報　文

Rapid headspace gas chromatography-mass spectrometry to analyze volatiles from *Arabidopsis thaliana* leaves

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Abstract

We used headspace gas chromatography-mass spectrometry (HS-GC-MS) to analyze volatiles collected from *Arabidopsis thaliana* leaves. Eleven compounds were identified from crushed leaves incubated for 30, 60, and 120 min at 50℃. 3-Pentanone, 1-Penten-3-one, Hexanal, (E)-2-pentenal, 1-Penten-3-ol, Heptanal, (E)-2-Hexenal, Methyl thiocyanate, (Z)-2-Penten-1-ol, 1-Hexanol and (Z)-3-Hexen-1-ol were identified. The selective ion monitoring (SIM) mode was used with HS-GC-MS in the analysis of the volatiles. We concluded there was less temperature stress to the plants using the SIM mode that provided much higher sensitivity to detect volatiles emitted from the crushed leaf of *Arabidopsis* where we were able to detect the 11 volatiles at 35℃ after 60 min.

Keywords: *Arabidopsis thaliana*, volatiles, Headspace gas chromatography-mass spectrometry, selective ion monitoring

Introduction

Plants produce numerous volatile metabolites from the derivatives of primary metabolism (Goff & Klee, 2006). Many plants species defend themselves against herbivorous insects indirectly by producing volatiles in response to herbivory and against many microbial pathogen species. These volatiles attract carnivorous predators of the herbivores and attract pollinators. And reports show the volatiles emitted from plants are used for plant-to-plant communication (van Poecke et al., 2001; Kessler & Baldwin, 2001; van Poecke & Dicke, 2002; Blee, 2002; Kishimoto et al., 2005; Baldwin et al., 2006). Further, many of these essential oils of plants are used as flavorings, preservatives, herbal remedies, and scents by humans.

Plants release various volatiles from several major biosynthetic pathways. In the past decade, a large number of pathways and enzymes for the synthesis of plant volatiles have been described (Pichersky et al., 2006). The terpenoids including the monoterpenes (C10) and sesquiterpenes (C15) are derived using the mevalonate pathway or the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. The phenylpropanoids and benzenoids are synthesized using the shikimate pathway. The volatiles that give the "green-note" flavor emitted by green leaves are derived from linoleic acid (hexanal) and linolenic acid (*cis*-3-hexenal, *cis*-3-hexenol, and *trans*-2-hexenal) using the lipoxygenase pathway (Bate et al., 1998; Bate & Rothstein, 1998; Salas et al., 2006; Pichersky et al., 2006). Floral scents are mixtures of volatile compounds including aromatics, terpenoids, and fatty acid derivatives (Peer & Murphy, 2003; Chen et al., 2003). Several genes coding for enzymes in the synthesis of these volatiles have been described (Nair et al., 2002; Aharoni et

al., 2003; Chen et al., 2003; Salas et al., 2006).

We used *Arabidopsis thaliana* as our model plant to describe plant volatiles. *Arabidopsis thaliana* is extensively used as a model plant in molecular biology studies because of its small genome size, short generation time, and complete genetic maps including many mutants (Bate et al., 1998; Nair et al., 2002; Chen et al., 2003; Peer & Murphy, 2003; van Poecke et al., 2001; van Poecke & Dicke, 2002; Kishimoto et al., 2005; Rohloff & Bones, 2005; Salas et al., 2006).

We used headspace gas chromatography-mass spectrometry (HS-GC-MS) to analyze volatiles from *A. thaliana*. And GC-MS analysis using selective ion monitoring (SIM) was used to obtain higher sensitivity.

Materials and Methods

Arabidopsis thaliana Plants

Arabidopsis thaliana wild-type, ecotype Columbia (Col-0), was used. The seeds of *A. thaliana* were grown in fertilized soil plug trays at 22℃ for 5-6 weeks using a controlled climate room (22±1℃, 50-70% RH) under a 16/8 h day/night regime with 3000 to 5000 lux fluorescent lights.

