

Graphical abstract

α -Humulene synthases were isolated from *Aquilaria crassna* cells. They and previously cloned δ -guaiene synthases are responsible for the production of the sesquiterpenes induced by MJ treatment.

**Characterization of α -humulene synthases responsible for the production of
sesquiterpenes induced by methyl jasmonate in *Aquilaria* cell culture**

Yukie Kumeta and Michiho Ito

Department of Pharmacognosy, Graduate School of Pharmaceutical Sciences, Kyoto
University, Sakyo, Kyoto, 606-8501, Japan

Corresponding author: Michiho Ito

e-mail address: michihoi@pharm.kyoto-u.ac.jp

Tel: +81-75-753-4506

Fax: +81-75-753-4507

Highlights

- Full-length cDNAs of sesquiterpene synthases were isolated from MJ-treated cells of *Aquilaria crassna*.
- Their recombinant proteins were functionally characterized as α -humulene synthases.
- α -Humulene synthases and previously cloned δ -guaiene synthases were involved in the production of sesquiterpenes induced by MJ treatment of cells.

Abstract

The resinous portions of *Aquilaria* and *Gyrinops* plants are known as ‘agarwood’, which has a peculiar odor. To examine the biosynthesis of these fragrant compounds, cell cultures of *Aquilaria crassna* were established in which the production of three sesquiterpenes (α -guaiene, α -humulene, and δ -guaiene) could be induced by methyl jasmonate (MJ). Cloning and characterization of the δ -guaiene synthase from MJ-treated cells showed that it is involved in the synthesis of these three compounds, but only very small amounts of α -humulene are produced. In the present study, cDNAs encoding α -humulene synthases were also isolated. Three putative sesquiterpene synthase clones (*AcHS1–3*) isolated from the MJ-treated cells had very similar amino acid sequences and shared 52% identity with δ -guaiene synthases. The recombinant enzymes catalyzed the formation of α -humulene as a major product. Expression of transcripts of the α -humulene synthase and δ -guaiene synthase genes in cultured cells increased after treatment with MJ. These results revealed that these α -humulene and

1 δ -guaiene synthases are involved in the synthesis of three sesquiterpenes induced by MJ
2 treatment.

4 **Keywords**

5 *Aquilaria crassna*, cell cultures, methyl jasmonate treatment, functional characterization,
6 terpene, α -humulene synthase

8 **1. Introduction**

9 Plants in the genera *Aquilaria* and *Gyrinops* in the family Thymelaeaceae are
10 large evergreen trees that are found mainly in Southeast Asia. The resinous portions of
11 their branches and trunks are known as agarwood, which has been used as natural
12 digestive, sedative, or anti-emetic medicines, and also as incense due to their unique
13 perfume. Agarwood is traded worldwide; however, its sources are facing serious
14 depletion because of uncontrolled collection of agarwood in forests and the rapid loss of
15 tropical rain forests. Consequently, *Aquilaria* and *Gyrinops* have been listed in
16 Appendix II of the Convention on International Trade in Endangered Species of Wild
17 Fauna and Flora (CITES) since 2005 (Ito and Honda, 2008), and the international
18 import and export of their products are strictly controlled. Recently, the cultivation of
19 *Aquilaria* and *Gyrinops* trees and more attempts to artificially produce agarwood from
20 these trees are being made in Southeast Asia. However, artificial production of
21 agarwood has not yet been successful because the mechanisms by which the fragrant
22 compounds are produced in trees have not been revealed.

1 The main fragrant compounds of agarwood are sesquiterpenes and
2 phenylethyl chromone derivatives; high-quality agarwood contains a great variety of
3 sesquiterpenes such as guaiane, eudesmane, and their oxidized forms (Varma et al.,
4 1963; Nakanishi et al., 1981; Hashimoto et al., 1985; Ishihara et al., 1993; Yagura et al.,
5 2003). Jinkoh-eremol and agarospirol, both of which are peculiar to agarwood, are
6 known to have sedative and analgesic effects (Okugawa et al., 1996, 2000). Our
7 previous studies on the effects of inhalation of the agarwood fragrance on mice suggest
8 that these compounds might have sedative effects (Takemoto et al., 2008). Agarwood is
9 thus feasible for development of therapeutic applications and is an important material
10 for terpenoid studies.

