Molecular cloning and characterization of a *Perilla frutescens* cytochrome P450 enzyme that catalyzes the later steps of perillaldehyde biosynthesis

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Abstract

Perilla produces the cyclohexanoid monoterpene perillaldehyde as a major constituent of an essential oil that is accumulated in its glandular trichomes. Perillaldehyde is a marker compound for quality control of *soyo* and has biological activities such as antibacterial, sedative, or vasodilatory effects. The predicted perillaldehyde formation involves the cyclization of geranyl diphosphate, hydroxylation, and oxidation, and cytochrome P450 plays a crucial role in perillaldehyde biosynthesis. In this study, a cytochrome P450-type enzyme with perillyl alcohol and perillaldehyde synthase activities was isolated by analyzing an expressed sequence tag library from several oil types of pure lines of perilla. A recombinant protein with a sequence that was highly specific for the type of perillaldehyde was expressed in *Saccharomyces cerevisiae* and evaluated by an *in vitro* enzymatic reaction. The recombinant protein catalyzed the hydroxylation and oxidation of limonene to perillyl alcohol and perillaldehyde. Cytochrome P450 limonene-7-hydroxylase cDNA from *Perilla frutescens* has been previously isolated. The cytochrome P450 isolated in this study shares 37% amino-acid identity with the previously isolated enzyme; however, it may have different characteristics.

Keywords

Perilla frutescens; Labiatae; Molecular cloning; Biosynthetic pathway; Cytochrome P450; Perillaldehyde synthase; Perillyl alcohol synthase; Monoterpenoid

1. Introduction

Monoterpenes are often major constituents of essential oils derived from plants. Many have biological activities, such as antibacterial or antitumor activities (Trombetta et al., 2005; Mills et al., 1995), and they are important and useful compounds in pharmaceutical sciences. Monoterpenes are also used in flavors and perfumes because they are fragrant and generally volatile at room temperature. Perilla, a common annual Asian herb, is the source of several oil types. The most popular type in Japan is type perillaldehyde (PA) (5), which contains perillaldehyde (5) as its major compound. Perilla leaves are also used as a natural medicine for Kampo prescriptions, and as per the Japanese Pharmacopoeia (JP), such preparations should contain at least 0.08% of PA (5), calculated by examination of dried preparations. JP also recommends that *soyo*, the leaves and branch tips of *Perilla frutescens* Britton var. *crispa* W. Deane, should be red on at least one side (The Ministry of Health, Labour and Welfare, Seventeenth ed, 2016). A library of genetically pure lines of perilla has been established and many studies has

been conducted showing that the syntheses of oil compounds are genetically controlled (Ito et al., 1999a; Ito et al., 1999b; Honda 1996; Ito et al., 2002). However, the details of the synthetic pathways await elucidation. The initial reaction step for monoterpene oil constituents is believed to be the dephosphorylation of geranyl diphosphate (GDP) (1), catalyzed by either geraniol or limonene synthases. PA (5) appears to be synthesized by hydroxylation at the C7 position of limonene (3) and subsequent oxidation to the aldehyde (5). Almost all monoterpene compounds found in perilla essential oil are considered to be synthesized from acyclic compounds derived from GDP (1), and the subsequent reactions of cyclization, oxidation, or reduction are considered to make up the different compound structures (Fig. 1). Oxidation/reduction steps of oil compounds in plants are often catalyzed by cytochrome P450, and previous studies in *Mentha* and *Catharanthus* have shown that limonene (3) and geraniol (2), both constituents of perilla oil, are employed as substrates for cytochrome P450 (Lupien et al., 1999; Collu et al., 2001).

Cytochrome P450 monooxygenases are heme-containing enzymes that catalyze a wide range of reactions, including oxidation, hydroxylation, epoxidation, and dealkylation. In plants, many cytochrome P450s mediate the biosynthesis of several secondary metabolites, including plant hormones, fatty acids, and defense compounds. The generation of diverse monoterpenes is usually initiated by cytochrome P450 hydroxylase.

Mau et al. (2010) isolated (–)-limonene-7-hydroxylase from perilla, showing that it catalyzes hydroxylation at the C7 position of limonene (3). Their study employed a hybridization strategy using previously cloned mint limonene hydroxylase cDNA as a probe. *P. frutescens*, *M. piperita*, and *M. spicata* are closely related species in the Labiatae family, and their limonene hydroxylases are thought to catalyze (–)-limonene (3) hydroxylation with different regiospecificities. A homology-based approach is often employed for cloning enzymes with identical or similar functions from closely related species. However, hydroxylation of limonene (3) appears to occur by different mechanisms for 7-hydroxylation and 3- and 6-hydroxylation, and Mau et al. were unsuccessful in isolating the full-length enzyme by a homology-based method. In the present study, cytochrome P450 enzymes relevant to PA synthesis were targeted, and their method of isolation depended on comparison of sequences expressed in pure strains with different oil types. In this context a library of pure strains of perilla have been obtained and achieved cloning of enzymes catalyzing the synthesis of oil compounds in perilla (Ito and Honda, 2007; Masumoto et al., 2010; Sato-Masumoto and Ito, 2014). The library has been established by collecting various types of perilla and

maintaining them for 10–25 years by self-pollination using paper pollination bags. With this library of pure strains of perilla, it is possible to compare expression levels of specific genes in different oil types and identify sequences relevant to the syntheses of oil compounds specific to the oil type. Herein described as the cloning and characterization of a cytochrome P450 enzyme mediating the synthesis of PA (5).

2. Results and Discussion

2.1. Isolation of a cytochrome P450 from *P. frutescens*

The PA synthase-encoding cDNA contig 7307 (named Pf-7307, GenBank ID; KU674339) was retrieved from a perilla expressed sequence tag (EST) library by comparing gene expression levels using the reads per kilobase of exon per million mapped reads (RPKM) index. RPKM of contig 7307 showed a high numerical value only for type PA among 12 strains of different oil types. The full-length cDNA of Pf-7307 was 1,497 bp in length and contained an open reading frame of 499 amino acids (Fig. 2). The known highly conserved regions of cytochrome P450s: a proline-rich region, an oxygen-binding pocket, and a heme-binding region, were conserved in Pf-7307. Based on the information registered on the website of Dr. D. R. Nelson (Cytochrome P450 Homepage), we obtained the corresponding nucleotide sequence of CYP71AT146. In the putative biosynthetic pathway of PA (5) in *P. frutescens*, PA (5) involves the cyclization of GDP (1) to limonene (3), hydroxylation at the C7 position (4), and oxidation to aldehyde (5) (Fig. 1). A recombinant protein of Pf-7307 is predicted to recognize perillyl alcohol (4) as a substrate. Pf-7307 shared 36%, 35%, 37%, and 23% identities in amino acid with peppermint (–)-limonene-3-hydroxylase (CYP71D3, GenBank ID; AF124816), spearmint (–)-limonene-6-hydroxylase (CYP71D18, GenBank ID; AF124815), perilla (–)-limonene-7-hydroxylase (Gen Bank ID; GQ120438), and perilla alcohol dehydrogenase (Gen Bank ID; JX629453). The sequence of Pf-7307 was derived from strain 32 and was found in strain 5647 with a high numerical value of RPKM. The same sequence of Pf-7307 was also found in another PA-type strain, 9. The presence of Pf-7307 in multiple strains of PA type perilla suggests that this P450 is closely associated with PA biosynthesis.