Reagents

Ethanol, 1-Butanol, (E)-2-pentenal and (E)-2-Hexenal were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Hexanol, 3-Pentanone, Hexanal, 1-Penten-3-ol, n-Heptanal, (Z)-2-Penten-1-ol, 1-Hexanol and (Z)-3-Hexen-1-ol were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Penten-3-one and Methylthiocyanate were from Fluka Chemie GmbH and Kanto Chemical (Tokyo, Japan), respectively. These reagents were control standard chemicals used for the qualitative analysis with headspace gas sampling by GC/MS and aided in the identification of the peaks detected from the *A. thaliana* leaves.

Volatile collection and analysis from *A. thaliana*

The leaves of *A. thaliana* were detached with a scissors; 1g was placed in a 20ml vial cooled with iced water; and crushed two min. with a glass rod (~ 360 times) . After crushing, the vial was immediately closed with an open face hole cap equipped with a PTFE/white silicone septum. The volatiles in the headspace above the crushed *A. thaliana* leaves in the vial were analyzed using GC-MS with a single quadrupole mass spectrometer QP5050A equipped with GC-17A and GCMS-QP5050A (Shimadzu, Japan).

Gas chromatography and mass spectrometry analysis (GC-MS)

GC-MS was performed using a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu GCMS-QP5050A mass spectrometer. The GC-MS was equipped with a Shimadzu AOC-5000 auto injector (Shimadzu, Kyoto, Japan). The volatiles were separated using a 60 m×0.32mm (i.d.) fused-silica capillary column coated with a 0.5μm film of polyethylene glycol(Stabilwax (RESTEK)). The carrier gas was helium using a splitting ratio of approximately 1:3 and a flow rate of about 1.7 ml/min. The column temperature program was 35℃ for 5 min followed by ramping from 35℃ to 220℃ at a rate of 2℃/min. The injector temperature was approximately 230℃. Shimadzu GCMS solution software (version 1.20) was used for data processing.

Data analysis

The components were identified using the comparisons of Kovats retention indices (KI) relative to C_9-C_{14} n-alkanes obtained on a polar Stabilwax column and of NIST mass

spectra libraries. Positive identification was assumed when a good match of mass spectrum and KI was achieved; otherwise, it was considered tentative (Davies, 1990; Orav, 2001; Acree and Arn, 2004; Rohloff & Bones, 2005). In addition, the reliable identification of the volatiles was confirmed using authenticated commercial standards.

Results and discussion

Calibration of the headspace GC-MS

Ethanol, 1-butanol, and, 1-hexanol (2 ml in 20 ml stoppered vial) were prepared at the concentrations shown in Figure 1. The vials were incubated for 30 min at 50℃ and the headspace was analyzed using GC-MS. The intensity of the peaks was plotted against the concentration of the standards. Using the least-square linear regression analysis, the calibration curves showed good linearity with correlation coefficients at > 0.999 for all three chemicals (Fig. 1). This indicated the headspace gas sampling method using GC-MS was very accurate for a quantitative analysis.

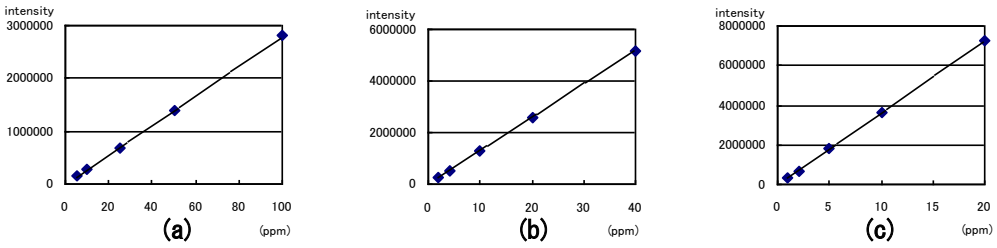

Figure 1. Calibration graphs of ethanol (a), 1-butanol (b) and 1-hexanol (c) obtained using the headspace gas sampling method with GC-MS.