11 Cell cultures of *Aquilaria crassna* were established to examine the
12 biosynthesis of their fragrant compounds at molecular level. Methyl jasmonate (MJ), an
13 elicitor of plant defensive responses, was administered to the cultures, and three species
14 of sesquiterpenes (α -guaiene, α -humulene, and δ -guaiene) were synthesized in the
15 cultured *A. crassna* cells (Ito et al., 2005; Okudera and Ito, 2009). The δ -guaiene
16 synthase genes responsible for the formation of these compounds were subsequently
17 cloned (Kumeta and Ito, 2010). However, the cloned δ -guaiene synthases produced only
18 a small amount of α -humulene although α -humulene was the main compound produced
19 in the cells 6 h after MJ treatment. We then predicted that another enzyme in agarwood
20 produces α -humulene as its main product, so we cloned the α -humulene synthase gene
21 to examine the mechanisms of sesquiterpene formation in cultured agarwood cells.

2. Results and Discussion

2.1. Cloning of sesquiterpene synthase cDNAs from MJ-treated cells

A degenerate primer (p5F) was designed with reference to the report of Jones et al. (Jones et al., 2008) to amplify sesquiterpene synthase cDNAs from MJ-treated *A. crassna* cells. The sequence of this primer was based on the conserved regions of several published angiosperm sesquiterpene synthase genes, namely, valencene synthase from *Vitis vinifera* (Lücker et al., 2004; GenBank accession No. AAS66358), germacrene D synthase from *Populus trichocarpa* × *deltoides* (Arimura et al., 2004; AAR99061), δ -cadinene synthase from *Gossypium hirsutum* (Davis et al., 1996; AAD51718), β -caryophyllene synthase from *Artemisia annua* (Cai et al., 2002; AAL79181), and δ -guaiene synthase from *A. crassna* (Kumeta and Ito, 2010; GU083696-GU083700). This primer was used for 3' Rapid Amplification of cDNA Ends (3'-RACE) PCR from total RNAs extracted from the MJ-treated cells. The 5' end of the sesquiterpene synthase coding sequences was obtained by subsequent 5'-RACE PCR. Eventually, three complete putative sesquiterpene synthase cDNA clones (*AcHS1*–3; GenBank accession No. KT893309-KT893311) were obtained. Their amino acid sequences were almost the same and they shared about 52% identity with δ -guaiene synthase (Figure 2). Each cDNA is comprised of an open reading frame of 1665 bp that encodes a protein of 555 amino acids and harbors motifs that are highly conserved among terpene synthases, such as an N-terminal RPx8 W motif (Bohlmann et al., 1998) and the DDxxD motif (Starks et al., 1997; Whittington et al., 2002), which is a divalent metal-ion substrate-binding site. As for the previously clone δ -guaiene

synthase, these enzymes have neither the N-terminal transit peptide that is usually found in monoterpene synthases nor sequences specific to diterpene synthases (Bohlmann et al., 1998).

2.2. Functional characterization of putative sesquiterpene synthases

The proteins encoded by cDNAs *AcHS1*–3 were expressed as C-terminal His-tagged fusion proteins in *Escherichia coli*. Recombinant proteins were purified using Ni²⁺ affinity chromatography and confirmed to be of the expected size of about 60 kDa by SDS-PAGE (data not shown).

The enzyme assays were performed using farnesyl pyrophosphate (FPP) as a substrate, and the reaction products were analyzed by GC-MS. The recombinant *AcHS1*–3 proteins generated α -humulene as a main product (ca. 95%) with a trace amount of β -caryophyllene (ca. 5%) (Figure 3 and Table 1), which revealed that these proteins have α -humulene synthase activity.

The kinetic parameters of *AcHS1*–3 are described in Table 1. *AcHS1*–3 showed similar kinetic profiles, while the previously cloned δ -guaiene synthases showed varied patterns among clones (Kumeta and Ito, 2010). The catalytic efficiencies (K_{cat}/K_m) of *AcHS1*–3 were 0.120–0.207 (those of δ -guaiene synthases were 0.035–2.404), and K_m values of *AcHS1*–3 were within the range of those previously reported for sesquiterpene synthases (0.1–10 mM; Cane, 1999) and δ -guaiene synthases.

In the present study, several clones of α -humulene synthases sharing very similar amino acid sequences and showing the same functionalities were obtained. Our

previous analyses of genomic DNA sequences of δ -guaiene synthases from *A. crassna* plants (Kumeta and Ito, 2011) identified multiple isoforms of the δ -guaiene synthase gene but also suggested that the mutations might have occurred during DNA replication in the cultured cells. It is conceivable that several isoforms of α -humulene synthase were obtained in the present study for the same reasons.

