

**Title** Two types of alcohol dehydrogenase from *Perilla* can form citral and perillaldehyde

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**Abstract**

Studies on the biosynthesis of oil compounds in *Perilla* will help in understanding regulatory systems of secondary metabolites and in elucidating reaction mechanisms for natural product synthesis. In this study, two types of alcohol dehydrogenases, an aldo-keto reductase (AKR) and a geraniol dehydrogenase (GeDH), which are thought to participate in the biosynthesis of perilla essential oil components such as citral and perillaldehyde, were isolated from three pure lines of perilla. These enzymes shared high amino acid sequence identity within genus *Perilla*, and were expressed regardless of oil type. The overall reaction from geranyl diphosphate to citral was performed in vitro using geraniol synthase and GeDH to form a large proportion of citral and relatively little geraniol as reaction products. The biosynthetic pathway from geranyl diphosphate to citral, the main compound of citral-type perilla essential oil, was revealed in this study.

**Keywords**

*Perilla*; Labiatae; aldo-keto reductase; geraniol dehydrogenase; geraniol; citral; sequential enzymatic reaction; biosynthetic pathway; Essential oil

## 1. Introduction

Japanese perilla plants are classified into four species including a cultivated species, *Perilla frutescens*, and three wild species, *P. citriodora*, *P. hirtella*, and *P. setoyensis* (Ito and Honda, 1996). Plants of each species contain essential oil that is composed principally of unique monoterpene or phenylpropene compounds. Each monoterpene (MT)-type oil can be further classified according to its principal constituent: C (citral, **1**), EK (elsholtziaketone, **2**), PA (perillaldehyde, **3**), PK (perillaketone, **4**), PL (perillene, **5**), PT (piperitenone, **6**), and SF (shisofuran, **7**) (Fig. 1) (Ito et al., 1999a). The synthesis of each of these types of oil is genetically controlled in perilla (Honda, 1996), and the functions of each gene can be determined by cloning the enzymes that catalyze the relevant reaction steps in the biosynthetic pathway. Elucidating the biosynthesis of these oil components should improve the engineering of useful secondary metabolites in plants and may also facilitate natural product syntheses involving similar reaction mechanisms. *P. frutescens*, the cultivated species, is hypothesized to have evolved as an amphidiploid of the two wild species, according to previous crossing and genotyping results (Honda and Ito, 1998; Ito and Honda, 2007). Progenies arising from interspecific crosses were subjected to natural selection during the process of forming the amphidiploid, and acquired both their characteristic morphological features and their biosynthetic pathways for secondary metabolites such as their essential oil constituents. Studies of the various oil types present in perilla might help elucidate how the cultivated species of perilla evolved from the wild species (Ito, 2008).

The initial reaction step in the putative pathway for the MT-type oil constituents is the dephosphorylation of geranyl diphosphate (GDP, **8**) catalyzed by either geraniol or limonene synthase. Linalool (**9**), which can be found in all perilla plants regardless of oil type and may be a dead-end compound in general monoterpene biosynthetic pathways (Pichersky et al., 1995), is also synthesized from GDP by linalool synthase. Our previous studies revealed that geraniol and linalool synthases from *P. citriodora*, *P. frutescens*, *P. hirtella*, and *P. setoyensis*, and also limonene synthases from *P. citriodora* and *P. frutescens* (Ito and Honda, 2007; Masumoto et al., 2010; Yuba et al., 1996) share very similar amino acid sequences. Enzymes in the cytochrome P450 family are also reported to function as limonene hydroxylase (Mau et al., 2010).

Geraniol (**10**) and citral are acyclic monoterpenes commonly found in a wide range of plants; these compounds accumulate in glandular trichomes and are emitted into the air. In perilla, citral is the main component in C-type essential oil. Both geraniol and citral are commercially important for perfumery, and for flavoring of food and medicine, and are pharmaceutically important for their antioxidant, antibacterial, and antitumor activities (Chen and Viljoen, 2010; Dudai et al., 2005). Geraniol is formed via dephosphorylation of GDP by geraniol synthase (Iijima et al., 2004), which has been cloned from four perilla species, as mentioned above (Ito and Honda, 2007; Masumoto et

al., 2010). Citral, a mixture of neral and geranial, has been proposed to be synthesized from geraniol by alcohol dehydrogenases. There are a few reports of the amino acid sequences of alcohol dehydrogenases that transform geraniol to citral in sweet basil, *Ocimum basilicum* (Iijima et al., 2005); the astigmatid mite, *Carpoglyphus lactis* (Noge et al., 2008); and a betaproteobacterium, *Castellaniella defragrans* (Lüddecke et al., 2012)).

Here we report the cloning and characterization of two different types of alcohol dehydrogenases that were identified by analysis of an expressed sequence tag (EST) library from perilla. Each of these cloned enzymes could, in combination with geraniol synthase, perform the sequential conversion of GDP to citral via geraniol in vitro.

## 2. Results and Discussion

### 2.1. Isolation of two types of alcohol dehydrogenases from perilla

A cDNA library was constructed using mRNA isolated from young perilla leaves and was sequenced using next-generation sequencing technology. Complete BLASTP searches were then performed in the EST database established from these sequenced cDNAs. Two different types of cDNAs encoding putative alcohol dehydrogenases were found. One cDNA showed the highest amino acid sequence similarity with aldo-keto reductase (AKR), and another eight AKR-like cDNAs were also retrieved from the EST database. Among these nine, one cDNA (*AKR1*) showed the highest amino acid sequence similarity to an AKR enzyme from *Salvia miltiorrhiza* (GenBank Accession No. EF666999). The sequence of one unique putative alcohol dehydrogenase was very similar to that of a geraniol dehydrogenase (GeDH) from *Ocimum basilicum* (GenBank Accession No. AY879284). The functions of these two dehydrogenases were further investigated.

Based on these sequences, two sets of primers for amplifying *AKR1* and *GeDH* were designed to clone these genes from *P. frutescens* [Strain No. 32], *P. citriodora* [Strain No. 87], and *P. setoyensis* [Strain No. 5031]. Consequently, we isolated the following cDNA clones (Pf, Pc, and Ps denotes *P. frutescens*, *P. citriodora*, and *P. setoyensis*, respectively): *PfAKR* and *PfGeDH* from *P. frutescens* (GenBank Accession Nos. JX629451 and JX855836); *PcAKR* and *PcGeDH* from *P. citriodora* (GenBank Accession Nos. JX629452 and JX855837); and *PsAKR* and *PsGeDH* from *P. setoyensis* (GenBank Accession Nos. JX629453 and JX855838) (Fig. 2). The cDNA sequences isolated for *PfAKR*, *PcAKR*, and *PsAKR* each consisted of 1062 nucleotides encoding a protein of 354 amino acids (Fig. 2), and the putative enzyme *PsAKR* had 66% amino acid identity to the AKR from *S. miltiorrhiza*. The 1080-nucleotide cDNA sequences of *PfGeDH*, *PcGeDH*, and *PsGeDH* encoding 360 amino acids (Fig. 2) shared up to 87.5% amino acid identity, in the case of *PcGeDH*, to the enzyme GeDH from *O. basilicum*.

There was extremely high amino acid identity (>98%) among the three *AKR1*s cloned from perilla, and the same was true among the *GeDH* cDNA sequences we cloned. However, the amino acid identity between the putative AKR and GeDH protein sequences isolated from one perilla strain was low.

## 2.2 Functional analysis of PsAKR and PcGeDH

### 2.2.1 Enzymatic reactions including components of C-type essential oil of perilla

Citral is a main component of the essential oil extracted from *P. citriodora* used in this study (No. 87) (Ito et al., 1999b), and is likely synthesized from GDP via geraniol (Fig. 1). Shisofuran, a main compound of the essential oil of *P. setoyensis* (No. 5031), is likely made from GDP via geraniol and citral (Fig. 1). According to the putative biosynthetic pathways for these oil compounds, both AKRs and GeDHs from *P. citriodora* and *P. setoyensis* were thought to be capable of converting geraniol to citral. Both AKR1 and GeDH shared greater than 98% amino acid sequence identity between these perilla species). The AKR from *P. setoyensis*, PsAKR, and the GeDH from *P. citriodora*, PcGeDH, were selected for further characterization. PsAKR and PcGeDH were heterologously expressed in *Escherichia coli*, and the resultant His-tagged proteins were purified and used for enzymatic reactions with geraniol as a substrate. Both PsAKR and PcGeDH transformed geraniol into citral (geranial and neral) and nerol (Figure 3B, E) by using NADP<sup>+</sup> as a cofactor. NADP<sup>+</sup> was chosen as the cofactor for all enzymatic oxidative reactions in this study because larger quantities of reaction products could be detected in reactions containing NADP<sup>+</sup> than in those containing NAD<sup>+</sup>. The control solutions without cofactors produced no reaction products (Figure 3A, D).