Effects of the incubation time prior to analysis

Arabidopsis thaliana leaves were incubated for 30, 60 and 120 minutes at 50℃; and the volatiles in the headspace were measured using GC-MS. **Figure 2** shows 11 peaks were detected at all incubation times. The intensity in each peak increases with a rise in the incubation temperature. The intensity of the individual peaks from one to 11 in Figure 2 was plotted against the incubation temperatures.

As shown in **Figure 3**, the intensity of 9 peaks (except peak No.2) showed a tendency to increase during incubation at 60 and 120 min than at 30 min. In addition, the peak intensity showed little tendency to decrease or increase after incubation for 120 min. The differences in the peak intensity using various incubation temperatures shows the concentration may be limited to the metabolic rate of the volatiles emitted from *A. thaliana* leaves. We concluded the incubation time to obtain enough volatiles in the vial for analysis using GC-MS was 60 min..

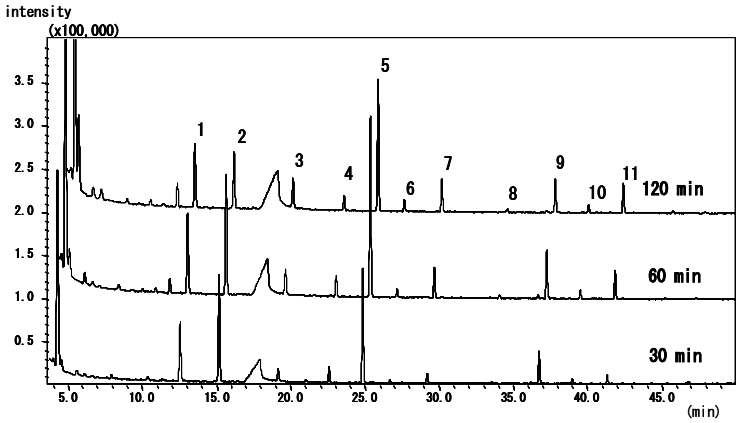

Figure 2. Volatile profiles of *Arabidopsis thaliana* leaf volitiles detected by HS-GC-MS. The leaves were crushed in a 20ml vial and incubated for 30, 60 and 120 minutes at 50℃ before analysis.

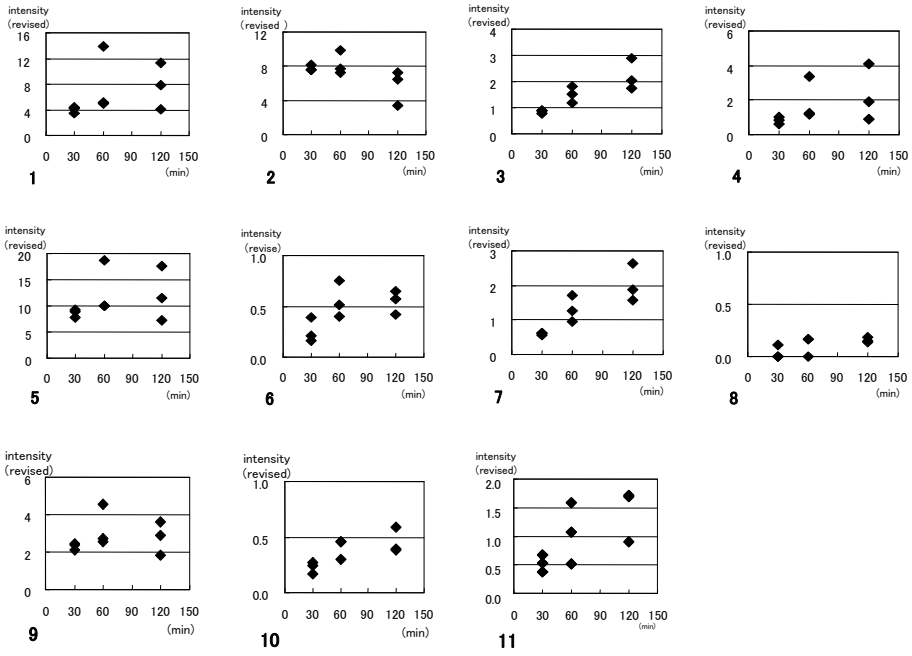

Figure 3. Changes in the peak intensity detected by HS-GC-MS analysis of the *Arabidopsis thaliana* leaves. Samples and analysis condition are the same as in Figure 2. The peak intensities obtained by the GC-MS analysis repeated three times were plotted. The numbers on the graphs correspond to those of each peaks on the chromatograms in Figure 2.