α -Humulene is one of the most common sesquiterpenes that is quite widely distributed among plant species; however, the cloning of enzymes that produce α -humulene as a main compound has previously been reported for only two species, *Zingiber zerumbet* (Yu et al., 2008) and *Picea glauca* (Keeling et al., 2011). The enzymes from those two species produce β -caryophyllene as a minor product, which was also true for AcHS1–3. Furthermore, our study revealed that the ratio of α -humulene to β -caryophyllene production by AcHS1–3 was almost the same as that by the enzyme from *Z. zerumbet* (ZSS1; α -humulene 95%, β -caryophyllene 5%). Although the enzymes AcHS1–3 each catalyze the same reaction as ZSS1, the amino acid sequences of AcHS1–3 share lower identity (40%) with ZSS1, whereas they share higher identity (52%) with δ -guaiene synthase from *A. crassna*. This result is consistent with previous reports that sequence similarities between some kinds of enzymes are based more on the taxonomic similarities of the plant species from which the genes were isolated rather than the type of products formed (Bohlmann et al., 1998).

2.3. Induction of terpene synthase mRNA expression by MJ treatment of cultured cells

Quantitative real-time PCR was performed to measure the mRNA expression levels of terpene synthase genes in MJ-treated cells. Primer pairs were designed specifically for α -humulene and δ -guaiene synthase genes, and for histone H2A as an internal control. The expression of the mRNAs for these two kinds of sesquiterpene synthases was elevated by MJ treatment, reached a maximum at 12 h, and then decreased (Figure 4). This result agrees with our previous study that the amount of three sesquiterpenes produced reached a maximum at 12 h after MJ treatment (Kumeta and Ito, 2010). Our studies show that α -humulene synthases together with δ -guaiene synthases contribute to the biosynthesis of three sesquiterpenes induced by MJ.

Interestingly, the increases in mRNA expression of these sesquiterpene synthase genes differed greatly. At 6 h after MJ treatment, the expression of the α -humulene synthase gene relative to the control had increased about 1.5-fold, whereas that of the δ -guaiene synthase gene had increased about 150-fold. The mRNA and protein expression levels of terpene synthase genes are not always directly correlated, which indicates the operation of post-transcriptional, translational, or post-translational regulatory mechanisms (Schnee et al., 2002; Yahyaa et al., 2015). Our study indicates that various post-transcriptional, translational, or post-translational regulatory mechanisms affecting the expression or activity of α -humulene and δ -guaiene synthases are possible.

3. Conclusions

New terpene synthase clones from MJ-treated cells of *A. crassna* were

isolated and characterized. These enzymes are involved in the synthesis of α -humulene as a main product and β -caryophyllene as a minor product. qPCR analyses of these α -humulene synthase genes and previously cloned δ -guaiene synthase genes (Kumeta and Ito, 2010) in cultured cells showed that the transcript levels of both genes increased to greatly different degrees after treatment with MJ. These results revealed that both α -humulene and δ -guaiene synthases are involved in the production of sesquiterpenes induced by MJ treatment. Furthermore, the expression of these genes might be subject to different transcriptional, post-transcriptional, or translational regulatory mechanisms.

4. Experimental

4.1. Cell cultures and MJ treatment

The methods used to prepare cultures of cells from *A. crassna* leaves were performed as described previously (Okudera and Ito, 2009). Cell suspension cultures were incubated with reciprocal shaking at 25 °C in Murashige-Skoog medium containing 3% w/v sucrose, 10^{-6} M 2,4-dichlorophenoxyacetic acid, and 10^{-6} M 6-benzyladenine, and were subcultured in fresh medium every two weeks. Just as in the previous study (Kumeta and Ito, 2010), cells cultured for 5 days after the inoculation were used for the experiments. MJ (Sigma Aldrich) was dissolved in DMSO to a concentration of 300 mM, and added to the culture at a final concentration of 0.1 mM.

4.2. Cloning of putative sesquiterpene synthase cDNAs using the RACE method

with a degenerate primer

A degenerate primer p5F (5'-TGGTGGGAARGAYTTIGAYTTY-3') was designed as a forward primer for 3'-RACE based on the WWK(E/D/S)(L/F)DF motif, referring to the report of Jones et al. (2008).