Citral is a mixture of geranial and neral, and at equilibrium, the ratio of geranial to neral is 1.5 (Kuwahara, 1983). The time courses for enzymatic reactions of PsAKR or PcGeDH with geraniol as a substrate were evaluated in terms of the geranial/neral ratio of the reaction products. Over short periods of time, both PsAKR and PcGeDH reactions showed a high ratio of geranial to neral in reaction products, and this ratio decreased to 1.5 with longer incubation times (data not shown).

Because PsAKR shared high amino acid sequence identity with known aldo-keto reductases, it might also catalyze the reverse reaction of geraniol oxidation, as other AKRs reportedly reduce aldehydes to alcohols (Jez, 1997). Both PsAKR and PcGeDH could reduce citral to geraniol and nerol when NADPH was added as cofactor (Figure 3C, F), but not when NADP<sup>+</sup> was the cofactor (data not shown). These results show that both PsAKR and PcGeDH can catalyze either oxidation or reduction.

These results suggest that geranial was formed from geraniol by enzymatic reaction of PsAKR or PcGeDH (Figure 3B, E) and was then isomerized to neral with or without an enzyme. Nerol might

then form from geranial or neral by the action of PsAKR or PcGeDH with NADPH generated from NADP<sup>+</sup> by the oxidation of geraniol.

Nerol has previously been reported as one of the oil constituents of C -type and PL-type perilla, which respectively contain a greater and lesser amount of citral in their essential oil (Ito et al., 1999a; Ito et al., 1999b). However, neither nerol nor citral could be found in the oils that contain perillaketone (PK), elsholtziaketone (EK), or shisofuran (SF) as main components, even though these primary constituents may have been formed via citral as an intermediate. It seems that the C- and PL-types of perilla do transform neither citral nor perillene to further oxidized compounds, whereas the PK-, EK-, and SF-types of perilla do convert citral into various oxidized compounds (Fig. 1). Nerol was not found in perilla types that oxidize citral, implying that the oxidation pathway is related to accumulation of citral and nerol.

### 2.2.2 Enzymatic reactions with compounds found in essential oils of other types of perilla

Strain No. 32 (PA-type) also carries and expresses genes for *PfAKR* and *PfGeDH*, even though the biosynthetic pathways for PA oil components do not include citral (Figure 1) (Ito et al., 1999a). This means that geraniol might not be a specific substrate for these two enzymes. Accordingly, some monoterpene alcohols other than geraniol were examined for their abilities to serve as a substrate for PsAKR and PcGeDH. A primary monoterpene alcohol, perilla alcohol (11), was converted into perillaldehyde by both PsAKR and PcGeDH using NADP<sup>+</sup> as cofactor (Figure 4B, D), whereas perillaldehyde was changed into perilla alcohol by PsAKR and PcGeDH using NADPH as cofactor (Figure 4C, F). When PcGeDH was incubated either with perilla alcohol and NADP<sup>+</sup> or with perillaldehyde and NADPH, *trans*-shisool was found in reaction products. However, *trans*-shisool was not detected in the reaction products of PsAKR. Shisool is an oil component of PA- and PP-type perilla essential oils, both of which contain perillaldehyde (Ito et al., 1999a), and might be synthesized from perilla alcohol or perillaldehyde by PcGeDH.

Secondary and tertiary monoterpene alcohols, namely, menthol and linalool, were not oxidized by either PsAKR or PcGeDH (cofactor: NADP<sup>+</sup>). Menthone (cofactor: NADPH) and limonene (12), which have no hydroxyl group (cofactor: NADP<sup>+</sup> or NADPH), were not transformed by any of the enzymes studied here. These results indicate that both PsAKR and PcGeDH transform only primary alcohols and aldehydes.

(-)-Isopiperitenol/(-)-carveol dehydrogenase and menthone reductase from peppermint (GenBank Accession Nos. AY641428 and AY288137) are monoterpene dehydrogenases previously cloned from plants in family Labiatae, and were respectively reported to oxidize secondary monoterpene alcohols and to reduce ketones (Davis et al., 2005; Ringer et al., 2005). Both of these enzymes are members of the short-chain dehydrogenase/reductase superfamily. On the other hand, AKRs from

perilla are more similar in amino acid sequence to the aldo-keto reductase superfamily, and GeDHs are homologous to the medium-chain dehydrogenase/reductase superfamily (Lüddecke et al., 2012). Accordingly, dehydrogenases from perilla are clearly distinguishable from those from peppermint by their structures (Joërnvall, 1999). These two groups differ further in substrate preference: (-)-isopiperitenol/(-)-carveol dehydrogenase from peppermint could not use perilla alcohol as a substrate (Ringer et al., 2005), whereas PsAKR and PcGeDH could catalyze reactions with perilla alcohol. These findings imply that these dehydrogenases from perilla and from peppermint are functionally different. However, isopiperitenol (**13**) was thought to be an intermediate in the biosynthetic pathways of oil components in perilla (Fig. 1), so an enzyme different from AKR1 or GeDH that is responsible for the (-)-isopiperitenol/(-)-carveol dehydrogenase reaction should exist in perilla.

### 2.3. Sequential enzymatic reactions of geraniol synthase and alcohol dehydrogenase (either AKR or GeDH) in vitro using GDP as a substrate

Biosynthetic reactions of natural products, especially regiospecific or stereospecific reactions, can be useful to synthetic chemists by providing intermediates along routes to natural products or offering hints to reaction mechanisms. Furthermore, these synthetic reactions could be used to produce useful compounds. Because we had obtained enzymes catalyzing two sequential steps in monoterpene synthesis in perilla, we were able to carry out sequential enzymatic reactions in vitro.

Heterologously expressed PcTps-C, a geraniol synthase previously cloned from *P. citriodora* (No. 87) (Ito and Honda, 2007) that catalyzes dephosphorylation of GDP to form geraniol with divalent metal ions ( $Mn^{2+}$  and  $Mg^{2+}$ ) as cofactors (Ito and Honda, 2007; Masumoto et al., 2010), and PsAKR or PcGeDH, which converts geraniol into citral, were used to perform in vitro conversion of GDP to citral. GDP could be transformed into citral by reactions using either combination of PcTps-C with PsAKR or PcTps-C with PcGeDH (Figure 5). However, the proportion of geraniol to citral in the resultant reaction products differed between combinations with PsAKR or PcGeDH. PsAKR reactions produced less citral than geraniol, whereas PcGeDH reactions produced much more citral than geraniol.

The ratio of geraniol to citral in reaction products from sequential reactions using PcTps-C with PcGeDH was comparable to that naturally occurring in perilla essential oil (Ito et al., 1999a, 1999b); namely, much less geraniol was detected than citral in the C-type oil. The amounts of geraniol in the SF-type and PK-type oils, whose main components are likely synthesized via citral, were also small.

As for the kinetics of these enzymes, both PsAKR and PcGeDH exhibited typical Michaelis-Menten kinetics with geraniol, and their  $k_{cat}/K_m$  values for geraniol were measured. The  $k_{cat}/K_m$

value of PcGeDH ( $2.07 \times 10^{-5} \text{ s}^{-1} \text{ M}^{-1}$ ) for geraniol was larger than that of PsAKR ( $3.35 \times 10^{-6} \text{ s}^{-1} \text{ M}^{-1}$ ), suggesting that PcGeDH converts larger amounts of geraniol into citral than does PsAKR in the same amount of time. This might explain the lower geraniol/citral ratio in the products of sequential reactions in vitro with PcGeDH than in those with PsAKR (Figure 5).

#### 2.4. Confirmation of *AKR1* and *GeDH* gene expression in C-, PA-, PK-, and PP-type perilla

Transcription levels of the *AKR* and *GeDH* genes in perilla Strains No. 87 (C type), No. 32 (PA type), and No. 6 (PK type) were compared by a semi-quantitative reverse transcription (RT)-PCR method. These three strains differ in oil constituents even though *AKR1* and *GeDH* transcripts can be detected in either strain (Figure 6). Although expression levels of the genes for these two enzymes were variable, that of No. 87, the C-type strain that accumulates a large proportion of citral, was the highest among these three perilla strains. The genes for these two enzymes were also expressed in PP-type perilla (data not shown), but their role in the biosynthetic pathways of oil components in PP-type perilla is unknown. These results demonstrated that both AKR and GeDH genes were actively expressed in many types of perilla, regardless of species or chemotype.

Genus *Perilla* exhibits various essential oil profiles among its wild and cultivated species, although the PA-type oil is found exclusively in the cultivated species *P. frutescens* (Ito, 2008). The differences in the essential oil types of perilla are significant because the PA-type oil is the one allowed for medicinal use in the Japanese Pharmacopeia (PMRJ, 2012). The species *P. frutescens* is hypothesized to have evolved as an amphidiploid of two wild species (Honda and Ito, 1998; Ito and Honda, 2007), and the PA-type essential oil profile is thought to have developed during the process of evolution of the amphidiploid. The lower substrate specificity of AKR and GeDH might have contributed to the diversity of oil types and oil components currently found in perilla.