2.2. Functional analysis of Pf-7307

2.2.1. Identification of a substrate

In the putative biosynthetic pathway of PA (5), the initial step is the dephosphorylation and cyclization of GDP (1) by limonene synthase to form limonene (3). The next step is expected to be its hydroxylation of limonene (3) at the C7 position to produce perillyl

alcohol (4) and oxidation to PA (5). This expectation is in agreement with an analysis of chemical composition by steam distillation in which limonene (3), perillyl alcohol (4), and PA (5) were detected only in oil from type PA among other monoterpene types (Ito et al., 1999a). However, the result of steam distillation may not match the true composition because unstable or highly reactive components may react with water or because of heating during steam distillation. A direct analysis of essential oils accumulating in the glandular trichomes was performed to confirm the composition, as follows: oil in a single glandular trichome was collected with a solid phase micro-extraction (SPME) fiber by breaking the cuticle layer of the trichome with SPME fiber under an optical microscope. Compounds were analyzed by GC-MS. PA (5) (61%) and limonene (3) (10%) were detected, but perillyl alcohol (4) was not. This analysis confirmed that PA (5) was formed not by oxidation during steam distillation but in glandular trichomes. Compounds with contents <6% following steam distillation cannot be detected by direct analysis, and perillyl alcohol (4) was detected at approximately 1% concentration by steam distillation. It is speculated that limonene (3), perillyl alcohol (4), and PA (5) were substrates for our target reaction.

2.2.2. Heterologous expression of cDNA in Saccharomyces cerevisiae *and enzymatic reaction*

To determine if Pf-7307 had the characteristic features of cytochrome P450, its expression vector was constructed and introduced into *S. cerevisiae*. The microsomal fraction was measured a reduced CO-difference spectra to estimate protein activity. There was no peak at 450 nm. An expression system in *S. cerevisiae* often does not show a characteristic peak at 450 nm owing to low expression. Therefore, an enzymatic reaction was performed to confirm enzymatic activity. The microsomal fraction was reacted with limonene (3), perillyl alcohol (4), or PA (5) as a substrate for 16 h. As a result, recombinant Pf-7307 showed limonene (3) conversion activity to produce PA (5), *trans*-shisool (6), and perillyl alcohol (4) (Fig. 3A). When perillyl alcohol (4) was used as a substrate, recombinant Pf-7307 clearly transformed it (4) into *trans*-shisool (6) and PA (5) (Fig. 3B); when PA (5) was used as a substrate, *trans*-shisool (6) and perillyl alcohol (4) were detected as reaction products (Fig. 3C). It could be predicted that the final product of a sequence pathway is *trans*-shisool (6) for all substrates used in this study, considering the putative biosynthetic pathway and relative ratio of accumulation of reaction products. As an additional enzymatic reaction, Pf-7307 was reacted with *trans*-shisool (6) as a substrate; no conversion activity was detected (Fig. 3D). When times limonene (3) or perillyl alcohol (4) were used as substrates, perillyl alcohol (4) and PA (5) were formed by oxidation. However, *trans*-shisool (6) was formed by reduction

when limonene (3), perillyl alcohol (4), and PA (5) were used as substrates, and perillyl alcohol (4) was formed by reduction when PA (5) was used as a substrate. In an expression system of cytochrome P450 *in vitro*, cytochrome P450 reductase (CPR) acts as an electron transfer partner (Paul, 2015). The presence of CPR allows cytochrome P450 to continuously act. In this study, Pf-7307 was ligated into a pGYR-Spe I vector (Sakaki et al., 1992) designed for the coexpression of the *S. cerevisiae* NADPH-P450 reductase gene and was expressed in *S. cerevisiae.* Production of perillyl alcohol (4) when PA (5) was used as a substrate and that of *trans*-shisool (6) when limonene (3), perillyl alcohol (4), and PA (5) were used as substrates may have been catalyzed by CPR. When control extracts prepared from *S. cerevisiae* with the vector plasmid alone were used in the enzymatic reaction, the control solutions yielded no product if limonene (3) and *trans*-shisool (6) were used as substrates (Fig. 3E and 3H). Control solutions yielded the same product as the reactions with Pf-7307 when perillyl alcohol (4) or PA (5) were used as substrates (Fig. 3F and 3G). Thus, *trans*-shisool (6) and perillyl alcohol (4), which are reduced forms of the substrate, were produced. These results strongly suggest that our hypothesis is accurate. Formation of perillyl alcohol (4) and *trans*-shisool (6) by reduction should be catalyzed by CPR. However, in perilla, *trans*-shisool (6) may be produced by the biosynthetic enzymes that catalyze reduction of perillyl alcohol (4) or PA (5). This may be because *trans*-shisool (6) is contained in only type PA, but not in strains that are not type PA, despite these strains showing PA production in the stem. Accordingly, it can be speculated that Pf-7307 is involved in transforming limonene (3) into perillyl alcohol (4) and PA (5). However, questions remain pertaining to the amount of production being too small and whether the synthetic pathway of PA (5) from limonene (3) is by direct synthesis from limonene (3) or via synthesis of perillyl alcohol (5).

The time courses of enzymatic reaction were evaluated. The result of Pf-7307 or vector control with limonene (3) as a substrate is illustrated in Figure 4. Perillyl alcohol (4) and PA (5) were detected from 30 min onward, and accumulation of perillyl alcohol (4) increased with time. There was no change in the accumulation of PA (5). This result indicated that limonene (3) was converted into perillyl alcohol (4) by Pf-7307, whereas the synthetic pathway of PA (5) derived from either limonene (3) or limonene-derived perillyl alcohol (4) is unclear. In other words, the enzymatic reaction with limonene (3) as a substrate did not show that Pf-7307 recognized perillyl alcohol (4) as a substrate, and Pf-7307 catalyzed two steps in the pathway from limonene (3) to PA (5) via perillyl alcohol (4).

Figure 5 shows the result of Pf-7307 or vector control with perillyl alcohol (4) as a

substrate. PA (5) and *trans*-shisool (6) were detected in the reaction product after enzymatic reaction for 30 min (Fig. 5B), the accumulation of PA (5) decreased and that of *trans*-shisool (6) increased. This result suggested that perillyl alcohol (4) was oxidized by Pf-7307 to PA (5), followed by reduction to *trans*-shisool (6). When PA (5) was used as substrate, the products of both Pf-7307 and the vector control were the same (Fig. 6). Thus, the formation of perillyl alcohol (4) and *trans*-shisool (6) from PA (5) should be catalyzed not by Pf-7307 but by CPR. Taking these observations together with the time course of the enzymatic reaction suggests that Pf-7307 is involved in the oxidation of limonene (3) to perillyl alcohol (4), and that of perillyl alcohol (4) to PA (5); in addition, they suggest that the biosynthetic pathway of PA (5) progresses from limonene (3) to PA (5) via perillyl alcohol (4) in perilla leaves.

The compositions of the products of steam distillation and reaction products in the reaction solutions *in vitro* were compared. In the steam-distilled essential oil, the content of PA (5) from the type of PA (5) was found to be approximately 50% and those from *trans*-shisool (6) and perillyl alcohol (4) were found to be low at 2%–3% (Ito et al., 1999a). Because perillyl alcohol (4) is a reaction intermediate in the formation of PA (5) from limonene (3), the content was expected to be low. PA (5) formed by the enzymatic reaction of Pf-7307 did not increase with the time course of the reaction in the reaction solution, but it was reduced to perillyl alcohol (4) or *trans*-shisool (6) by coexpression of CPR. The difference in the pattern of accumulation was apparently influenced by coexpression of CPR. To act continuously, cytochrome P450 requires CPR for transfer of electrons to it. In this study, *S. cerevisiae*-derived CPR-coding sequence that was transfected into a vector was employed for coexpression with a target gene, although *P. frutescens*-derived CPR was isolated (Mau et al., 2010). CPR is an electron donor to cytochrome c and causes reduction. CPR has been isolated from fish and mammals as well as bacteria (Urenjak et al., 1987; Porter and Kasper, 1985), and the full amino-acid sequence of yeast reductase showed 33% and 32% identity with those of rat and rabbit, respectively. Despite the low similarity among full amino-acid sequences, the possible functional domains related to binding of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and nicotinamide adenine dinucleotide phosphate (NADPH) were well conserved among these species (Yabusaki et al., 1988). Regardless of the types of CPRs that are available for the enzymatic reaction *in vitro* or the functional domains they have, CPRs can become partners in electron transfer to cytochrome P450. However, the full amino-acid sequences are highly divergent except in the conserved domain, and unexpected effects may occur. In a previous study, mutants lacking N-terminal amino acids of yeast-derived CPR retained the activity of native CPR, whereas human-derived