Total RNA was extracted from the cultured cells that had been incubated with MJ for 4 h. First-strand cDNA for 3' RACE was synthesized from total RNA using an oligo dT-adapter primer (5'- CCACGCGTCGACTAC (T)₁₅-3'; adapter sequences are underlined) and ReverTra Ace® (Toyobo). The resulting cDNA was used as a template for PCR with the degenerate primer p5F and adapter primer, which generated a 1000-bp fragment. The reaction mixture contained 2 µL of 10× reaction buffer (TaKaRa), 0.2 mM of each dNTP, 0.5 U of ExTaq polymerase (TaKaRa), 3 µM of p5F primer, 0.3 µM of adapter primer, 0.2 µL of DMSO, and 1 µL of template cDNA in a final volume of 20 µL. The thermal cycling conditions for PCR were as follows: a denaturing step at 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min 30 s, and a final elongation at 72 °C for 2 min. Following gel purification, the PCR product was cloned into the pCR 4-TOPO vector (Life Technologies) and sequenced.

For 5' RACE PCR, three reverse primers specific to the sequences of the 3' RACE PCR product (A: 5'-GCTTCAGGTTCCCATCTCTCAACTGCA-3'; B: 5'-GGGGTTCATATTGGACGCCCAAATCC-3'; and C: 5'-GTAACACTCAACCAACCGATCTC-3') were designed. First-strand cDNA for 5' RACE was synthesized from total RNA using reverse primer A, as described above, and a poly-C sequence was added at the 5'-end of the cDNA. The resulting poly-C-tailed

cDNA was used as a template for nested PCR with the oligo dG-adapter primer (5'-GGCCACGCGTCGACTAGTACGGG(I)(I)GGG(I)(I)GGG(I)(I)G-3') and reverse primer B (for the first PCR), and then the adapter primer and reverse primer C (for the second PCR). A ~900-bp PCR product was obtained. The reaction mixtures for nested PCR were as follows: 5 µl of 10×KOD plus ver. 2 buffer (Toyobo), 0.2 mM of each dNTP, 1 U of KOD plus polymerase (Toyobo), 0.24 µM of primers, 1.5 mM of MgSO₄, 1 µl of cDNA template (first PCR) or first PCR product (second PCR) in final volumes of 50 µL. Nested PCR conditions were as follows: a denaturing step at 94 °C for 40 s, followed by 30 cycles (first PCR) or 35 cycles (second PCR) of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 1 min, and a final elongation at 68 °C for 2 min. Following gel purification, the second PCR product was cloned into the pCR 4-Blunt TOPO vector (Life Technologies) and sequenced.

The full-length open reading frame of a putative sesquiterpene synthase gene was amplified by PCR from cDNA template in 3' RACE using forward primer (5'-CACCATGTCTCCAGCTCAGGCCCCCCTAA-3') and reverse primer (5'-AATCGTGAAAGGATGAACTAAC-3'). The reaction mixture contained 2 µL of 10×KOD plus ver. 2 buffer (Toyobo), 0.2 mM of each dNTP, 1 U of KOD plus polymerase (Toyobo), 0.3 µM of forward and reverse primers, 1.25 µL of MgSO₄, and 1 µL of template cDNA in a final volume of 20 µL. PCR conditions were as follows: a denaturing step at 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 68 °C for 1 min 40 s, and a final elongation at 68 °C for 3 min 20 s. Following gel purification, the resultant PCR product (about 1670 bp) was cloned into the

pET101/D-TOPO directional expression vector (Life Technologies) and sequenced. The sequences described in this work have been deposited into GenBank under the accession numbers KT893309, KT893310, and KT893311.

4.3. Heterologous expression and enzyme assays

Heterologous expression in *E. coli* and enzyme assays were performed as described previously (Kumeta and Ito, 2010), except for SPME-GC-MS analysis of the reaction products of the enzyme assays.

SPME-GC-MS (Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry) analysis was performed using the 7890GC/5975MSD system (Agilent) fitted with a DB-WAX column (0.25 mm × 0.25 μm × 60 m, GL Science). The helium flow rate was 1 mL min⁻¹, ionization voltage was 1500 V, and column oven program was the same as used in our previous report (Kumeta and Ito, 2010). The reaction products were extracted onto SPME fiber (100 μm bonded polydimethylsiloxane coating, Supelco), and the fiber was inserted directly into the injection port (250 °C) of the GC apparatus for 10 min. Enzyme products were identified by comparison of retention times and mass spectra to authentic standards for α-humulene (Sigma) and β-caryophyllene (Tokyo Chemical Industry).