### 3. Conclusion

Two different types of alcohol dehydrogenase, namely, AKR and GeDH, from *P. frutescens* (No. 32), *P. citriodora* (No. 87), and *P. setoyensis* (No. 5031) were isolated and characterized. Three AKRs shared extremely high amino acid sequence similarity (>98%), as did the GeDHs that we identified. Both PsAKR and PcGeDH could convert geraniol into citral and nerol, and could also convert perilla alcohol into perillaldehyde. On the other hand, these enzymes did not oxidize secondary and tertiary monoterpene alcohols such as linalool and menthol, suggesting that PsAKR and PcGeDH can act on only primary monoterpene alcohols as substrates. Sequential in vitro enzymatic reactions of PcTps-C and PcGeDH with GDP as the substrate predominantly produced citral, and a small amount of geraniol. The proportions of citral and geraniol in the resultant

reaction products were very similar to those in the C-type essential oil of perilla (Ito et al., 1999a, 1999b).

In this study, we have revealed the biosynthetic pathway from GDP to citral, a main component of the C-type perilla essential oil (Figure 1). The genes for the AKR and GeDH enzymes were expressed in perilla regardless of chemotype, and the enzymes showed broader substrate specificity than did monoterpene synthases such as geraniol synthase. The lower substrate specificity of the enzymes and oil-type-independent expression of the *AKR* and *GeDH* genes are characteristics that might have contributed to the diversity of oil types observed in perilla as the cultivated species was developed from an amphidiploid.

## 4. Experimental

### 4.1. General experimental procedures

Authentic geraniol and citral, and all chemical reagents and solvents were purchased from either Nacalai Tesque Inc. (Kyoto, Japan) or Wako Pure Chemical Industries Co. (Osaka, Japan). Agarose gel and plasmid extraction kits were purchased from Takara (Shiga, Japan), the His-tagged protein purification kit was purchased from Qiagen (Valencia, CA), and vectors and *E. coli* competent cells were obtained from Invitrogen (Carlsbad, CA). Solid-phase microextraction (SPME) fiber (100  $\mu$ m polydimethylsiloxane) was from Supelco (Bellefonte, PA). Polymerase chain reactions were performed on a Thermal Cycler Personal (Takara). GC-MS measurements were carried out on a G-9000M gas chromatograph (Hitachi, Tokyo, Japan) connected to an M-7000 system (Hitachi) or GC-MS (6850GC/5975MSD; Agilent Tech., Santa Clara, CA). GC-FID measurements were performed on a G-5000 gas chromatograph (Hitachi). DNA sequencing was performed by Bio Matrix Research Inc. (Chiba, Japan) and FASMAC Co., Ltd. (Kanagawa, Japan).

### 4.2. Plant materials

All perilla plants used in this study were grown at the Experimental Station for Medicinal Plant Research, Graduate School of Pharmaceutical Sciences, Kyoto University. They have been bred and kept as pure lines as described in a previous study (Ito and Honda, 2007). Strain numbers, oil types, and species names of perilla used in this study were as follows: *P. frutescens* [Strain No. 6, PK type, Strain No. 12, PP type, and Strain No. 32, PA type]; *P. citriodora* [Strain No. 87, C type]; and *P. setoyensis* [Strain No. 5031, SF type].

### 4.3. Construction of EST database and cloning of alcohol dehydrogenases from perilla

Construction of cDNA libraries and sequencing of cDNAs to develop an EST database were carried out by Kazusa DNA Research Institute. Total RNA was extracted from young perilla leaves using the RNeasy Plant Mini Kit (Qiagen), and was processed according to the mRNA-Seq protocol (Illumina, San Diego, CA). The cDNA library was sequenced using the Illumina's next-generation sequencing instrument Genome Analyzer IIx with 100 bp paired-end (PE) reads. The Illumina reads were assembled using the commercially available CLC Genomics Workbench version 4.7.2 (CLC bio, Cambridge, MA). Assembled contigs were used as queries against the non-redundant (nr) protein database using the BLASTX algorithm.

BLAST searches revealed two types of alcohol dehydrogenases, and two pairs of primers were designed for homology-based PCR approaches: 233AKR-f1 (5'-ATGGCTACGGCGGCGATGACGGT-3') as forward primer and 233AKR-r (5'-TACTCCATCGTCAATGTGGTTCTCTAGTTT-3') as reverse primer for cloning of PcAKR, PfAKR, and PsAKR; GeDH-f (5'-ATGGCGAAAACCCAGAAACAGAGCAC-3') as forward primer with GeDH-r (5'-GTGAGCATTGAAAGATTTGTTAACATC-3') as reverse primer for cloning of *PcGeDH*, *PfGeDH*, and *PsGeDH*. Total RNA was isolated from fresh young leaves of perilla (Strain No. 5031 was used to isolate *PsAKR* and Strain No. 87 was used to isolate *PcGeDH*), reverse transcribed to cDNA by ReverTra Ace (Toyobo, Osaka, Japan). PCR for the determination of *AKR* sequences was performed in a reaction mixture containing 0.5 units ExTaq polymerase (Takara), 0.2 mM dNTPs, 0.3  $\mu$ M forward primer and reverse primer, and 1.5% DMSO with a temperature program starting at 94 °C for 100 s, followed by 27 cycles of 94 °C for 30 s, 57 °C for 43 s, 72 °C for 80 s, and a final elongation at 72 °C for 80 s. For determination of *GeDH* sequences, PCR was performed using Blend Taq (Toyobo) instead of ExTaq polymerase, with a temperature program starting at 94 °C for 100 s, followed by 33 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 70 s, and a final elongation at 72 °C for 70 s. Amplified *AKR* and *GeDH* sequences were cloned into pCR T7/CT-TOPO (Invitrogen) and pET101/D-TOPO (Invitrogen), respectively, sequences were confirmed, and each clone was expressed in an *E. coli* expression system, as described below.

#### 4.4. Heterologous expression of cDNAs in *E. coli* and His-tagged protein purification

Geraniol synthase used in this study was PcTps-C (GenBank Accession No. DQ088667) previously cloned from *P. citriodora* (Ito and Honda, 2007). Each plasmid harboring *PcTps-C*, *PsAKR*, or *PcGeDH* was transformed into BL21(DE3)pLysS (Invitrogen) competent cells. Cultures initiated from single colonies were incubated in LB medium and cultured at 37 °C for 6-8 h, followed by inoculating 40 mL of LB (*PsAKR* and *PcGeDH*) or TB (*PcTps-C*) medium containing 0.1 mg/ml of ampicillin with the culture. After 1.5-2 h incubation at 37 °C with shaking at 170 rpm, isopropylthio- $\beta$ -galactoside (IPTG) was added to the culture to a final concentration of 1 mM for

expression of PcTps-C and PsAKR proteins, while 1 mM IPTG and 10  $\mu$ M ZnCl<sub>2</sub> were added to cultures for expression of PcGeDH protein. The cultures were then incubated for 26-41 h at 16 °C with shaking at 220 rpm. Isolation and purification of His-tagged proteins were performed as described in a previous study (Kumeta and Ito, 2010).

#### 4.5. Enzymatic assays and GC-MS analysis

**Enzymatic reactions:** Reaction mixtures were composed of 50 mM Tris buffer (pH 7.0), 10% (v/w) glycerol, 1 mM dithiothreitol, and purified recombinant proteins. In the PcTps-C reaction, 28  $\mu$ M GPP, 0.5 mM MnCl<sub>2</sub> and 20 mM MgCl<sub>2</sub> were added to the mixture, and in the PsAKR and PcGeDH reactions, 0.1 mM substrate and 1 mM cofactor (NADP<sup>+</sup> or NADPH) were added. When the coupled reactions of geraniol synthase and alcohol dehydrogenases were performed, reaction mixtures included 50 mM Tris buffer (pH 7.0), 10% (v/w) glycerol, 1 mM dithiothreitol, 28  $\mu$ M GPP, 0.5 mM MnCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, 1 mM NADP<sup>+</sup>. Purified PcTps-C and either PsAKR or PcGeDH alcohol dehydrogenase were added to final concentrations of 10% or 20% (v/v), respectively. A preparation devoid of cofactors (NADP<sup>+</sup> or NADPH) was used as a control for enzyme assays. Complete reaction mixtures were incubated at 30 °C for 16 h for enzymatic reactions.

