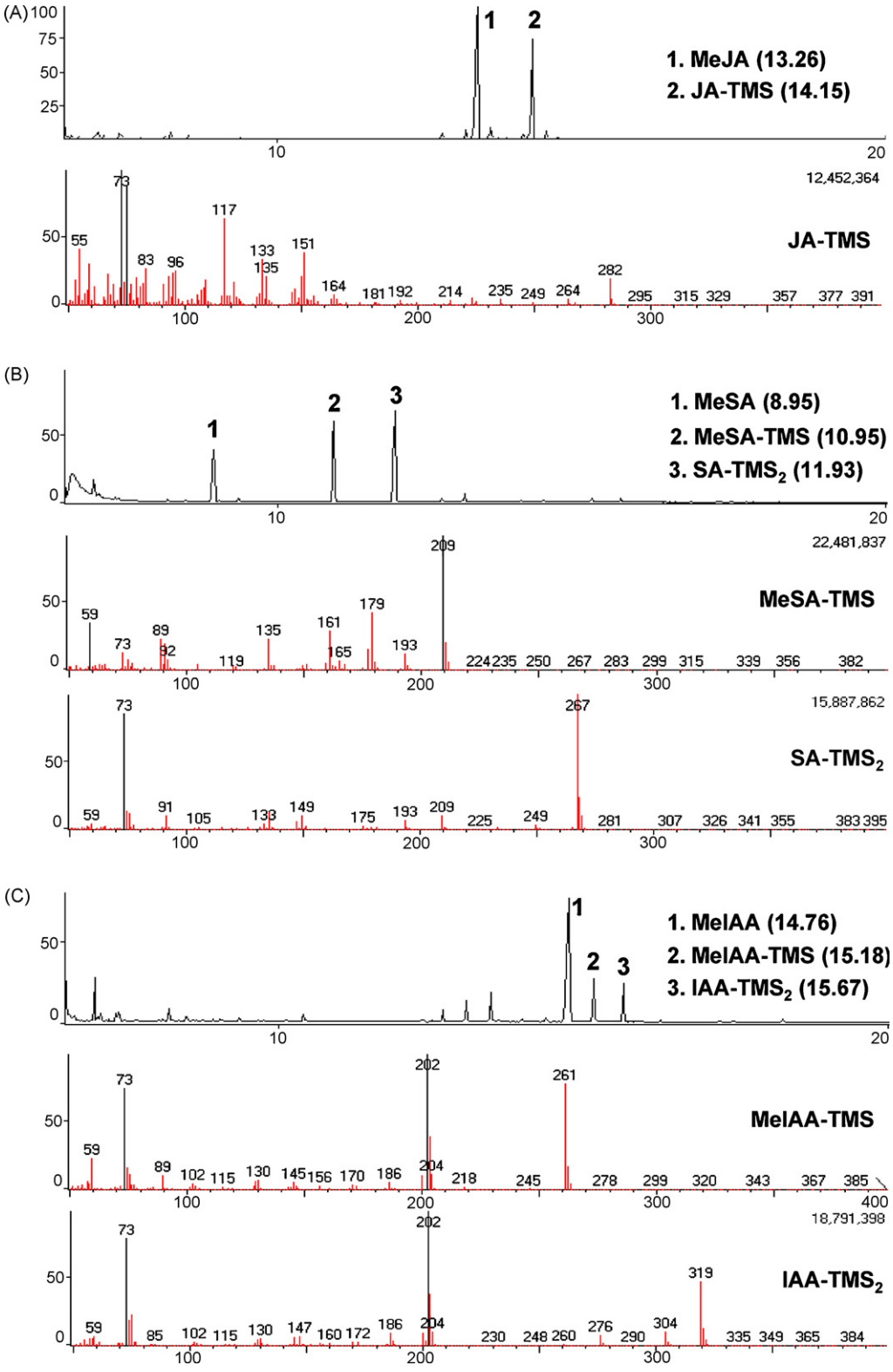


Fig. 2. GC–MS chromatogram of methyl esters and silylated JA (A), SA (B), and IAA (C). Separation was performed through a DB-5 column under conditions described in Experimental section. Silylated methyl SA and silylated methyl IAA are also shown.