4.4. Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells incubated for 0 (control), 6, 12, or 24 hours after MJ treatment using the method described above. Reverse transcription of 2

1 μ g RNA into cDNA was carried out with incubation at 42 °C for 60 min with 1 U of
 2 ReverTra Ace (Toyobo) and 0.25 mM oligo dT₁₅ primer (Takara) in a reaction volume
 3 of 20 μ L. After the cDNA solution was purified on a Nucleospin gel and PCR Clean-up
 4 column (Macherey-Nagel) and eluted with 40 μ L of elution buffer, 2 μ L of purified
 5 cDNA solution was used as template for qPCR. PCR was performed using a
 6 StepOnePlus Real-Time PCR System (Applied Biosystems) in a reaction mixture
 7 containing THUNDERBIRD SYBER qPCR Mix (Toyobo) and 0.3 μ M forward and
 8 reverse primers using the following cycling parameters: 95 °C for 1 min followed by 40
 9 cycles of 95 °C for 15 s, 64 °C for 30 s, and 72 °C for 30 s, followed by a melting curve
 10 analysis comprised of 95 °C for 15 s, 64 °C for 30 s, then ramping by 0.3°C s⁻¹ to 95 °C.
 11 Transcript abundance was normalized to the transcript abundance of the histone H2A
 12 gene and was calculated from three technical replicates of three biological replicates.
 13 Relative transcript abundances were calibrated against the transcript abundance of
 14 control samples. The primer pairs used for qPCR were as follows: Histone (product
 15 size: 144 bp): 5'-AAGGTCGTTATTCGGAGCGTGTC-3' (forward) and
 16 5'-CAGGATCATAACCGAGACATGTTC-3' (reverse); α -humulene synthase (156 bp):
 17 5'-GCTCCATATGCGACCAGAGAATC-3' (forward) and
 18 5'-CTCGGGAGTTCATTGCTACATG-3' (reverse); and δ -guaiene synthase (133 bp):
 19 5'-GCTTCGACAATCCCAGAAATGGTC-3' (forward) and
 20 5'-GGTGTTACATGAGGGAGTTTG-3' (reverse).

Acknowledgement

We thank Ms. Kaori Nakano for her technical help in cloning α -humulene synthases.

This work was partially supported by the Takeda Science foundation (funds to M. Ito).

Figure legends

Fig. 1. Putative biosynthetic pathways for sesquiterpenes found in agarwood and cell suspension cultures.

Fig. 2. Alignment of deduced amino acid sequences of sesquiterpene synthases from cultured cells of *A. crassna*. AcC2 is δ -guaiene synthase (GU083697) cloned in our previous study. Shading indicates levels of sequence conservation (100%: black on white; over 50%: black on grey; under 50%: black on white). The conserved RP_xW and DDxxD motifs are underlined. The WWK(E/D/S)(L/F)DF motif, which was used for designing the degenerate primer, is surrounded by a box.

Fig. 3. GC-MS profiles. (A) Total ion chromatogram of the products formed by sesquiterpene synthase (AcHS1) with FPP as a substrate. (B) Mass spectra of the sesquiterpenes and their authentic standards.

Fig. 4. Real-time PCR analysis of the expression of the α -humulene synthase gene (A) and δ -guaiene synthase gene (B) in MJ-treated cultured cells of *A. crassna*. For both genes, level of each transcript in the control cells was set as 1. Error bars indicate SD of three technical replicates of three biological replicates.

Table

Table 1 Products of sesquiterpene synthases expressed in *E. coli*.

Clone name	Total products (%)		Kinetic parameters		
	α -humulene	β -caryophyllene	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
AcHS1	94.6	5.4	6.17 $\times 10^{-4}$	3.07	0.201
AcHS2	95.0	5.0	8.56 $\times 10^{-4}$	4.14	0.207
AcHS3	93.5	6.5	9.38 $\times 10^{-4}$	2.54	0.357

References

- Arimura, G., Huber, D.P.W., Bohlmann, J., 2004. Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa* $\times$ *deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1. *Plant J.* 37, 603-616.
- Bohlmann, J., Meyer-Gauen, G., Croteau, R., 1998. Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc. Natl. Acad. Sci. USA* 95, 4126-4133.
- Cai, Y., Jia, J.W., Crock, J., Lin, Z.X., Chen, X.Y., Croteau, R., 2002. A cDNA clone for β -caryophyllene synthase from *Artemisia annua*. *Phytochemistry* 61, 523-529.
- Cane, D.E., 1999. Sesquiterpene biosynthesis: cyclization mechanisms. In *Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and*

1 Steroids, Vol 2. Pergamon, Oxford, pp 155–200

2 Davis, E.M., Tsuji, J., Davis, G.D., Pierce, M.L., Essenberg, M., 1996. Purification of
3 (+) -delta-cadinene synthase, a sesquiterpene cyclase from bacteria-inoculated cotton
4 foliar tissue. *Phytochemistry* 41, 1047-1055.