Effects of the incubation temperature before analysis

Figure 4 shows chromatograms for leaves incubated for 60 min at 40 and 50℃. The number of peaks detected from the leaves incubated at 40℃ was less than at 50℃. In this study, hereafter, the GC-MS analysis was carried out after incubation of the vial for 60 min at 50℃ and the peaks on the chromatogram were identified.

The identification of volatiles detected from *A. thaliana* leaves

Figure 5 shows a typical gas-chromatographic profile of the headspace above *A. thaliana* leaves incubated for 60min at 50℃. Eleven components were identified using their mass spectral characteristics, GC-MS retention indices (RI), and co-elution with standards. All of the identified volatiles are listed according to the elution order from the GC in **Table 1** (Acree, T. and Arn, H. 2004; Salas and Aparicio, 2006): 3-Pentanone, 1-Penten-3-one, Hexanal, (E）-2-pentenal, 1-Penten-3-ol, Heptanal, (E)-2-Hexenal, Methyl thiocyanate, (Z)-2-Penten-1-ol, 1-Hexanol and (Z)-3-Hexen-1-ol were identified, respectively; and all of the identified volatile compounds have been shown in previous reports concerning *A. thaliana* leafs. Bate et al.,(1988) report *Arabidopsis* leaf tissue contains C_6-aldehydes hexanal, and trans-2-hexenal as well as the C_6-alcohols: hexanol, and 3-hexenol. As shown in **Figure 6,** the lipoxygenase pathway is responsible for the production of six carbon (C6) volatile compounds. These volatiles are perceived by humans and other animals as the "green-note" flavor associated with many fruits and vegetables (Hatanaka et al. 1987, Hatanaka 1993).

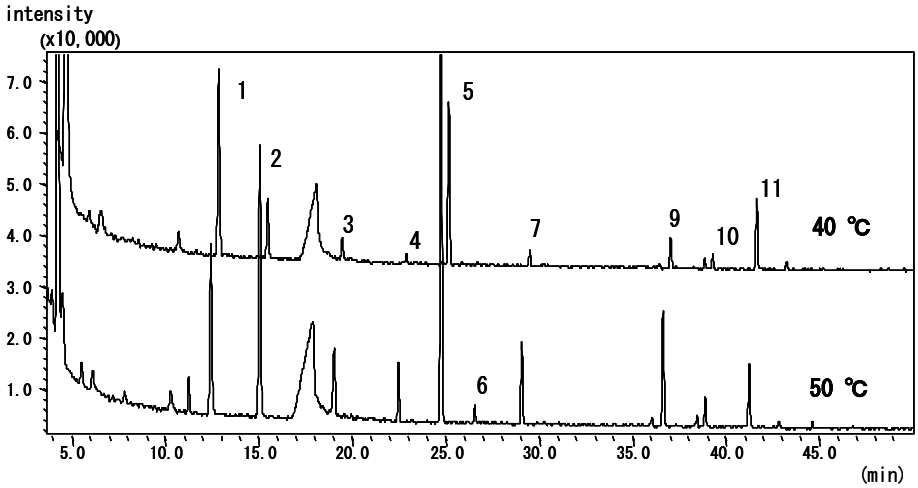

Figure 4. Volatile profiles from *Arabidopsis thaliana* leaves detected by HS-GC-MS. The leaves were crushed in a 20ml vial and incubated for 60 minutes at 40 and 50℃ before analysis.