**SPME-GC-MS analyses:** Enzymatic reactions were performed in a solution of 250  $\mu$ l in a 4 ml glass vial sealed with a polytetrafluoroethylene-coated silicone rubber septum. After the reaction, SPME fiber (100  $\mu$ m polydimethylsiloxane; Supelco) was inserted into the headspace of the vial at room temperature for 30 min and was then transferred to the injector of a G-9000M gas chromatograph connected to an M-7000 system (Hitachi). The SPME fiber was heated for 10 min at 200 °C for desorption of compounds. Compounds were separated on a TC-WAX column (60 m  $\times$  0.25 mm, 0.25  $\mu$ m film thickness; GL Sciences Inc., Tokyo, Japan) with a temperature program starting at 100 °C, holding at this temperature for 5 min, then increasing at 5 °C min<sup>-1</sup> to 200 °C, and holding at this temperature for 5 min. Helium was used as carrier gas, and column flow was set to 1.0 ml min<sup>-1</sup>. Eluted compounds were identified by comparisons to ion spectra and relative retention times of authentic standards.

**GC-MS analyses:** Enzymatic reactions were prepared in screw-capped glass tubes in a 500- $\mu$ l volume. After incubation, the reaction mixture was extracted three times with pentane, while 12 nmol of limonene in 10  $\mu$ l of hexane was added to each tube as an internal standard just before extraction. The pentane fractions were combined and concentrated under a N<sub>2</sub> gas stream to be injected into the GC-MS (6850GC/5975MSD; Agilent Tech.) system equipped with a DB-WAX column (60 m  $\times$  0.25 mm, 0.25  $\mu$ m film thickness; Agilent Tech.). Operation conditions were as follows: injector, 160 °C; oven program starting at 50 °C, holding at this temperature for 5 min,

then increasing at 5 °C min<sup>-1</sup> to 200 °C, and holding at this temperature for 5 min. Helium was used as carrier gas, and column flow was set to 1.0 ml min<sup>-1</sup>. Reaction products were identified by comparing their retention times and mass spectra with those in a MS data library (NIST11; National Institute of Standards and Technology).

**GC-FID analyses for kinetic analyses:** Purified enzymes were incubated at 30 °C for 6 h in the case of PsAKR, and for 3 h in PcGeDH in 1 mL reaction volumes with GDP ranging from 25 to 250 μM. After incubation, extraction of the reaction mixture was accomplished in the same manner as for analyses by GC-MS. The pentane fractions were injected into the GC-FID G-5000 gas chromatograph (Hitachi) system equipped with an InertCap WAX column (60 m × 0.25 mm, 0.25 μm film thickness; GL Sciences Inc.). Operation conditions were as follows: injector, 160 °C; FID, 210 °C; oven program starting at 50 °C, holding at this temperature for 5 min, then increasing at 3 °C min<sup>-1</sup> to 200 °C, and holding at this temperature for 5 min. Assays were repeated three separate times.

#### 4.6. Semi-quantitative RT-PCR

Total RNA was isolated from young perilla leaves (Strain Nos. 87, 32, and 6) using the method described above, and first-strand cDNAs were synthesized using ReverTra Ace (Toyobo) and an oligo(dT) primer (Takara) following the manufacturers' protocols. PCR was performed with two pairs of primers to amplify *PcAKR* or *PcGeDH* for quantitative analysis. Histone provided the internal control for PCR using the forward primer 5042histone-f (5'-TAAGAAGAAGCCGGTTACCCGCT-3') and reverse primer 5042histone-r (5'-CTTGGTTCTGAATCAAAAGCAC-3').

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## Figure legends

**Figure 1.** Putative biosynthetic pathways of oil constituents (MT-type) in perilla. (1) Citral; (2) elsholtziaketone; (3) perillaldehyde; (4) perillaketone; (5) perillene; (6) piperitenone; (7) shisofuran; (8) geranyl diphosphate; (9) linalool; (10) geraniol; (11) perilla alcohol; (12) limonene; (13) isopiperitenol.

**Figure 2.** Alignment of the amino acid sequences of alcohol dehydrogenases described in this study.

PfAKR: aldo-keto reductase from *Perilla frutescens* [Strain No. 32, PA type] (GenBank Accession No. JX629451);

PcAKR: aldo-keto reductase from *P. citriodora* [Strain No. 87, C type] (GenBank Accession No. JX629452);

PsAKR: aldo-keto reductase from *P. setoyensis* [Strain No. 5031, SF type] (GenBank Accession No. JX629453);

PfGeDH: geraniol dehydrogenase from *P. frutescens* [Strain No. 32, PA type] (GenBank Accession No. JX855836);

PcGeDH: geraniol dehydrogenase from *P. citriodora* [Strain No. 87, C type] (GenBank Accession No. JX855837);

PsGeDH: geraniol dehydrogenase from *P. setoyensis* [Strain No. 5031, SF type] (GenBank Accession No. JX855838).

Black background indicates 100% amino acid identity among six clones, and gray color indicates greater than 50% amino acid identity.

**Figure 3.** GC charts of the reaction products of PsAKR and PcGeDH when either geraniol or citral was utilized as substrate. (A), (B), (D), and (E) are total ion chromatograms.

(A) Control assay of PsAKR devoid of cofactor (NADP<sup>+</sup>); (B) enzymatic reaction of PsAKR with geraniol; (C) enzymatic reaction of PsAKR with citral; (D) control assay of PcGeDH devoid of cofactor (NADP<sup>+</sup>); (E) enzymatic reaction of PcGeDH with geraniol; (F) enzymatic reaction of PcGeDH with citral.

Closed black circles indicate neral, open circles indicate geranial, gray circles indicate nerol, and closed squares indicate geraniol.

**Figure 4.** GC charts of the reaction products of PsAKR and PcGeDH with either perilla alcohol or perillaldehyde used as substrate. All charts are total ion chromatograms.

(A) Control assay of PsAKR devoid of cofactor ( $\text{NADP}^+$ ); (B) enzymatic reaction of PsAKR with perilla alcohol; (C) enzymatic reaction of PsAKR with perillaldehyde; (D) control assay of PcGeDH devoid of cofactor ( $\text{NADP}^+$ ); (E) enzymatic reaction of PcGeDH with perilla alcohol; (F) enzymatic reaction of PcGeDH with perillaldehyde.

Closed circles indicate perillaldehyde, open circles indicate perilla alcohol, and gray circles indicate *trans*-shisool.

**Figure 5.** GC charts of the sequential reaction products of PsAKR and PcGeDH with GDP as substrate. These charts are extracted ion chromatograms of  $m/z = 134$  to  $137$ .

(A) Sequential reaction of PcTps-C followed by PsAKR; (B) sequential reaction of PcTps-C followed by PcGeDH.

Closed black circles indicate neral, open circles indicate geranial, gray circles indicate nerol, and closed squares indicate geraniol.

**Figure 6.** Semi-quantitative RT-PCR analysis of alcohol dehydrogenases from perilla strains characterized by various oil types (Strain Nos. 6, 32, and 87). *AKR*, aldo-keto reductase; *GeDH*, geraniol dehydrogenase. Amplification of the histone fragment was used as the internal PCR control.

#### Supplementary data

**Figure 1S.** MS patterns of the reaction products of PcGeDH with geraniol (A, B, C) or perilla alcohol (D) as a substrate. (A) Reaction product marked with a closed black circle in GC charts in Figure 3E; (B) reaction product marked with an open circle in GC charts in Figure 3E; (C) reaction product marked with a gray circle in GC charts in Figure 3E; (D) reaction product marked with a closed circle in GC charts in Figure 4E; (E) reaction product marked with a gray circle in GC charts in Figure 4E. These mass spectra were compared with those in the MS library (NIST11).