3. Results and discussion

3.1. Identification of reaction products of methyl esterase by silylation

Silylation is a derivatization method that vaporizes many chemicals, which can then be detected by GC–MS. There are three different available functional groups that can be derivatized with the trimethylsilyl (TMS) group in JA, SA, and IAA: carboxyl, hydroxyl, and nitrile groups. The substrates MeSA and MeIAA were derivatized to MeSA-TMS and *N*-trimethylsilyl-indole-3-acetic acid methyl ester (MeIAA-TMS), respectively (Fig. 1). The products of the esterase reaction, JA, SA, and IAA, were converted to JA-TMS, SA-TMS_2, and IAA-TMS_2, respectively. All TMS products showed different times of retention to their substrates and also showed the expected mass patterns when analyzed by GC–EI–MS in total ion monitoring mode (Fig. 2).

In the cases of SA and IAA, there were two functional groups for TMS reaction: the phenol and carboxylic acid group in SA, and the amine and carboxylic acid group in IAA. The reactivity for TMS was different between these functional groups, and intermediates exchanged at one group were produced when the TMS reaction proceeded for short times. These intermediates were completely converted to double TMS products when the TMS reaction was conducted for 30 min at 80 °C. In the cases of SA and IAA, none of the single exchange products were seen on the chromatogram, confirming that the TMS reactions were completed.

3.2. Quantitative analysis of silylated phytohormones

Chemical ionization (CI) mode was used in GC–MS analysis for determination of the molecular weights of chemicals. In this study, we detected some chemicals with much higher sensitivity than others. Detection in selected ion mode is a recommended method in quantitative analysis in GC–MS. Thus, GC–CI–MS in selected ion monitoring mode was used for the analysis of reaction products and plant samples. To analyze the products of the esterase reaction, we optimized the reaction time for complete conversion to final derivatives (Fig. 3A). After 90 min, 5 μg of standards were shown to be maximally silylated. Furthermore, 90–95% of the acids were derivatized at 30 min. Thus, all subsequent silylation reactions were conducted for 30 min. Standard curves were determined twice with three replicates for each added amount of compound. The overall recovery of the analytical procedure was almost entirely determined by the recovery of the extraction with diethyl ether. Thus, the recovery of the method was determined by measuring the difference between standard acids reacted with silylation reagent and silylated products of standards extracted with diethyl ether (Fig. 3B–D). Recovery of the extracted and silylated samples was over 97% for all compounds in the linear range of the standard curve. The estimation curves were linear over the range of 1–500 ng of silylated compound for JA and IAA, with correlation coefficients of 0.998 and 0.999, respectively; the estimation curve for SA was linear over the range of 0.2–200 ng, with a correlation coefficient of 0.999.