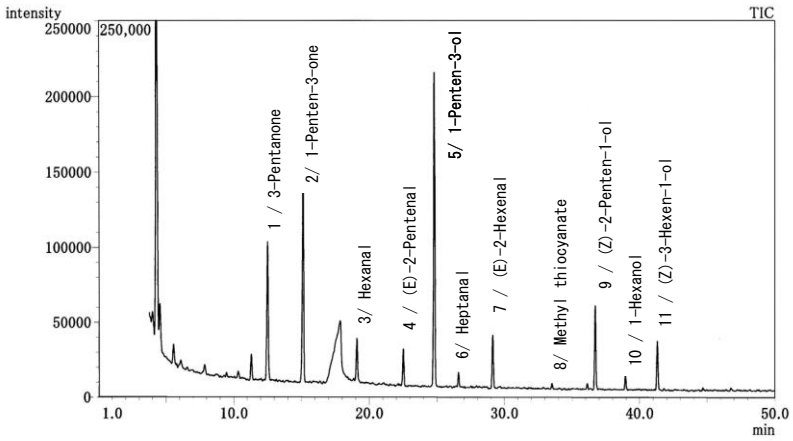

Figure 5. Identification of the volatiles in *Arabidopsis thaliana* leaves detected by HS-GC-MS. The leaves were crushed in a 20ml vial and incubated for 60 minutes at 50℃ before analysis.

Table 1. The identification of volatiles from *Arabidopsis thaliana* leaves. The number column (No.) corresponds to the peak number shown in Figure 2.

No.	Compound	RT[a]	RI[b]	method of identification[c]	odor note
1	3-Pentanone	12.45	986	1,2,3	ether[d]
2	1-Penten-3-one	15.08	1028	1,2	sweet, strawberry[e]
3	Hexanal	19.07	1090	1,2,3	grass, tallow, fat[d]
4	(E)-2-Pentenal	22.50	1139	1,2,3	strawberry, fruit, tomato[d]
5	1-Penten-3-ol	24.79	1170	1,2	butter, pungent[d]
6	Heptanal	26.59	1195	1,2	fat, citrus, rancid[d]
7	(E)-2-Hexenal	29.12	1230	1,2,3	apple, green[d]
8	Methyl thiocyanate	33.54	1289	1,2	
9	(Z)-2-Penten-1-ol	36.71	1333	1,2	banana[e]
10	1-Hexanol	38.96	1364	1,2,3	resin, flower, green[d]
11	(Z)-3-Hexen-1-ol	41.31	1396	1,2,3	grass[d]

[a] Retention time on Stabilwax column.

[b] Linear retention index on Stabilwax column.

[c] 1,tentative identification using mass spectrometry and MS databases; 2,authentic standard injection; 3,coincidence between calculated retention index and known retention index of compounds.