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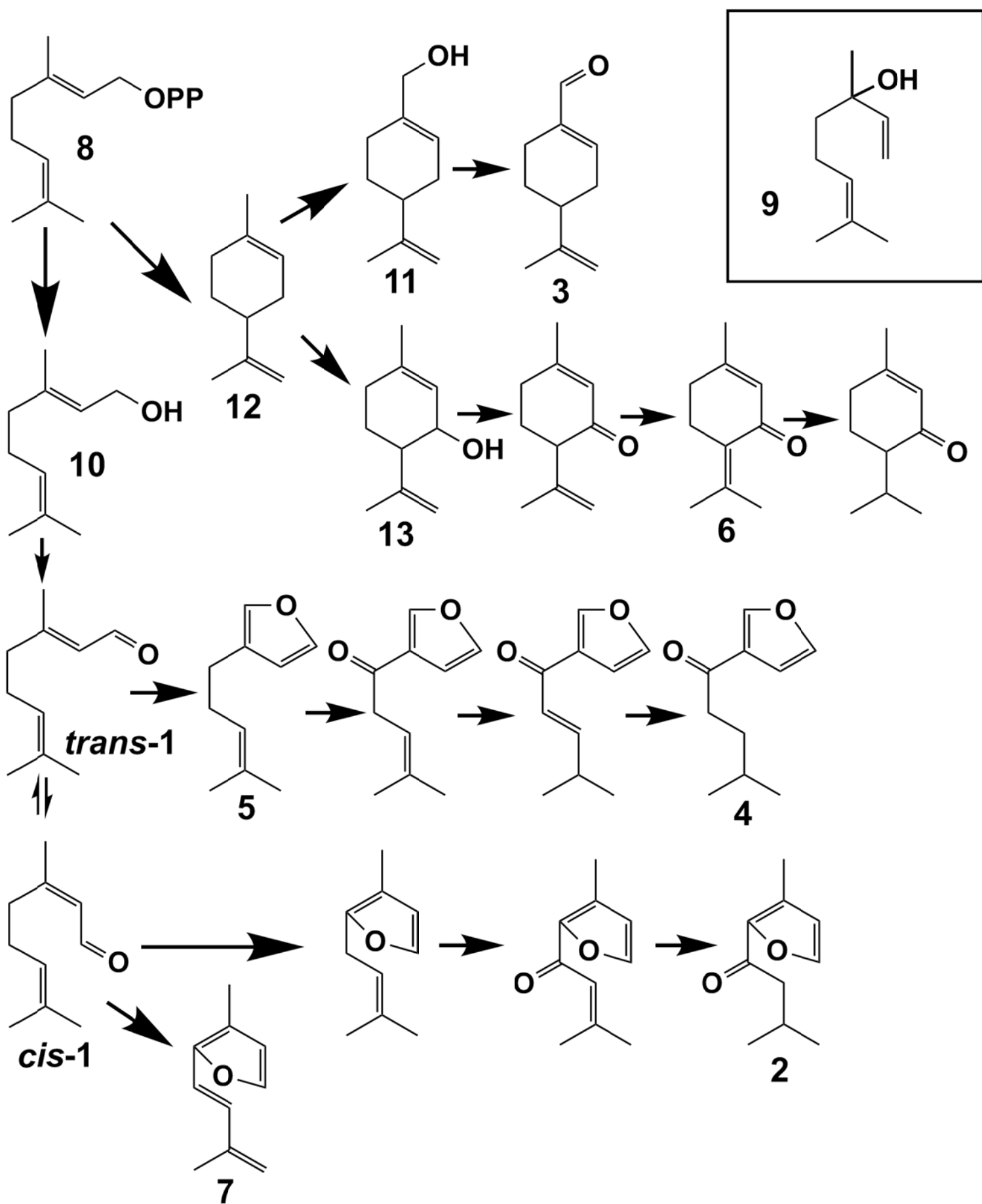
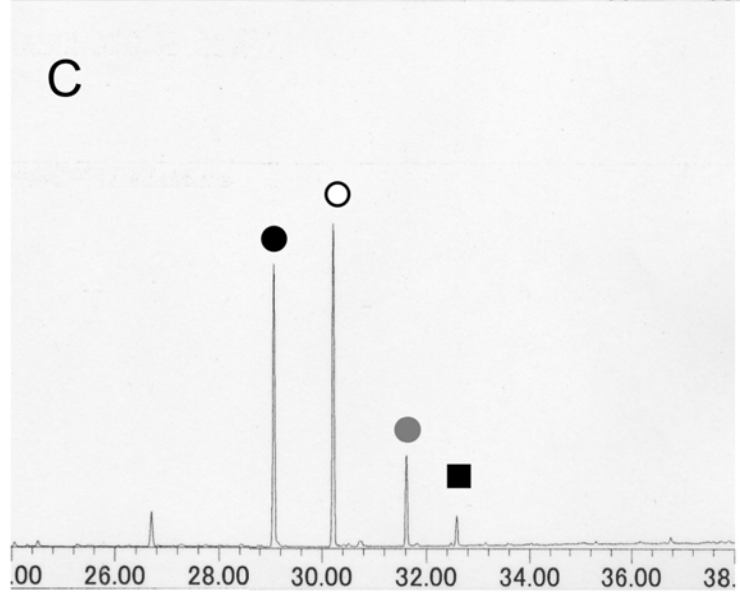
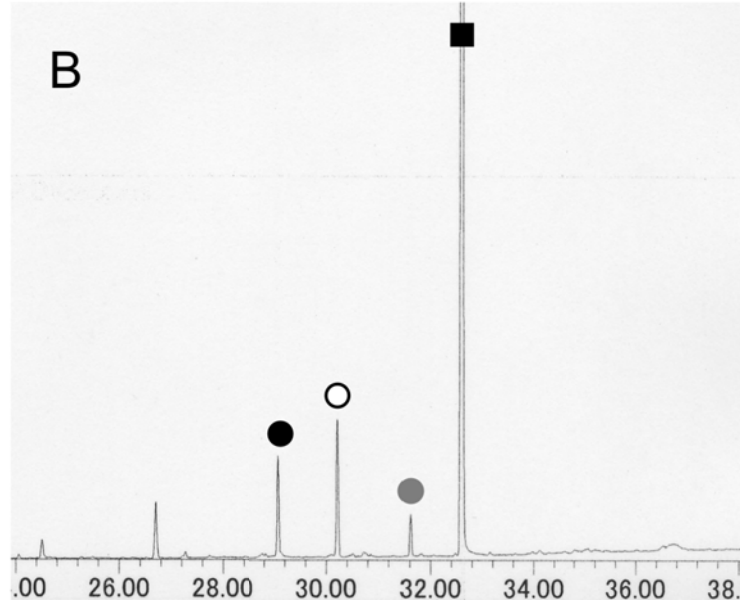
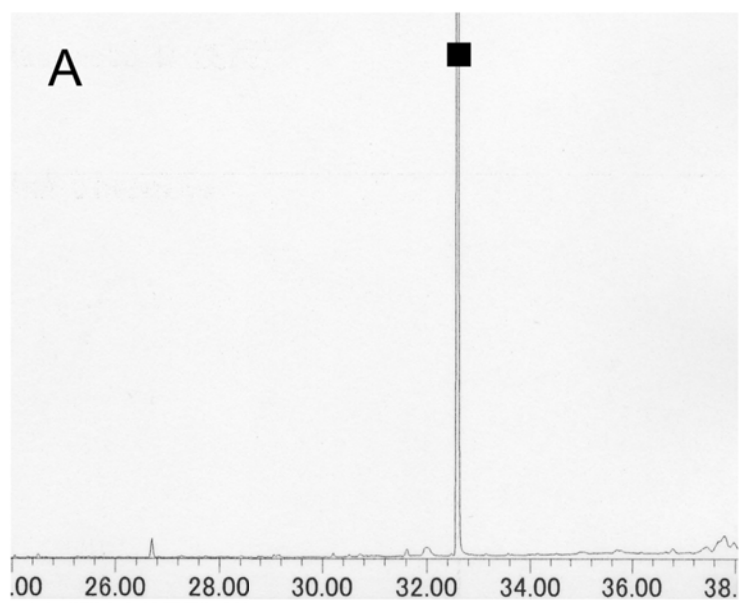