CPR was inactive (Venkateswarlu et al., 1998). In another study, a chimeric protein in which the C-terminal hydrophobic sequence of cytochrome b5 replaced the N-terminal hydrophobic sequence of CPR was constructed. This hybrid flavoprotein retained the catalytic properties of the native CPR and was able to reconstitute fatty acid and steroid hydroxylation activities with CYP4A1 and CYP17A. However, the hybrid protein was much less effective than CPR for reconstituting activity with CYP3A4 (Gilep et al., 2001). In view of these results, it can be hypothesized that differences in partial sequences reflect unique and specific information essential for the recognition needed to establish electron transfer from CPR. In the present study, the enzymatic reaction with cytochrome P450 used monoterpenes, which are C10 compounds comprising two isoprene units, as substrates, resulting in an unexpected reduction reaction. There is no precedent for this reduction in other research using the same vector. Previous studies employed more complex compounds than monoterpenes, such as flavonoids and alkaloids (Nazir et al., 2011; Ikezawa et al., 2009). This suggests that monoterpenes, with simple structure and high reactivity, are better recognized by CPR and undergo unexpected reactions. Because composition analysis by steam distillation showed that PA (5) accumulates in perilla leaves despite its tendency to get easily reduced, it is believed that the enzyme, which is expressed in perilla leaves, including CPR, does not recognize PA (5); alternatively, if it recognizes PA (5), oxygenase is preeminent among perilla leaf enzymes.

2.3. Comparison of Pf-7307 and other cytochrome P450

It is proposed that Pf-7307 is a cytochrome P450 because it contains regions well conserved among all cytochrome P450s and 499 amino-acid residues, nearly equal to the approximately 500 amino-acid residues of cytochrome P450. Pf-7307 shows approximately 35% amino-acid identity with limonene hydroxylase of mint. This is very low despite their recognition of a common substrate and catalysis of a similar hydroxylation reaction. Cytochrome P450 is classified by amino-acid sequence identity. Accordingly, it can be speculated that Pf-7307 belongs to a family that is different from other limonene hydroxylases. The most similar amino-acid sequence, CYP71AT92 (GenBank ID; KP337662) derived from *Salvia miltiorrhiza*, shares 65% identity with Pf-7307. This sequence was obtained from three tissue types of *S. miltiorrhiza* using RNA-Seq, and functional characterization of the gene has not been performed (Chen et al., 2014). According to a chemical composition report of the essential oils in leaves of *S. miltiorrhiza* by hydrodistillation, saturated fatty acids, sesquiterpenes, and diterpenes [e.g., hexadecanoic acid (17%), germacrene D (9.1%), phytol (8.9%), and ß-caryophyllene (7.1%)] are the main components; monoterpenes were not found in oil (Li and Wang,

2009). In addition, in the analysis of flowers, the sample was dominated by sesquiterpenes (71%), including β-caryophellene and β-caryophellene oxide as the main components, with monoterpenes not detected in the aboveground parts (Liang et al., 2009). It is thought that they have low levels of reaction patterns despite the high identity of their amino-acid sequences. The CYP71A1 and CYP83B1 families share at least 50% of amino-acid sequence identity with Pf-7307; however, none of the family members have yet been identified, and no information is available on the reactions they catalyze.

The amino-acid identities was then compared with reactionally similar cytochrome P450 hydroxylase for monoterpene—CYP76C family derived from *Arabidopsis thaliana* and CYP76B family derived from *Helianthus tuberosus* or *Catharanthus roseus*. Catalytic activities of these genes are similar to that of Pf-7307 in terms of their similar substrates and reactions. They recognize nerol, citronellol, linalool, lavandulol, and α-terpineol as substrates and are involved in hydroxylation or oxidation (Hofer et al., 2014). However, the CYP76C and CYP76B families exhibit low amino-acid identity (25%–36%) with Pf-7307. Terpineol-10-hydroxylase, which catalyzes the introduction of hydroxyl group into α-carbon of a cyclohexene ring, closely resembles the reaction catalyzed Pf-7307. CYP76C1, CYP76C4, and CYP76B4 are involved in this reaction and have 35%, 33%, and 34% amino-acid identity with Pf-7307, respectively; however, none of these were found to have a characteristic domain common with Pf-7307.

Comparison of amino-acid identity between Pf-7307 and cytochrome P450 with respect to similar substrate or reaction indicated no correlation between the amino-acid sequence and the pattern of reaction. It is thus concluded that Pf-7307 may have characteristics different from other cytochrome P450s.

3. Conclusion

The perillyl alcohol and PA synthase cloned in this study is the first functionally characterized cytochrome P450 from *P. frutescens*. It mediates two steps, hydroxylation of limonene (3) at the C7 position and subsequent oxidation of perillyl alcohol (4). Pf-7307 is probably a novel cytochrome P450 differing from the cytochrome P450 involved in the synthesis of monoterpenes, because Pf-7307 shows low amino-acid identity with perilla limonene-7-hydroxylase, which was isolated by hybridization based on other limonene hydroxylase and cytochromes P450; these enzymes catalyze reactions similar to those catalyzed by Pf-7307, such as hydroxylation and oxidation, or recognize similar substrates.

Monoterpenes are generally volatile and fragrant, and they play important roles in

defense against bacteria and parasitic worms and help pollinators. Many more monoterpenes are used for food flavoring or perfumery, and a few have antitumor activity and thus are essential for life. For the compounds focused on in this study, clinical studies have been conducted. Limonene (3) was evaluated for its breast cancer preventive and therapeutic effects, and a phase-II study of the effectiveness of perillyl alcohol in treating patients with metastatic prostate cancer and pancreatic cancer has been conducted (ClinicalTrials.gov. The U.S. National Institutes of Health). Furthermore, Elegbede et al. reported that PA (5) inhibited the proliferation of a human carcinoma cell line *in vitro* (Elegbede et al., 2003). These compounds have lately attracted attention as base compounds for anticancer agent development. Isolation of an enzyme that catalyzes the biosynthesis of perillyl alcohol (4) and PA (5) of perilla essential oil is a key to generating valuable products for various fields such as pharmaceutical science, food science, and cosmetic chemistry.

PA (5) is an important compound from the viewpoint of quality control of *soyo*. The JP prescribes that *soyo* must contain not <0.08% of PA (5), calculated by examination of dried *soyo preparations*. Old books state that superior leaves have strong fragrance. In the quality assurance of herbal medicine, it is important to precisely identify the biosynthetic pathway of a standard compound. The strategy of identifying specific genes expressed in certain oil types using comparison of gene expression levels based on pure lines is applicable for elucidation of other biosynthetic pathways of perilla oil types that can be determined by genetic control. With this approach, it can be expected to increase the understanding of the biosynthesis of complex secondary metabolism orchestrated by hybridization and selection.