[d] Acree and Arn,2004

[e] Salas et al.,2006

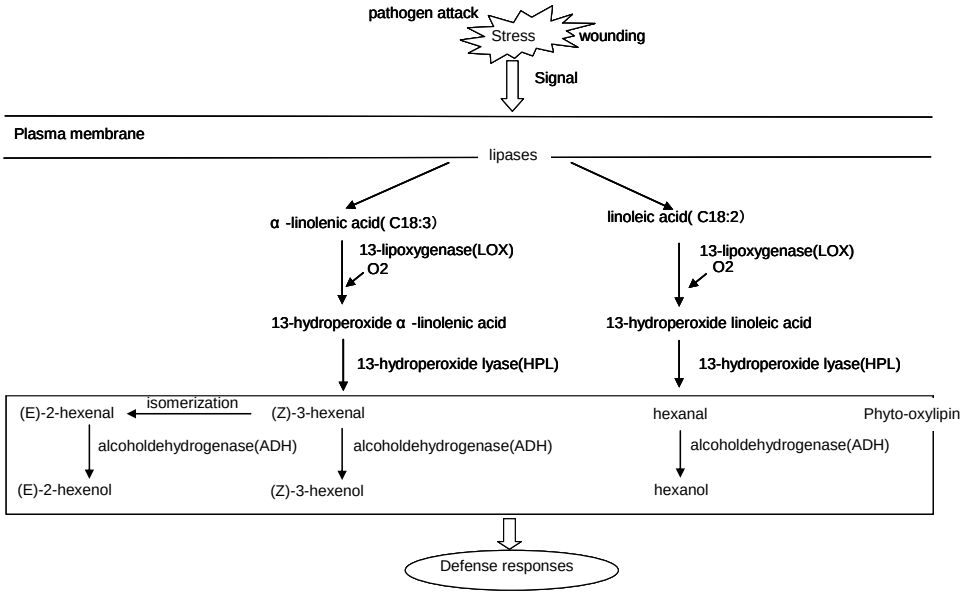

Figure 6. Diagrams showing the pathway to biosynthesis of C_6-volatiles (Bate & Rothstein, 1998).

Detection of peaks using selective ion monitoring (SIM)

The GC-MS technique can be performed using two methods: the full-scan mode or SIM mode. SIM detection using GC-MS detects known compounds at lower concentrations than does the full-scan technique (Rohloff and Bones, 2005). We used the headspace gas for GC-MS in SIM mode to analyze the volatiles from *A. thaliana* leaves. **Figure 7** shows chromatograms from crushed leaves incubated for 60min at 35,40 and 50℃, respectively. Comparing the GC-MS analysis using full-scan mode to the SIM mode shows SIM enhanced the sensitivity of the GC-MS method to detect the volatiles (Fig. 4). When using SIM mode interference was reduced. SIM allowed detection at much lower concentrations. Our data shows GC-MS using SIM provides much higher sensitivity to detect volatiles emitted from crushed leaves of *Arabidopsis*. This technique was sensitive at a gentle incubation of 35℃ for 60 min that would not be a stress temperature for the monitoring of healthy plants.

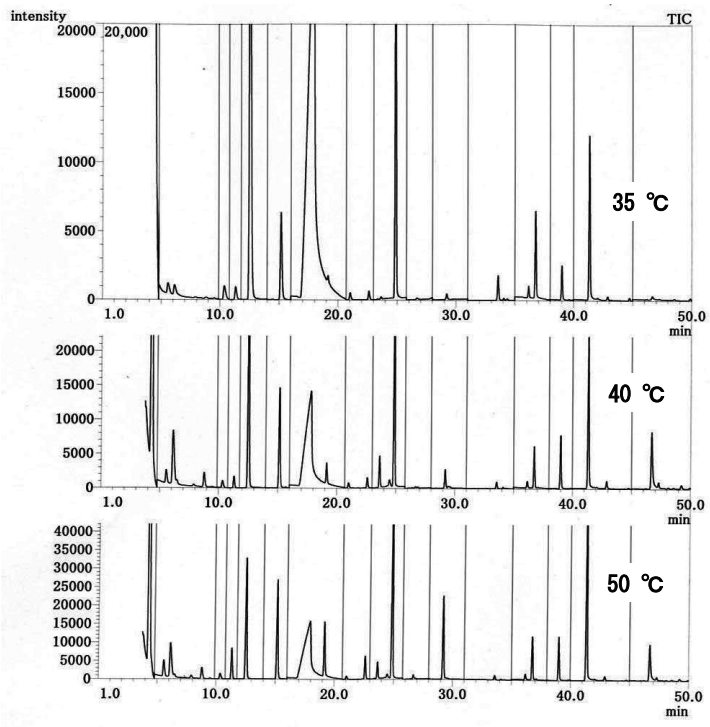

Figure 7. Gas chromatogram of the headspace above the *Arabidopsis thaliana* leaves obtained in SIM mode. The leaves were crushed in a 20ml vial and incubated for 60 minutes at 35, 40 and 50℃ before analysis.

Acknowledgements

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Title：ヘッドスペース・ガスクロマトグラフ質量分析法によるシロイヌナズナ葉の揮発性物質の迅速分析法

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