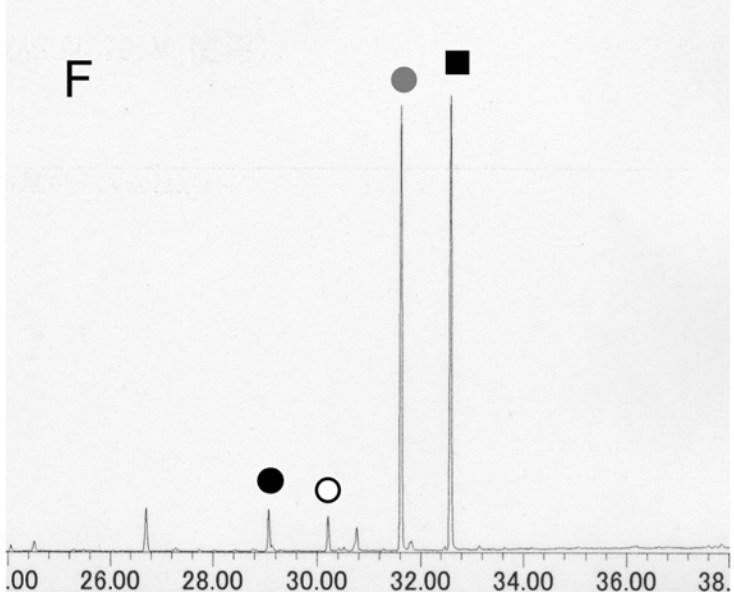
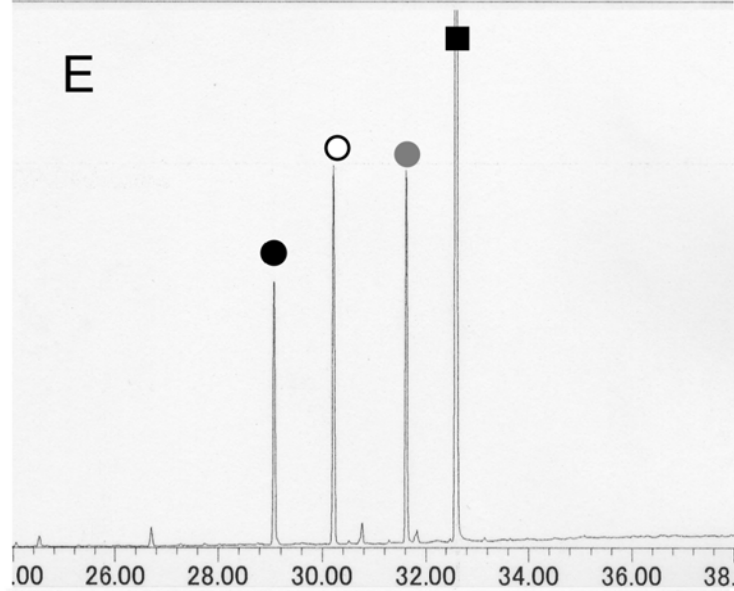
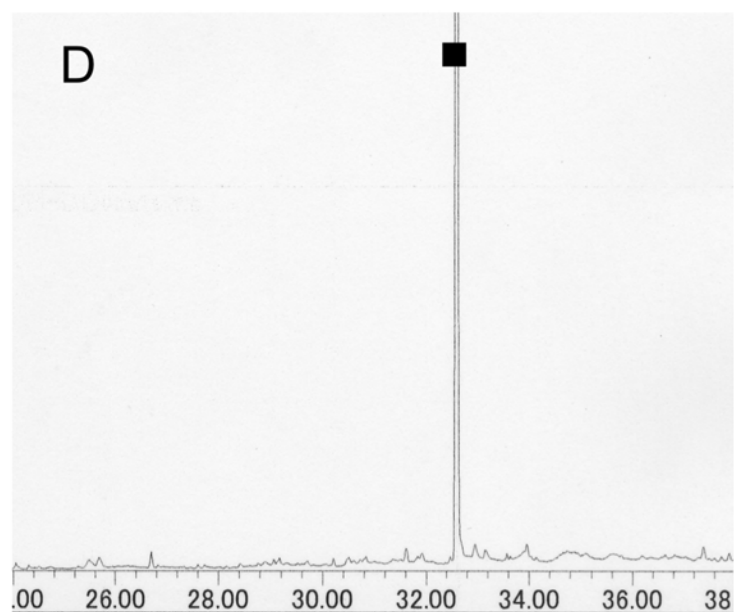
Figure 1

Figure 2

|        |            |             |            |            |            |            |            |            |
|--------|------------|-------------|------------|------------|------------|------------|------------|------------|
|        | 10         | 20          | 30         | 40         | 50         | 60         | 70         | 80         |
| PfAKR  | MATAAMTVAV | PRIKLGVDGL  | EVSKIGLGCV | GMSETYGPPR | PEPEMVQLIH | HAVDSGVTFF | DTSDSYGPHT | NEILLGRALK |
| PcAKR  | MATAAMTVV  | PRIKLGMDGL  | EVSKIGLGCV | GMSETYGPPR | PEPEMVQLIH | HAVDSGVTFF | DTSDFYGPHT | NEILLGRALK |
| PsAKR  | MATAAMTVAV | PRIKLGVDGL  | EVSKIGLGCV | GMSETYGPPR | PELEMVRLIH | HAVDSGVTFF | DTSDFYGPHT | NEILLGRALK |
| PfGeDH | -MAKTPETEH | PVKAFGWAAR  | DNSGT-LSPF | NFSRRDTGER | -DVQFKVLYC | GVCHSDLHMV | KNE--RGVTH | YPVVPGEIV  |
| PcGeDH | -MAKTPETEH | PVKAFGWAAR  | DNSGT-LSPF | NFSRRDTGER | -DVQFKVLYC | GVCHSDLHMV | KNE--RGVTH | YPVVPGEIV  |
| PsGeDH | -MAKTPETEH | PLKAFGWAAR  | DNSGT-LSPF | NFSRRDTGER | -DVQFKVLYC | GVCHSDLHMV | KNE--WGVTH | YPVVPGEIV  |
|        | 90         | 100         | 110        | 120        | 130        | 140        | 150        | 160        |
| PfAKR  | GMREKVQIAT | KFGARLKGDV  | VEICGHPAYV | REACEASLKR | LETNYIDLY  | --YIHRIDTR | VPIEITMGEL | KKLVEEGKIK |
| PcAKR  | GMREKVQIAT | KFGARLKGDV  | VEICGHPAYV | REACEASLKR | LETNYIDLY  | --YIHRIDTR | VPIEITMGEL | KKLVEEGKIK |
| PsAKR  | GMREKVQIAT | KFGARLKGDV  | VEICGHPAYV | REACEASLKR | LETNYIDLY  | --YIHRIDTR | VPIEITMGEL | KKLVEEGKIK |
| PfGeDH | GVVTEVGSKV | EKVKAGDKVG  | VGIVVGSCRQ | CDQCSNDLEN | YCSKQILTYG | APYIDGTITR | GGYSNIMVAD | EHFIIR-WPE |
| PcGeDH | GVVTEVGSKV | EKVKAGDKVG  | VGIVVGSCRQ | CDQCSNDLEN | YCSKQILTYG | APYIDGTITR | GGYSNIMVAD | EHFIIR-WPE |
| PsGeDH | GVVTEVGSKV | EKVKAGDKVG  | VGIVVGSCRQ | CDQCSNDLEN | YCSKQILTYG | APYIDGTITR | GGYSNIMVAD | EHFIIR-WPE |
|        | 170        | 180         | 190        | 200        | 210        | 220        | 230        | 240        |
| PfAKR  | HVGLSEACPS | TISRAHAVHP  | LAAVELEWSL | WSR--DSEDE | IIPTCRELGI | GIVAYSPLGR | GFLAAGPSFV | ENLSESDFRK |
| PcAKR  | HVGLSEACPS | TIRRAHAVHP  | LAAVELEWSL | WSR--DSEDE | IIPTCRELGI | GIVAYSPLGR | GFLAAGPSFV | ENLSDSDFRK |
| PsAKR  | HVGLSEACPS | TIRRAHAVHP  | LAAVELEWSL | WSR--DSEDE | IIPTCRELGI | GIVAYSPLGR | GFLAAGPSFV | ENLSESDFRK |
| PfGeDH | NLPLDAGAPL | LCAGITTYSP  | LRYYGLDKPG | LSVGVNGLGG | LGHVAVKFAK | AFGKTVTVIS | TSLGKKKEAI | EHLGVDEFLV |
| PcGeDH | NLPLDAGAPL | LCAGITTYSP  | LRYYGLDKPG | LSVGVNGLGG | LGHVAVKFAK | AFGKTVTVIS | TSLGKKKEAI | EHLGVDEFLV |
| PsGeDH | NLPLDAGAPL | LCAGITTYSP  | LRYYGLDKPG | LSVGVNGLGG | LGHVAVKFAK | AFGKTVTVIS | TSLGKKKEAI | EHLGVDEFLV |
|        | 250        | 260         | 270        | 280        | 290        | 300        | 310        | 320        |
| PfAKR  | R-FPRFQPEN | IEQNKKIYER  | LCEMAARREC | SPAQLALAWV | LARGDDVCPI | PGTTKIDNLN | QNMEAFLLEL | TPEEKADLES |
| PcAKR  | R-FPRFQPEN | IEQNKKIYER  | LCEMAARREC | SPAQLALAWV | LARGDDVCPI | PGTTKIDNLN | QNMEAFLLEL | TPEEKAELES |
| PsAKR  | R-FPRFQPEN | IEQNKKIYER  | LCEMAARREC | SPAQLALAWV | LARGDDVCPI | PGTTKIDNLN | QNMEAFLLEL | TPEEKAELES |
| PfGeDH | SSDPQQMQAA | VGTLDGIIIDT | VSAEHPLVPL | MSLLKPHGKL | IVVGAPQKPL | QLHAFSLIQG | RKTVAGSAIG | GMKETQEMID |
| PcGeDH | SSDPQQMQAA | VGTLDGIIIDT | VSAEHPLVPL | MSLLKPHGKL | IVVGAPQKPL | QLHAFSLIQG | RKTVAGSAIG | GMKETQEMID |
| PsGeDH | SSDPQQMQAA | VGTLDGIIIDT | VSAEHPLVPL | MSLLKPHGKL | IVVGAPQKPL | QLHAFSLIQG | RKTVAGSGIG | GMKETQEMID |
|        | 330        | 340         | 350        | 360        | 370        | 380        | 390        | 400        |
| PfAKR  | YASPDVMVKG | RHAFMSQTWI  | NSETPQLSN  | ---WKLENHI | DDGV---    | ...        | ...        | ...        |
| PcAKR  | YASPDVMVKG | RHAFMSQTWI  | NSETPQLSN  | ---WKLENHI | DDGI---    | ...        | ...        | ...        |
| PsAKR  | CASPDVMVKG | RHAFMSQTWI  | NSETPQLSN  | ---WKLENHI | DDGV---    | ...        | ...        | ...        |
| PfGeDH | FAAKKNIIPD | -VEVIPIDYI  | NTAMDRLKLS | DVKYRFVIDV | NKSFNAH    | ...        | ...        | ...        |
| PcGeDH | FAAKKNIIPD | -VEVIPIDYI  | NTAMDRLKLS | DVKYRFVIDV | NKSFNAH    | ...        | ...        | ...        |
| PsGeDH | FAAKKNIIPD | -VEVIPIDYI  | NTAMDRLKLS | DVKYRFVIDV | NKSFNAH    | ...        | ...        | ...        |