4. Experimental

4.1. General experimental procedures

Authentic limonene (3), perillyl alcohol (4), perillaldehyde (PA) (5), and all chemical reagents and solvents were purchased from Nacalai Tesque Inc. Kyoto, Japan. or Wako Pure Chemical Industries Co. Osaka, Japan. or Tokyo Kasei Co., Ltd. Tokyo, Japan. Agarose gel and plasmid extraction kits were from macherey-nagel. SPME fiber (100-μm polydimethylsiloxane) was from Supelco, St. Louis, MO USA. Polymerase chain reactions were performed on a Thermal Cycler personal (Takara Bio. Shiga, Japan). GC-FID measurements were performed using a G-5000 gas chromatograph (Hitachi High-Technologies Corporation. Tokyo, Japan), whereas GC-MS measurements employed 6850GC/5975MSD (Agilent Technologies Japan, Ltd. Tokyo, Japan.). DNA sequencing was performed on FASMAC Co., Ltd. Kanagawa, Japan. and Eurofins

Genomics. K.K. Tokyo, 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 Science, Kyoto University. They were bred and kept as pure lines by repeated self-pollination requiring bagging flowers with paper pollination bags; the bags were then opened and seeds were collected after they matured. Strain numbers and oil types used in this study for cloning and characterization of Pf-7307 were strain 32, type PA. Those used for comparison of expression pattern were strain 87, type citral (C); strain 1861, type elsholtziaketone (EK); strain 4931, type C; strain 5031, type shisofuran (SF); strain 5073, type perillaketone (PK); strain 5601, type piperitone (PT); strain 5640, type SF; and strain 5647, type PA. Both strain 32, type PA and strain 9, type PA were used to confirm the expression of Pf-7307.

4.3 Preparation of substrates

Trans-shisool (6) was prepared as follows. Commercially available (*S*)-(–)-PA (5) (7.5 g, 50 mmol) was reduced as described (Fronza et al., 2004). This procedure provided a 1:1 mixture of dihydroperillaldehyde isomer. A portion of product (0.60 g) was isomerized for transformation by mixing with K_2CO_3 (0.080 g):MeOH (39 mL), and *trans*-dihydroperillaldehyde (0.12 g) was reduced by $NaBH_4$ (0.031 g):EtOH (4.0 mL), followed by purification by column chromatography on silica gel to give *trans*-shisool (6) as a pale yellow oil (0.18 g). Its structure was determined by the interpretation of its ^1H, ^{13}C, DEPT, INEPT, HMQC, COSY, and GC-MS spectra. ^1H NMR (500 MHz, $CDCl_3$): δ_H 1.02 (2H, qd, J = 12.2 and 3.4 Hz, H-2_{ax}, H-6_{ax}), 1.22 (2H, qd, J = 12.3 and 2.6 Hz, H-3_{ax}, H-5_{ax}), 1.46 (1H, m, H-1), 1.72 (3H, s, -CH_3), 1.84 (5H, m, H-2_{eq}, H-6_{eq}, H-3_{eq}, H-5_{eq}, H-4), 3.46 (2H, d, J = 6.3 Hz, -CH_2OH), 4.68 (2H, s, =CH_2), ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 20.9 (CH_3), 29.4 (C-2. C-6), 31.1 (C-3, C-5), 40.2 (C-1), 45.4 (C-4), 68.5 (CH_2OH), 108.0 (=CH_2), 150.7 (-C (CH_2) (CH_3)), and GC-MS (*m/z*): 154.

4.4. Construction of EST library and cloning of Pf-7307

cDNA libraries were constructed, and cDNA was sequenced to construct an EST database at Kazusa DNA Research Institute. Total RNA was extracted from fresh young perilla leaves using the RNeasy Plant Mini Kit (Qiagen K.K. Tokyo, Japan.) and was processed according to the mRNA-Seq protocol (Illumina K.K. Tokyo, Japan.). The cDNA library was sequenced using a Genome Analyzer IIx sequencer (Illumina) with 100-base paired-end (PE) reads. The Illumina reads were assembled using CLC Genomics Workbench version 4.7.2 (CLC Bio).

The gene expression levels of contigs were compared among oil types using calculated

RPKM. Comparative analysis revealed a specific gene, contig 7307, expressed only in type PA. A conserved region of cytochrome P450, the proline-rich region, oxygen-binding pocket, and heme-binding region were highly conserved in Pf-7307. Randomly amplified cDNA ends (RACE) methods determined the complete sequence of Pf-7307. Total RNA was isolated from fresh young leaves of strain 32 perilla using an RNeasy Plant Mini Kit, and reverse transcription with primer add2 (5′-CCACGCGTCGACTACTTTTTTTTTTTTTTT-3′) by RevTra Ace (Toyobo Co., Ltd. Osaka, Japan.). The synthesized cDNA was used as a template for RT-PCR for 3′-RACE performed in a reaction mixture containing 0.025 U/μL B-Taq (Toyobo), 0.2 mM dNTPs, 0.2 μM primer amm {5′-GGCCACGCGTCGACTAC-3′}, and 0.2μM primer 7307-f {5′-ATGATGCTTCTACTACTACTGGATAG-3′} with a temperature program starting at 94°C for 100 s, followed by 30 cycles of 94°C for 30 s, 44°C for 30 s, 72°C 90 s, and final elongation at 72°C for 90 s. The reaction products were electrophoresed in agarose gel, cut out for extraction from the gel, purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG. Düren, Germany.), and ligated to the vector pTA2 (Toyobo). Sequences were confirmed using FAMAC Co., Ltd.

4.5. Heterologous expression of Pf-7307 in S. cerevisiae

To determine the activity of Pf-7307, a yeast expression vector was constructed in pGYR-SpeI with the *S. cerevisiae* NADPH-P450 reductase gene and SpeI-cloning site. Full-length Pf-7307 cDNA was amplified by PCR with the following primers to introduce a SpeI site (underlined): 7307speI-f primer {5′-<u>ACTAGT</u>ATGATGCTTCTACTACTACTG-3′}, 7307speI-r primer {5′-<u>ACTAGT</u>TTAAACACCATATTTCTTAGG-3′}. Reaction mixture containing 0.02 U/μL KOD-Plus (Toyobo), 0.2 mM dNTPs, 0.3 μM primer 7307speI-f, and 0.3 μM primer 7307-speI-r with a temperature program starting at 94°C for 100 s, followed by 25 cycles of 94°C for 15 s, 52°C for 30 s, 68°C for 90 s, and final elongation at 68°C for 90 s. After subcloning in pTA2 vector and confirmation of nucleotide sequence, full-length cDNA of Pf-7307 was cut with SpeI and then ligated into the SepI site of pGYR-SpeI to generate yeast expression vector. The sequence was confirmed. Pf-7307 was introduced into *S. cerevisiae* strain AH22 by the LiCl method, transgenic *S. cerevisiae* cells were cultured in concentrated SD medium at 30°C and 220 rpm for 20–24 h, and cultures were transferred to 600 mL medium and incubated for 40–48 h with shaking for protein expression. Cultured *S. cerevisiae* cells were harvested by centrifugation at 4°C, 8000×g for 10 min, and preparation of the microsomal fraction was performed as previously described (Ikezawa et al., 2003). A microsomal fraction was suspended in buffer (100 mM HEPES/NaOH pH 7.5) and stored at –80°C until needed. A reduced CO-difference

spectrum that was characteristic of cytochrome P450 (Omura and Sato, 1964) was measured to confirm the criterion for the enzyme activities.

4.6. Enzymatic assay and GC-MS analysis

Enzymatic reactions were performed in a solution of 250 μL in a 4 mL glass vial sealed with a polytetrafluoroethylene-coated silicone rubber septum. Reaction mixtures comprised 100 mM HEPES/NaOH, 1 mM NADPH, 1 μM substrate, and enzyme preparation. The reaction mixtures were incubated at 30°C for 16 h. An enzymatic reaction to investigate temporal changes was performed 6 times: 0 min, 30 min, 2 h, 4 h, 8 h, and 16 h. After the reaction, SPME fiber (100 μm polydimethylsiloxane, Supelco) was inserted into the headspace of the vial at room temperature for 5 min and was then transferred to the injector of a GC-MS (6850GC/5975MSD, Agilent Tech). The SPME fiber was heated for 10 min at 160°C to desorb the compounds. The compounds were separated on a DB-WAX column (60 m × 0.25 mm, 0.25 μm film thickness; Agilent Tech.). The temperature program began at 50°C for 5 min, increased at 5°C/min to 200°C, increased at 10°C/min to 240°C, and remained at 240°C for 3 min. He was used as carrier gas, and the column flow was 1.0 mL/min. The eluted compounds were identified by comparison of their ion spectra and relative retention times with authentic standards or the MS data library (NIST11; National Institute of Standards and Technology).