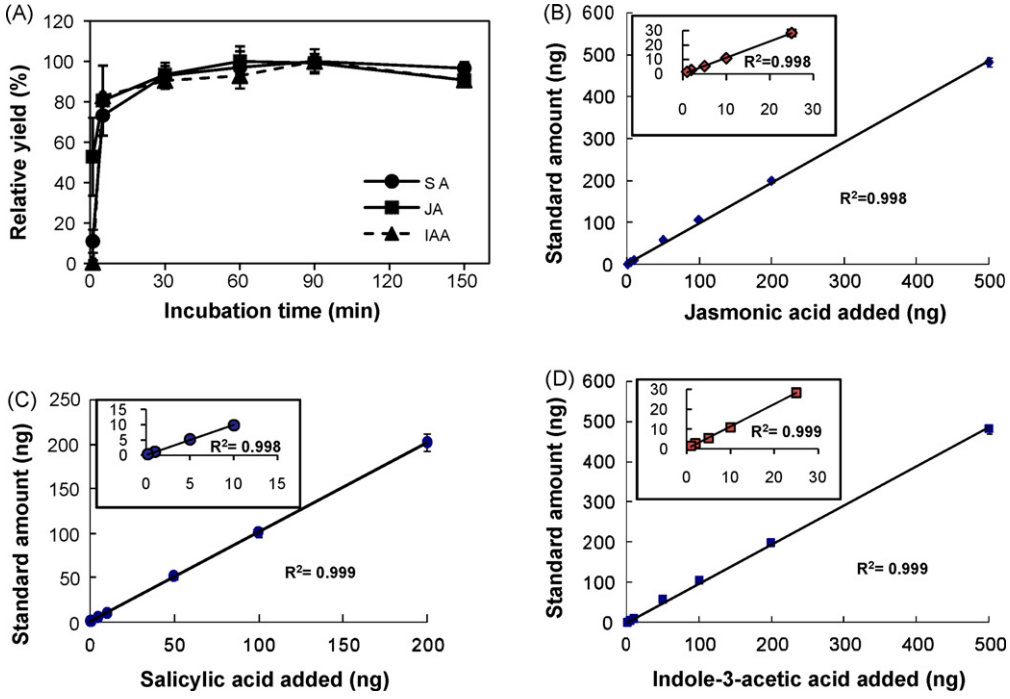


Fig. 3. The accuracy and precision of the silylation and GC–MS detection method. (A) Samples (5 μg each) of SA, JA, and IAA were silylated and analyzed by GC–CI–MS in selected ion monitoring mode at each time point indicated. Relative yields are shown as the percentage of molar responses at the maximum recovery at 90 min. Values are the means of four independent experiments and bars indicate standard deviations. Standard curves of JA (B), SA (C) and IAA (D). To simulate enzyme reaction, reaction mixture containing indicated amount of each phytohormone product instead of substrate methyl ester were extracted with diethyl ether and quantified by silylation and GC–MS. R^2 is the correlation coefficient. Insets are enlarged versions of part of the standard curves.

3.3. Enzymatic properties of recombinant methyl esterase

We used the silylation method to determine the enzyme characteristics of an esterase isolated from *Arabidopsis*. A protein Basic Local Alignment Search Tool (BLAST) search revealed that the predicted amino acid sequence of the esterase candidate (At2g23600) had about 52% identity to tobacco salicylic acid binding protein 2 (SABP2) and about 44% identity to tomato methyl jasmonate esterase (MJE). This candidate was named *Arabidopsis* methyl esterase (*AtME8*) and the cDNA clone U12118 was obtained from the *Arabidopsis* stock center. *AtME8* was expressed in *E. coli* as a glutathione-*S*-transferase (GST)-fusion protein and was purified by glutathione-agarose affinity chromatography to near homogeneity (Fig. 4). The molecular mass of the GST-fusion AtME8 protein was estimated on the gel to be 52 kDa, which was close to the expected size (54.9 kDa).

The enzyme fraction was reacted with the substrates MeJA, MeSA, and MeIAA, and the reaction products were analyzed by TLC (Fig. 5A). One microgram of purified AtME8 was reacted with 1 mg of each substrate for 1 h. The reaction mixtures were extracted with diethyl ether and the concentrated extracts were loaded on silica plates. From the TLC experiment, it was confirmed that all three methyl esters were good substrates for AtME8 (Fig. 5A).