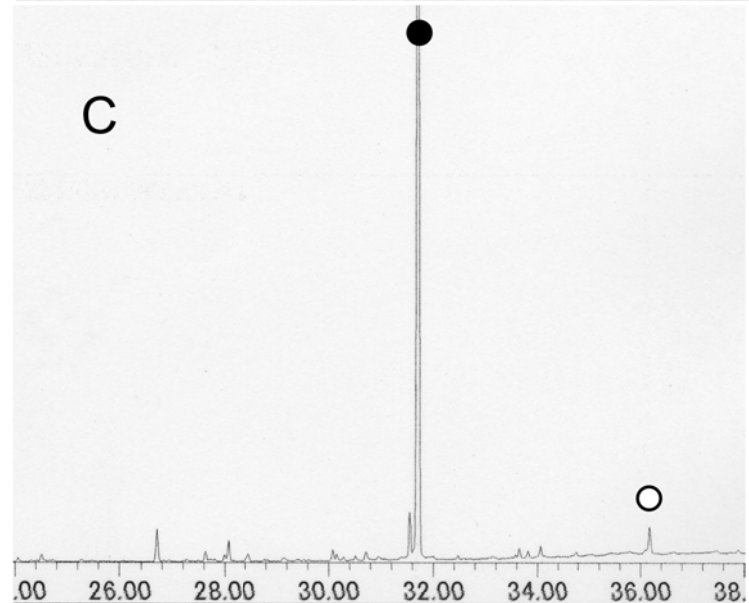
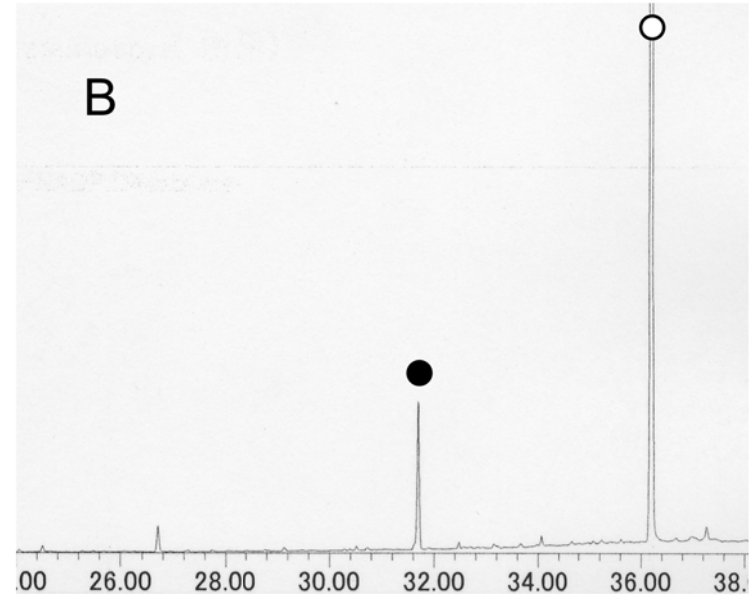
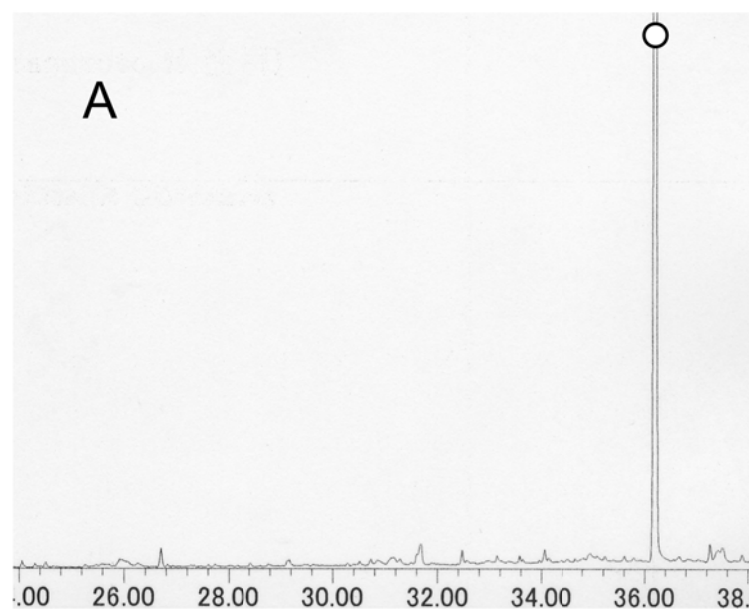


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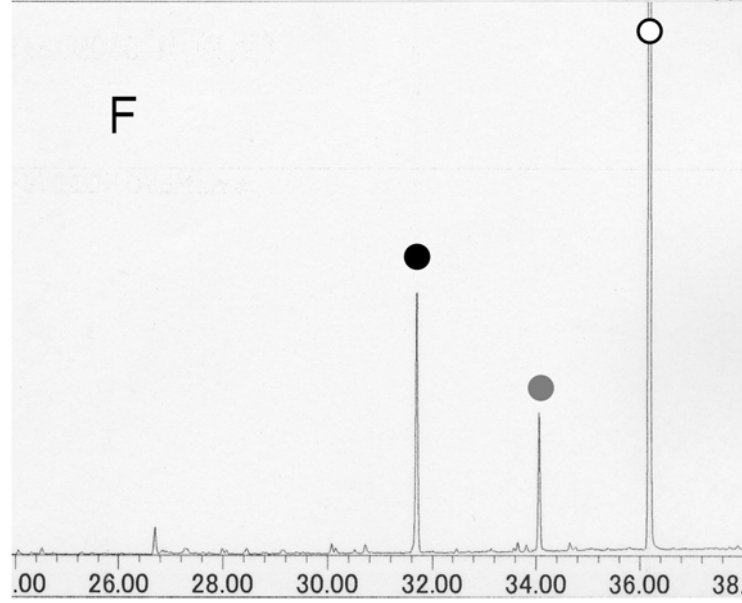
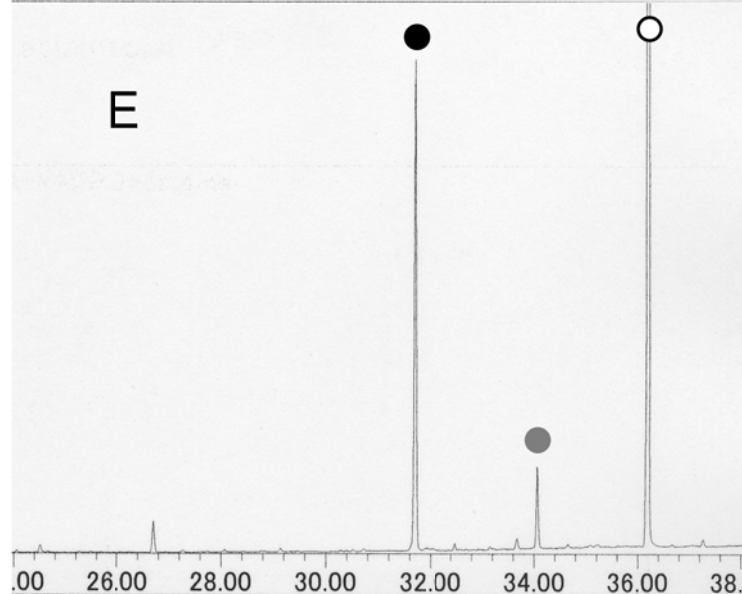
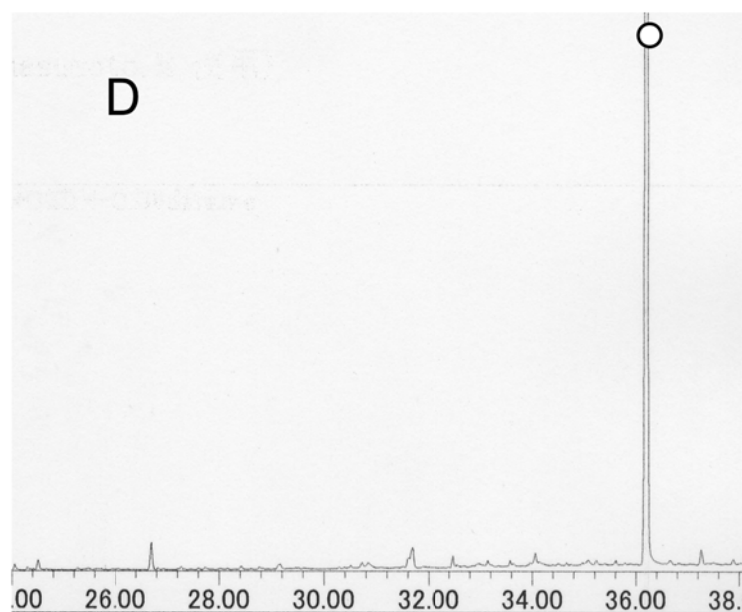