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Figure legends

Fig.1 Putative biosynthetic pathway of oil constituents in MT type perilla. *Trans*-shisool (6) may be related to MT biosynthesis. However the step is unclear.

Fig.2 Alignment of the amino-acid sequences of Pf-7307 described in this study, limonene-7-hydroxylase from *Perilla frutescens*, CYP71AT92 from *Salvia miltiorrhiza*, CYP76C1 from *Arabidopsis thaliana* and CYP76B6 from *Catharanthus roseus* in previous study. Black color of the background shows 100% amino-acid identity between the five clones.

Fig. 3 GC charts of the reaction product of Pf-7307 (A–D) and vector control (E–H) with substrate. These charts are extracted ion chromatograms of m/z = 136–154. (A) Pf-7307 with limonene (3), (B) Pf-7307 with perillyl alcohol (4), (C) Pf-7307 with perillaldehyde (5), (D) Pf-7307 with *trans*-shisool (6), (E) vector control with limonene (3), (F) vector control with perillyl alcohol (4), (G) vector control with perillaldehyde (5), and (H) vector control with *trans*-shisool (6). Open circles are limonene (3), closed circles are perillaldehyde (5), open squares are *trans*-shisool (6), and closed squares are perillyl alcohol (4).

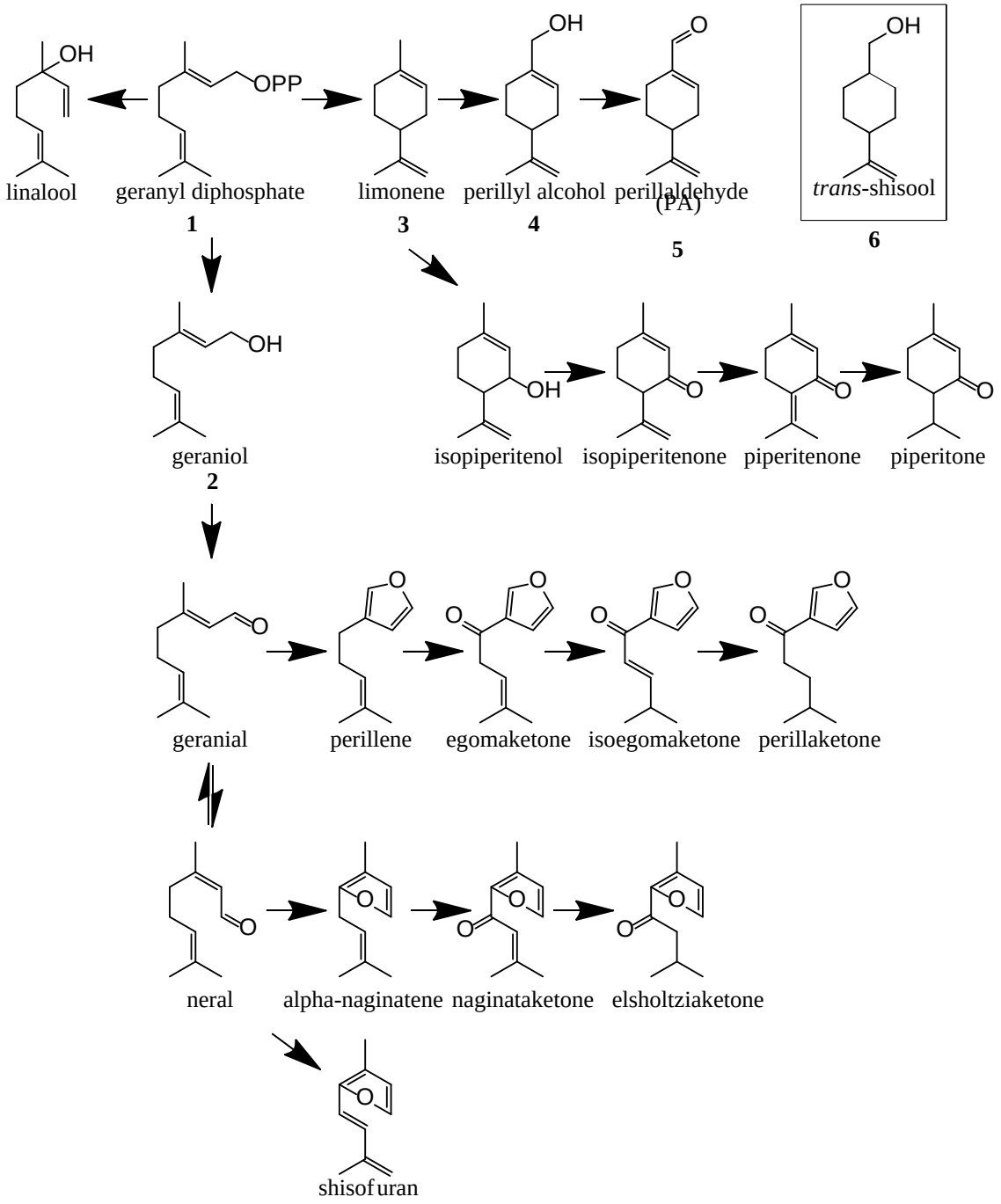
Fig. 4 GC charts of the sequential reaction product of Pf-7307 (A–F) and vector control (G–L) with limonene (3) as substrate. These charts are extracted ion chromatograms of m/z = 136–154. (A, G) 0 min for enzymatic reactions. (B, H) 30 min for enzymatic reactions. (C, I) 2 h for enzymatic reactions. (D, J) 4 h for enzymatic reactions. (E, K) 8 h for enzymatic reactions. (F, L) 16 h for enzymatic reactions. Open circles are limonene (3), closed circles are perillaldehyde (5), open squares are *trans*-shisool (6), and closed squares are perillyl alcohol (4).

Fig. 5 GC charts of the sequential reaction product of Pf-7307 (A–F) and vector control (G–L) with perillyl alcohol (4) as substrate. These charts are extracted ion chromatograms of m/z = 150–154. For enzymatic reactions (A, G) 0 min, (B, H) 30 min, (C, I) 2 h, (D, J) 4 h, (E, K) 8 h, (F, L) 16 h. Closed circles are perillaldehyde (5), open

squares are *trans*-shisool (6), and closed squares are perillyl alcohol (4).

Fig. 6 GC charts of the sequential reaction product of Pf-7307 (A–F) and vector control (G–L) with perillaldehyde (5) as substrate. These charts are extracted ion chromatograms of m/z = 150–154. For enzymatic reactions (A, G) 0 min, (B, H) 30 min, (C, I) 2 h, (D, J) 4 h, (E, K) 8 h, (F, L) 16 h. Closed circles are perillaldehyde (5), open squares are *trans*-shisool (6), and closed squares are perillyl alcohol (4).