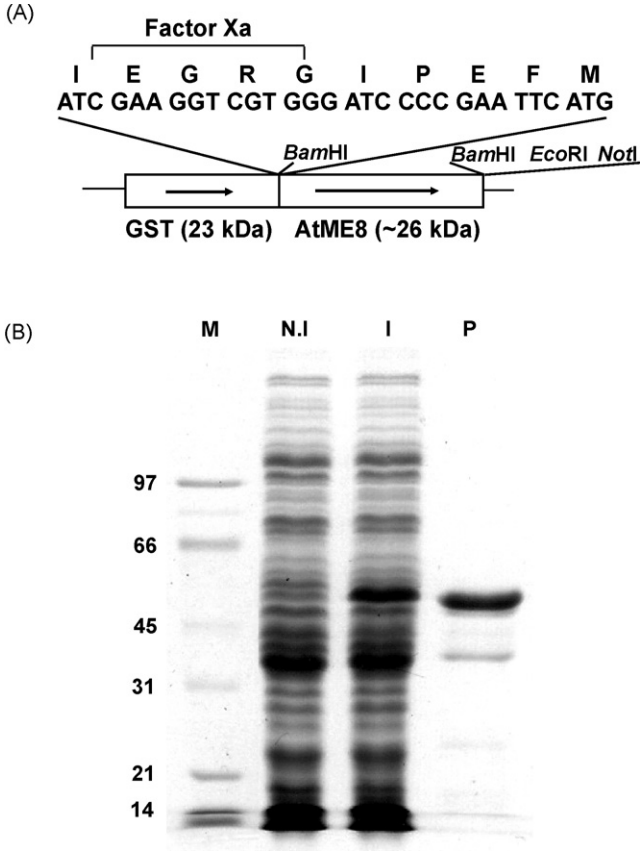


Fig. 4. Construction and expression of recombinant AtME8 in *E. coli*. (A) Recombinant DNA construction map for over-expression of AtME8 in *E. coli*. The cDNA of *AtME8* was fused with the N-terminal GST sequence in the pGEX-5X-1 vector. Junction nucleotides and the amino acid sequence inserted between GST and AtME8 are shown. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified AtME8. The abbreviations M, N.I, I, and P indicate size marker, no induction, induction with 1 mM IPTG, and purified, respectively. The sizes of the marker proteins (in kDa) are indicated in the left column.

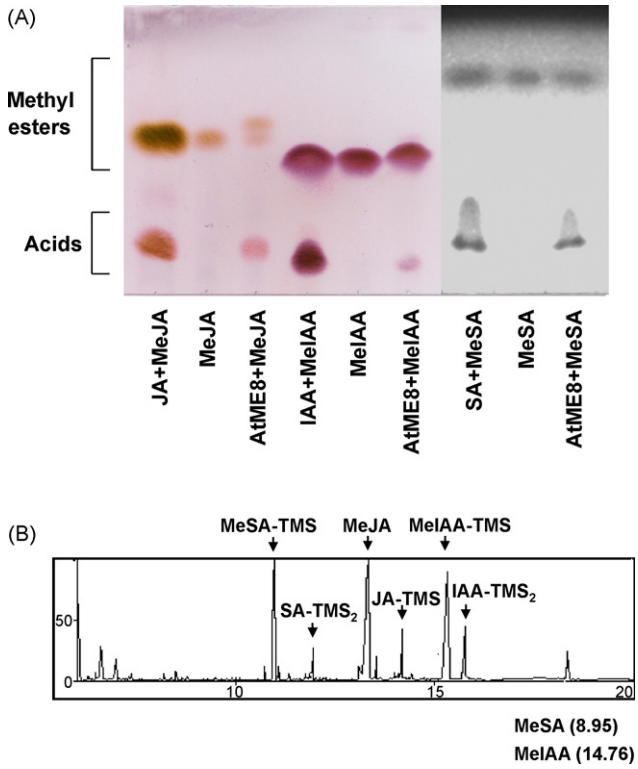
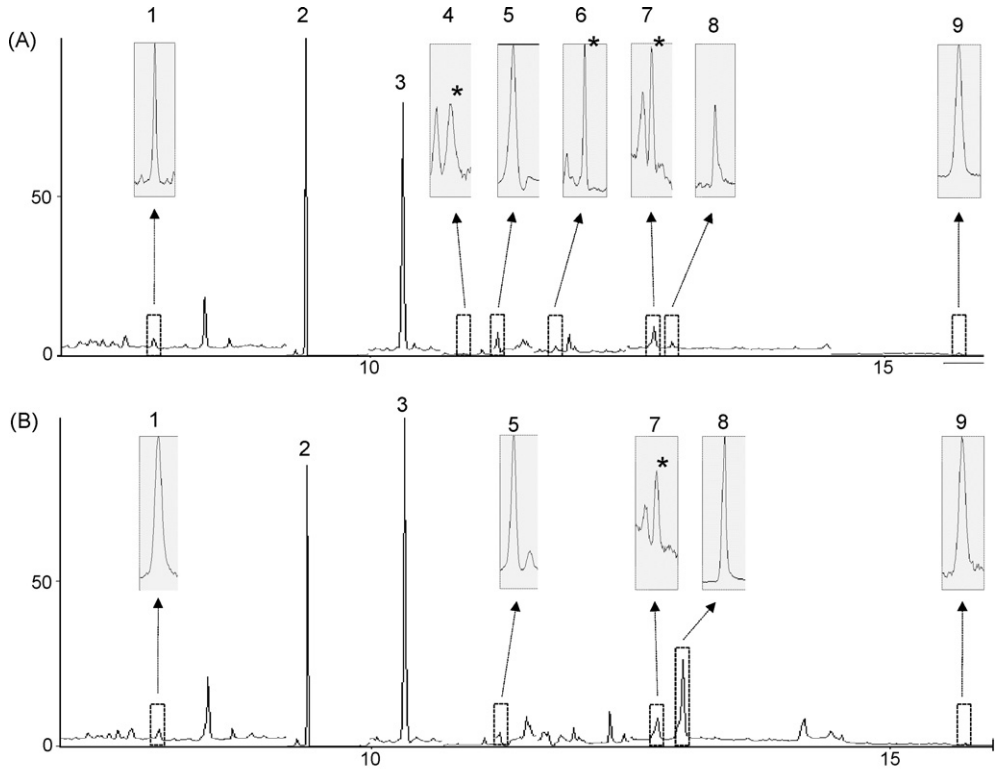