Retention time (min)

Figure 3



Retention time (min)



Retention time (min)

Figure 4

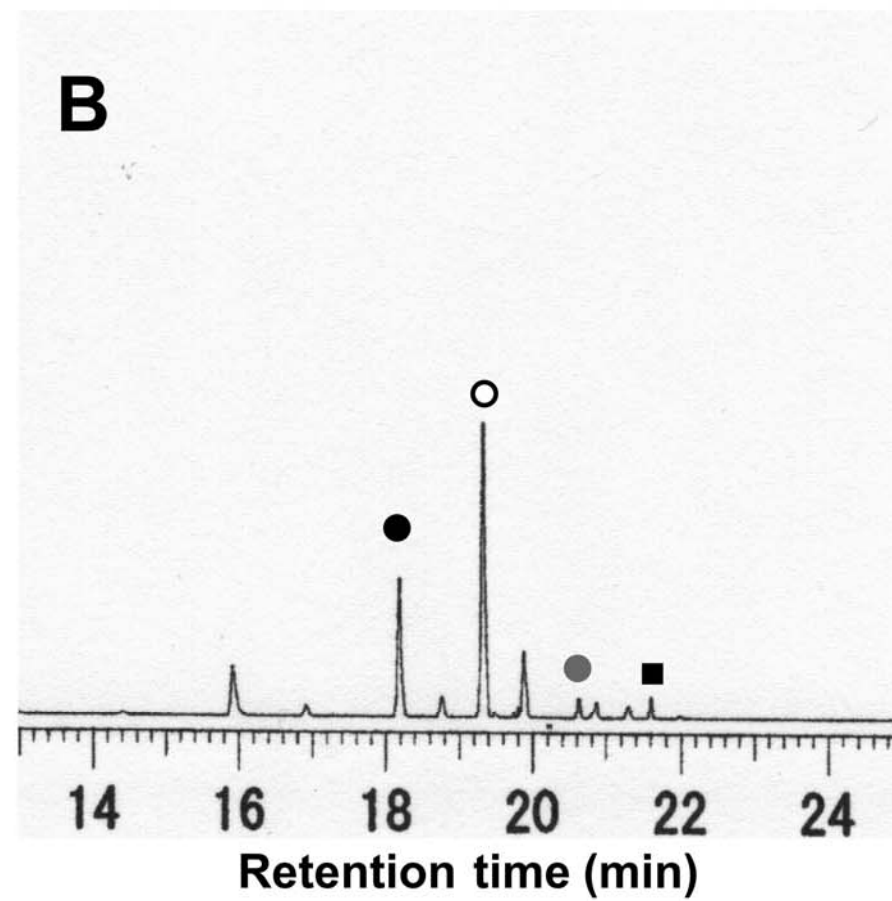
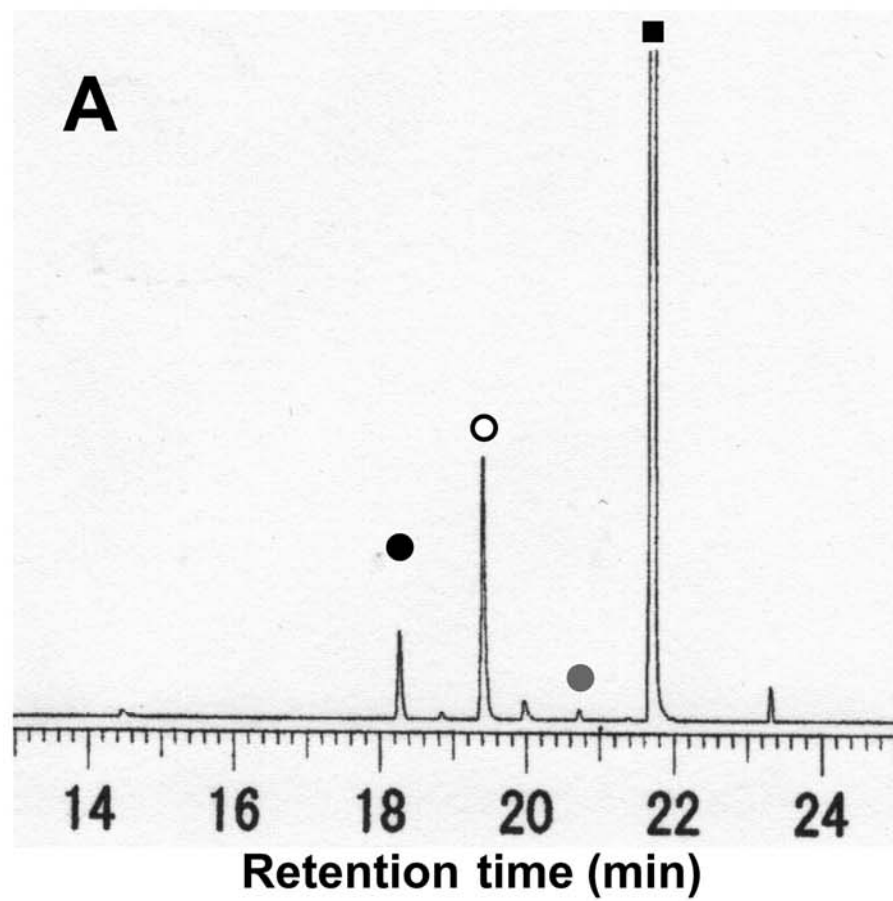


Figure 5

Figure 6

# Strain Number

32

6

87

**AKR**



**GeDH**



**histone**



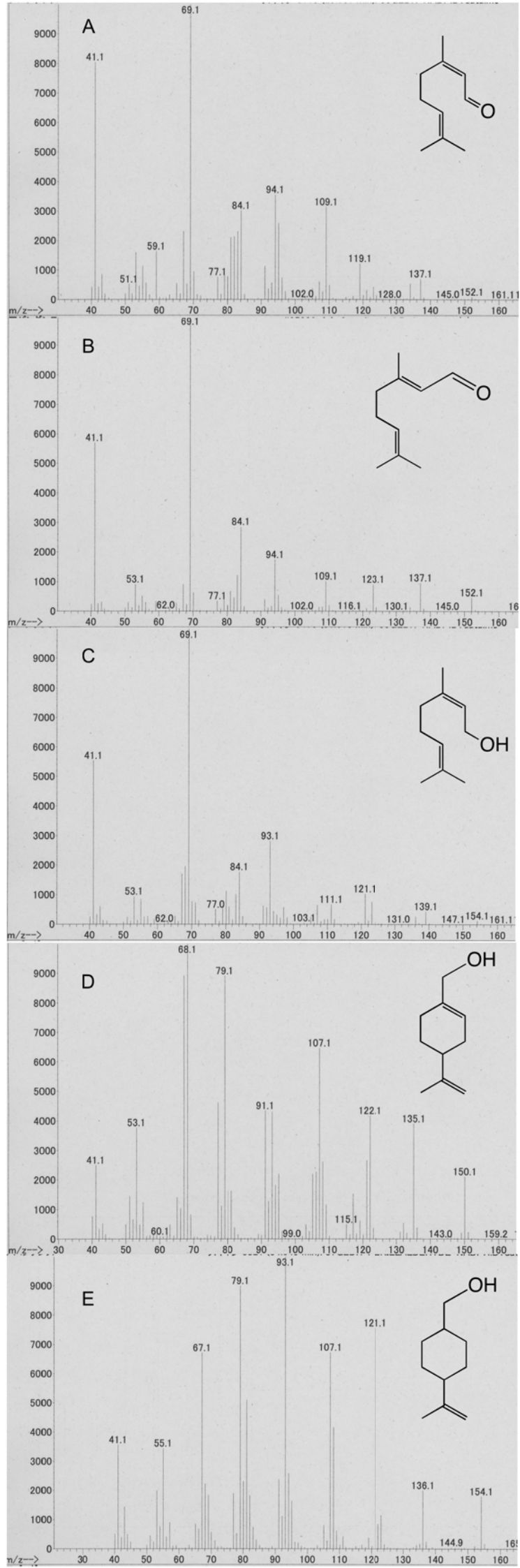


Figure 1S