Fig. 7 GC charts of the sequential reaction product of Pf-7307 (A–F) and vector control (G–L) with *trans*-shisool (6) as substrate. These charts are extracted ion chromatograms of m/z = 150–154. For enzymatic reactions (A, G) 0 min, (B, H) 30 min, (C, I) 2 h, (D, J) 4 h, (E, K) 8 h, (F, L) 16 h. Open squares are *trans*-shisool (6).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OPP
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O
OH
linalool
geranyl diphosphate
limonene
perillyl alcohol
perillaldehyde (PA)
trans-shisool
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3
4
5
6
OH
OH
O
O
O
geraniol
isopiperitenol
isopiperitenone
piperitenone
piperitone
2
O
O
O
O
O
O
O
geranial
perillene
egomaketone
isoegomaketone
perillaketone
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neral
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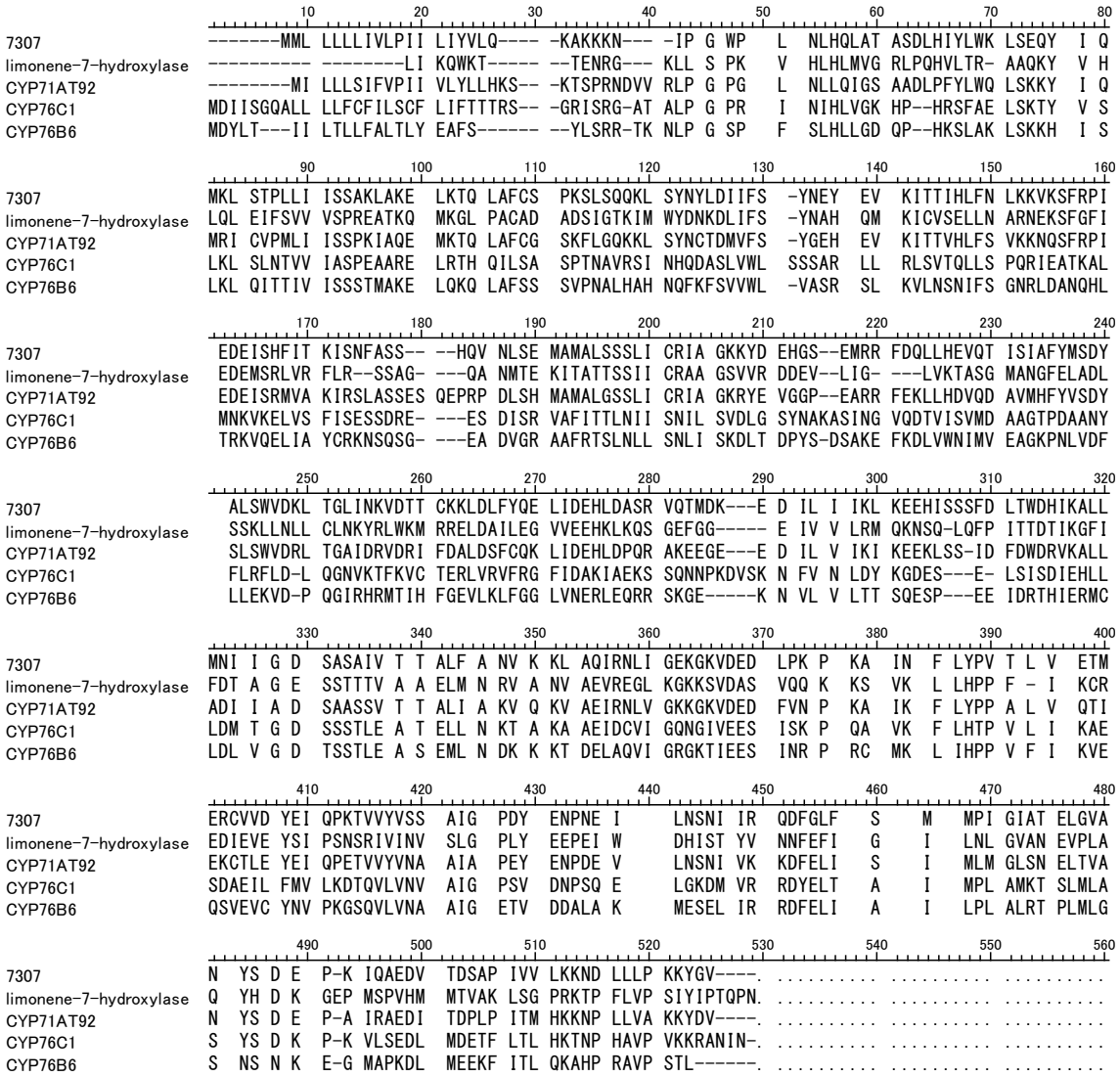
O
shisofuran

Figure 1



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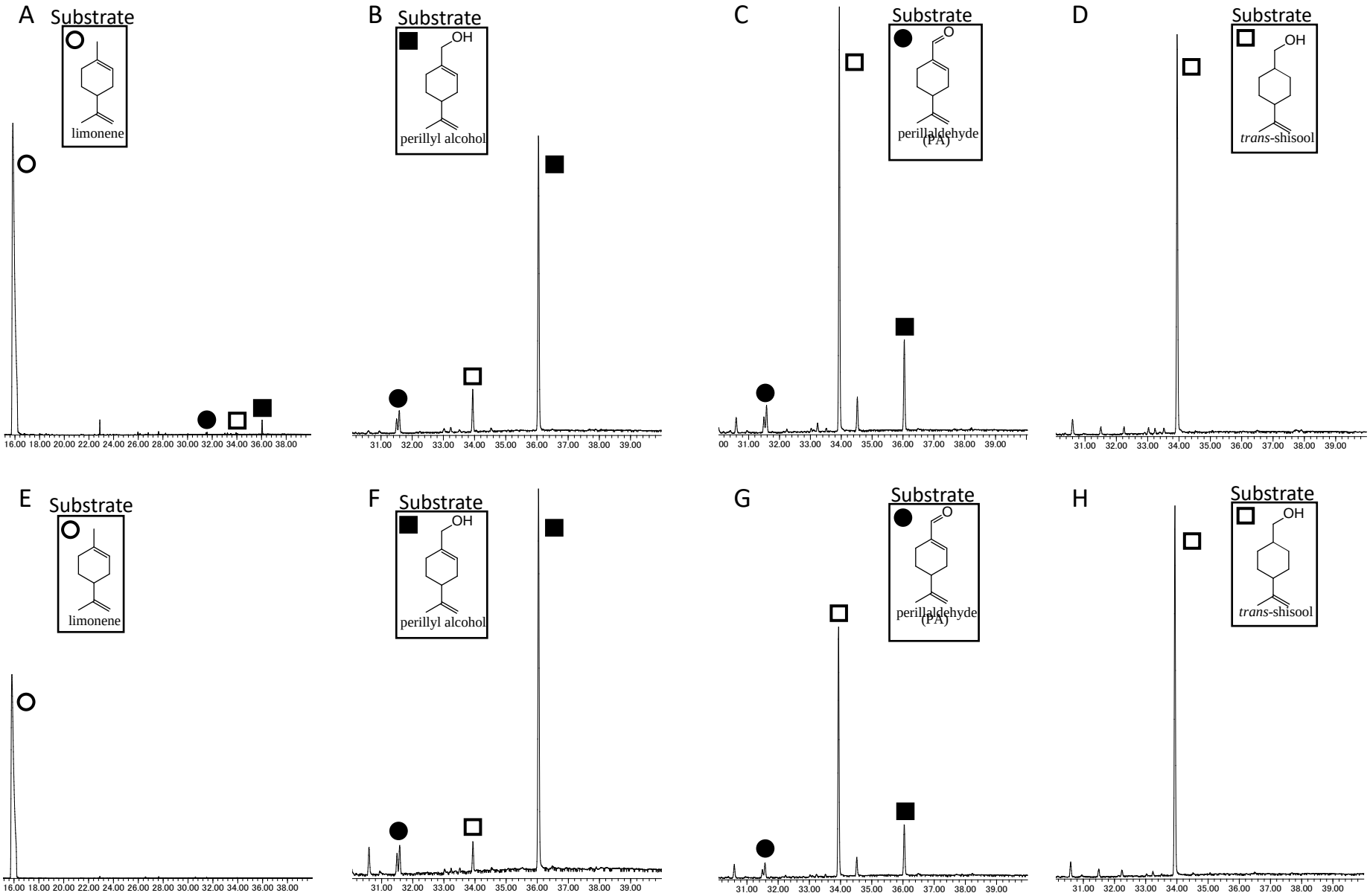
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Substrate
limonene
B
Substrate
OH
perillyl alcohol
C
Substrate
O
perillaldehyde
(PA)
D
Substrate
OH
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E
Substrate
limonene
F
Substrate
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G
Substrate
O
perillaldehyde
(PA)
H
Substrate
OH
trans-shisool