Fig. 5. Detection of enzyme reaction products. (A) Methyl esterase activity of AtME8 was analyzed by thin-layer chromatography. Methyl esters of JA, IAA, or SA were incubated with purified AtME8. The reaction products were extracted, concentrated, and loaded on the TLC plate. Reaction products were visualized by "burning" (left panel) or UV irradiation (right panel). (B) The enzymatic activity of AtME8 was analyzed by GC–MS. MeJA, MeSA, and MeIAA were each incubated with purified AtME8. The reaction products were extracted, silylated with TMS, and analyzed by GC–MS.

Enzyme activity was qualitatively demonstrated by GC–MS analysis on a chromatogram (Fig. 5B). As confirmed in the TLC experiment, AtME8 synthesized SA, JA, and IAA from MeSA, MeJA, and MeIAA, respectively. Furthermore, this method was applied to determine the kinetic parameters of AtME8 (Table 1). The K_m value of AtME8 for the substrate MeJA was 394 μM. This value is high, compared with that of tomato MJE, which was 15.1 μM [18]. The turnover number was 0.047 s^{-1}, which was five times lower than that of tomato MJE. On the other hand, the K_m value of AtME8 for MeSA was 38.02 μM and the turnover number was 0.24 s^{-1}. These values also represent relatively lower esterase activity, compared with tobacco SABP2, which showed a K_m value of 8.6 μM and a turnover number of 0.45 s^{-1} for MeSA [7]. The K_m value for MeJA was not within

Table 1
Kinetic parameters of AtME8

Substrate	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
MeJA	394	2.79	0.047	0.0001
MeSA	38.02	2.64	0.24	0.0064
MeIAA	294.37	20.24	1.86	0.0063



(C)

Number	1	2	3	4	5	6	7	8	9
Target Mol.	MeBA	BA	MeOAA	MeSA	OAA	SA	MeJA	JA	IAA
m/z	137	195	167	225	225	283	225	283	320