Figure 3

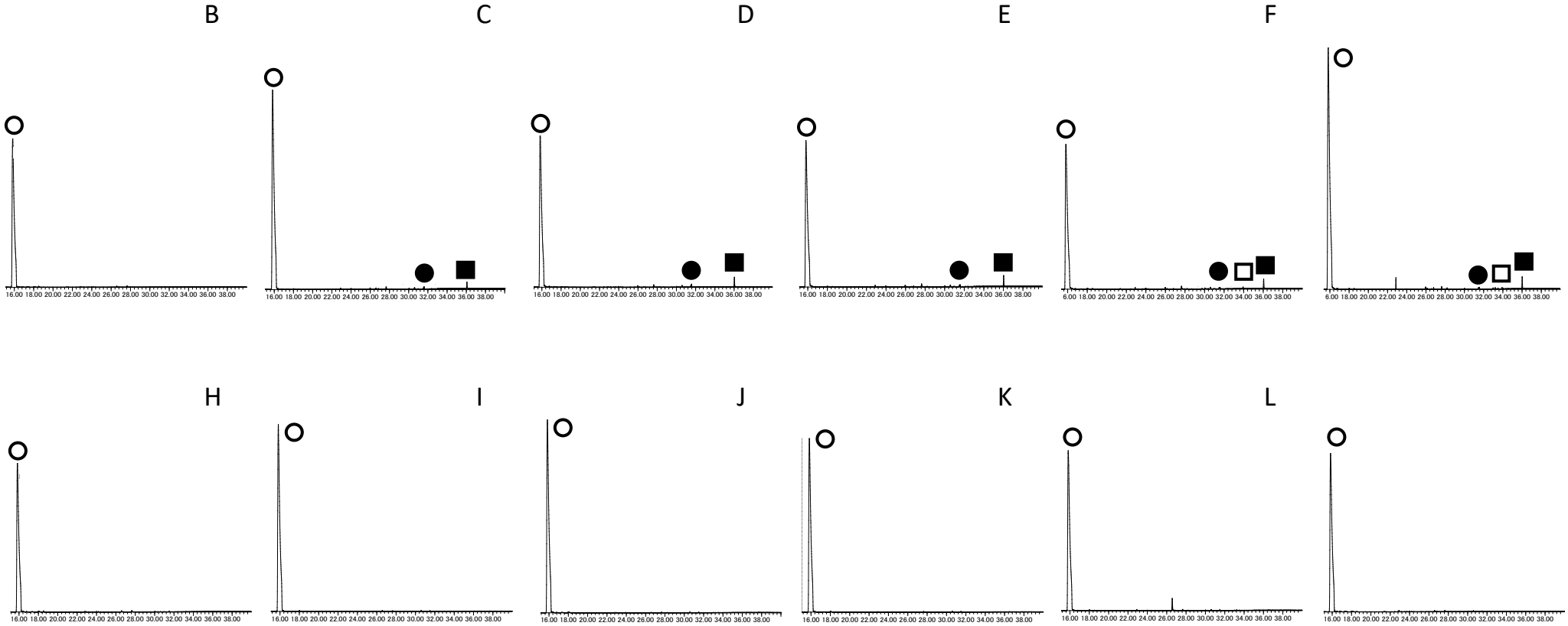

Figure 4

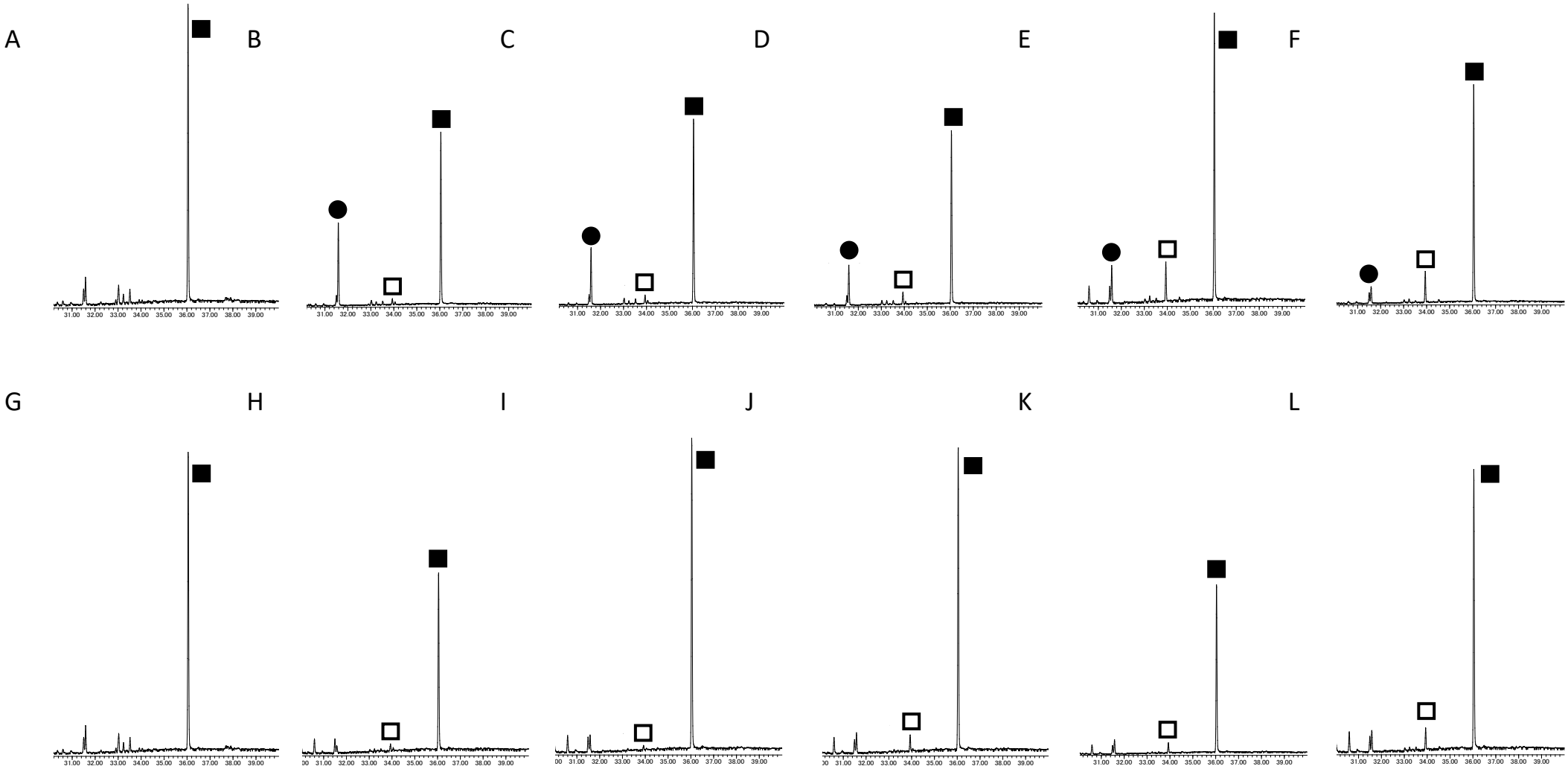

Figure 5

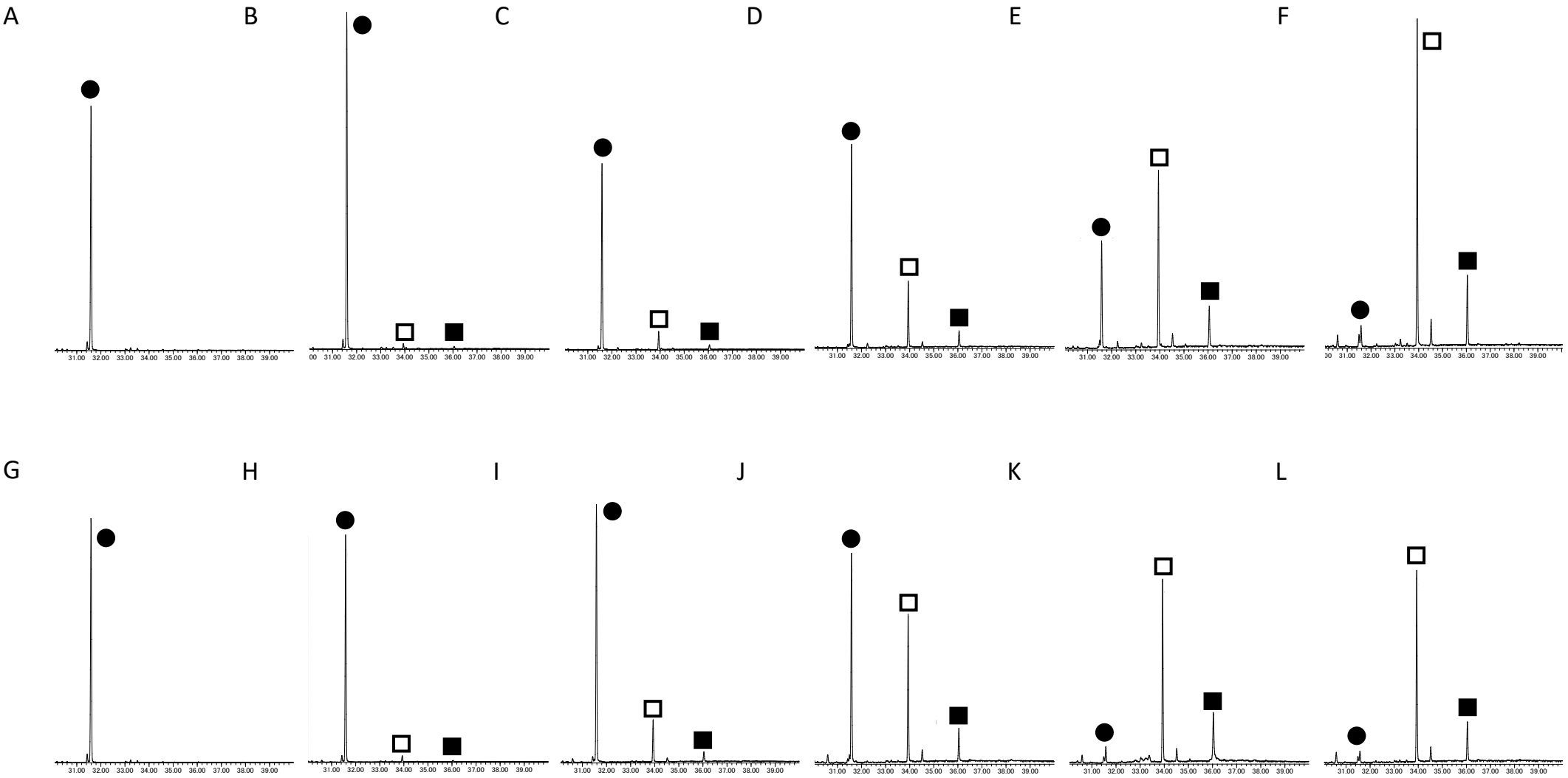

Figure 6

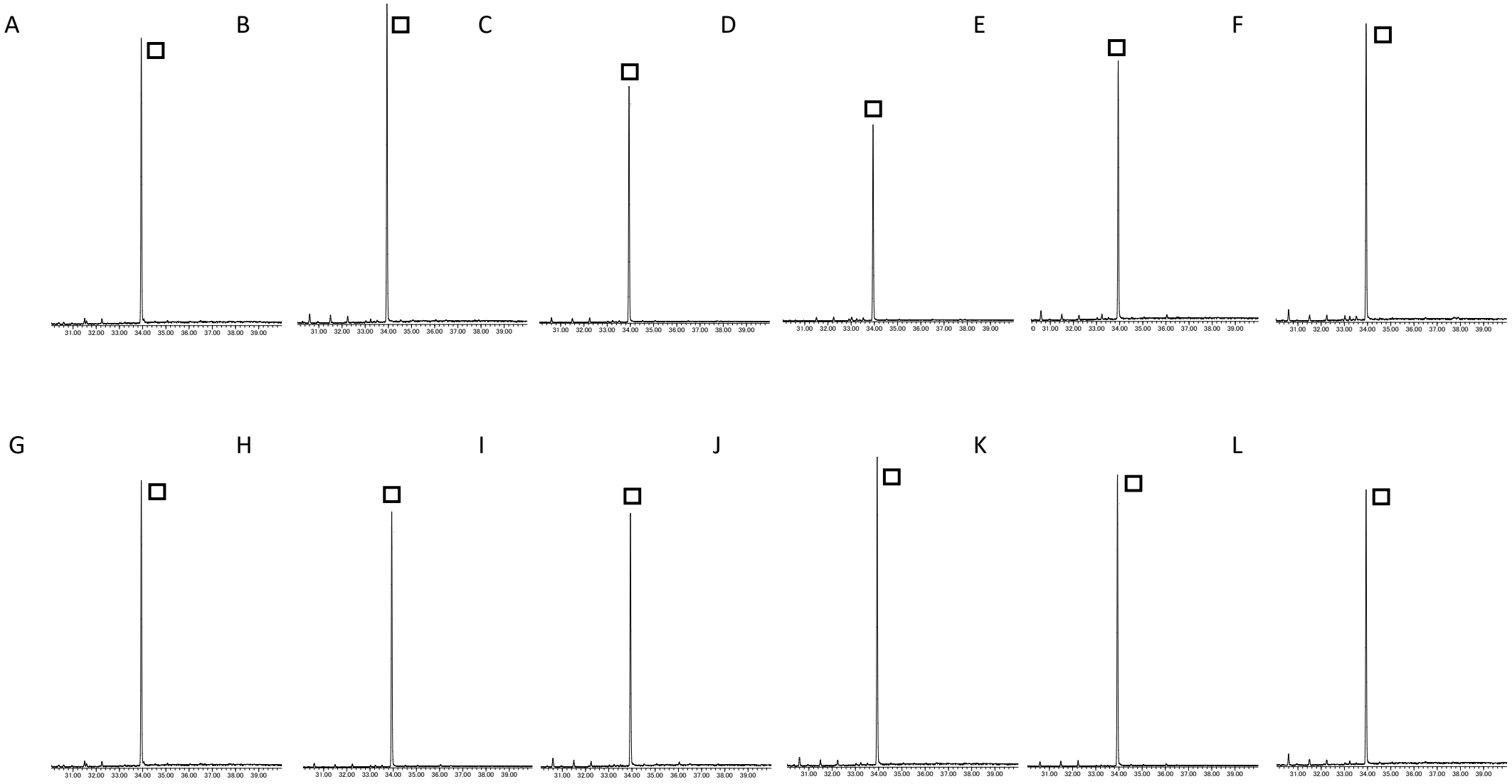

Figure 7