Fig. 6. The GC–MS chromatogram of the plant extracts. Petals of lily (A) and rose (B) were extracted and analyzed by GC–CI–MS in selected ion monitoring mode. The enlarged chromatograms of selected masses of each compound are shown in the insets. Asterisks indicate the identified peaks of each molecule. Detected target molecules and monitored mass of each peak on the chromatogram are shown (C). MeOAA and OAA were used as internal standards for esters and acids, respectively.

the range of the reported *in vivo* MeJA concentration for *Arabidopsis* [9]. However, the K_m value for MeSA was within the range of the *in vivo* MeSA concentration of *Arabidopsis* infected with *Pseudomonas syringae* [8].

3.4. Quantification of phytohormones in plant samples

JA, SA, and IAA were successfully analyzed by methylation with diazomethane [21,22]. However, trimethylsilylation using BSTFA showed a high molar response to GC–MS detection [19]. Thus, we applied this silylation method to the direct detection of plant samples for analyzing JA, SA, IAA, and their methyl esters. Flower perfumes include a variety of volatile compounds, and MeSA and MeJA are major components. They are emitted from the nectary or petals of the flower. Thus, petals are a good source for direct determination of phytohormones and methyl esters. The simple sample preparation method, which used an adsorbent Super Q, was applied to sample extraction [16]. In this extraction method, a very high recovery rate was obtained for methylated JA and SA. Silylated derivatives were also recovered at ≥90% for all silylated compounds (data not shown). About 100 mg of plant material was taken and treated as described in Section 2. As shown in Fig. 6, the major phytohormones and the methyl esters were analyzed on a single chromatogram. Benzoic acid (BA) and benzoic acid methyl ester (MeBA) were included as control compounds in this analysis (Fig. 6). The lily petal possessed most of the phytohormones and their methyl esters; however, in the case of the rose, the amounts of MeSA and SA were too small to be detected as major compounds. The acid forms of phytohormones and their methyl esters were extracted in a single preparation and successively analyzed on a single chromatogram. To our knowledge, this is the first reported method for analyzing acids and the methyl esters simultaneously. The recovery rates of silylated compounds were very high, compared with those for the methylated derivatives.

Compared with previous methods for determination of esterase activity, silylation and GC–MS analysis method employed in this work is relatively simple and more sensitive. Enzymatic re-methylation with radioactive methyl donor, ^{14}C-AdoMet, was also reported [7]. But methyltransferase enzymes for each substrate phytohormone are necessary. Compared with the methylation or ethylation derivatives, the TMS derivatives of

phytohormones showed more intense signals in GC–MS analysis [19]. It makes the method more sensitive and accurate.

Most of previous methods quantificated acidic form of phytohormones. However, it has been known that the methylated phytohormes also have distinct roles in plant and plant environments [7–11]. By using this TMS derivatization method acidic form and methylated form of phytohormes can be separated by a single run of GC–MS. Simultaneous detection of acidic and methylated phytohormones is an advantage of this method.

4. Conclusions

We describe a simple GC–MS analysis method, which included a silylation step. Silylation is a well-known technique in GC–MS analyses, because of the easy volatilization of the compounds and amplification of detection sensitivity [20,23]. Methylation is also a well-known derivatizing method, routinely used for analysis of phytohormones with GC–MS [24,25]. However, the methyl ester forms of these hormones cannot be detected separately from their acid forms. Thus, the method developed here is advantageous for analyzing methyl ester and acid forms by a single preparation on a single chromatogram.

This method was used to test the enzyme activity of methyl esterases. To apply it to the enzyme activity test, we cloned an enzyme possessing methyl esterase activity, AtME8, which exhibits broad substrate specificity. The products of the reactions, the acid forms of the phytohormones, were quantitatively detected with high sensitivity. Compared with previous methods, this method was simple and accurate, because there is no additional enzyme reaction or overlapping of products with the substrate. Furthermore, the absence of radioisotopes in the enzyme reaction is another advantage of this method.

Acknowledgments

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