5 Hashimoto, K., Nakahara, S., Inoue, T., Sumida, Y., Takahashi, M., Masada, Y., 1985. A
6 new chromone from agarwood and pyrolysis products of chromone derivatives. *Chem.*
7 *Pharm. Bull.* 33, 5088-5091.

8 Ishihara, M., Tsuneya, T., Uneyama, K., 1993. Fragrant sesquiterpenes from agarwood.
9 *Phytochemistry* 33, 1147-1155.

10 Ito, M., Okimoto, K., Yagura, T., Honda, G., 2005. Induction of sesquiterpenoid
11 production by methyl jasmonate in *Aquilaria sinensis* cell suspension culture. *J. Essent.*
12 *Oil Res.* 17, 175-180.

13 Ito, M., Honda, G., 2008. Agarwood-its sedative effect on mice and current state in the
14 production sites. *Aroma Research* 34, 122-127.

15 Jones, C.G., Keeling, C.I., Ghisalberti, E.L., Barbour, E.L., Plummer, J.A., Bohlmann,
16 J., 2008. Isolation of cDNAs and functional characterisation of two multi-product
17 terpene synthase enzymes from sandalwood, *Santalum album* L.. *Arch. Biochem.*
18 *Biophys.* 477, 112-130.

19 Keeling, C.I., Weisshaar, S., Ralph, S.G., Jancsik, S., Hamberger, B., Dullat, H.K.,
20 Bohlmann, J., 2011. Transcriptome mining, functional characterization, and phylogeny
21 of a large terpene synthase gene family in spruce (*Picea* spp.). *BMC Plant Biol.* 11: 43.

22 Kumeta, Y., Ito, M., 2010. Characterization of δ -guaiene synthases from cultured cells

1 of *Aquilaria*, responsible for the formation of the sesquiterpenes in agarwood. Plant
2 Physiol. 154, 1998-2007

3 Kumeta, Y., Ito, M., 2010. Genomic organization of δ -guaiene synthase genes in
4 *Aquilaria crassna* and its possible use for the identification of *Aquilaria* species. J. Nat.
5 Med. 65, 508-513

6 Lückner, J., Bowen, P., Bohlmann, J., 2004. *Vitis vinifera* terpenoid cyclases: functional
7 identification of two sesquiterpene synthase cDNAs encoding (+)-valencene synthase
8 and (-)-germacrene D synthase and expression of mono- and sesquiterpene synthases in
9 grapevine flowers and berries. Phytochemistry 65, 2649-2659.

10 Nakanishi, T., Yamagata, E., Yoneda, K., Miura, I., 1981. Jinkohol, a prezizane
11 sesquiterpene alcohol from agarwood. Phytochemistry 20, 1597-1599.

12 Okudera, Y., Ito, M., 2009. Production of agarwood fragrant constituents in *Aquilaria*
13 calli and cell suspension cultures. Plant Biotech. 26, 307-315.

14 Okugawa, H., Ueda, R., Matsumoto, K., Kawanishi, K., Kato, A., 1996. Effect of
15 jinko-eremol and agarospirol from agarwood on the central nervous system in mice.
16 Planta Med. 62, 2-6.

17 Okugawa, H., Ueda, R., Matsumoto, K., Kawanishi, K., Kato, K., 2000. Effects of
18 sesquiterpenoids from "Oriental incenses" on acetic acid-induced writhing and D2 and
19 5-HT_{2A} receptors in rat brain. Phytomedicine 7, 417-422.

20 Schnee, C., Köllner, T.G., Gershenzon, J., Degenhardt, J., 2002. The maize gene *terpene*
21 *synthase 1* encodes a sesquiterpene synthase catalyzing the formation of
22 (*E*)- β -farnesene, (*E*)-nerolidol, and (*E,E*)-farnesol after herbivore damage. Plant Physiol.

1 130, 2049-2060.

2 Starks, C.M., Back, K.W., Chappell, J., Noel, J.P., 1997. Structural basis for cyclic
3 terpene biosynthesis by tobacco 5-epi-aristolochene synthase. *Science* 277, 1815-1820.

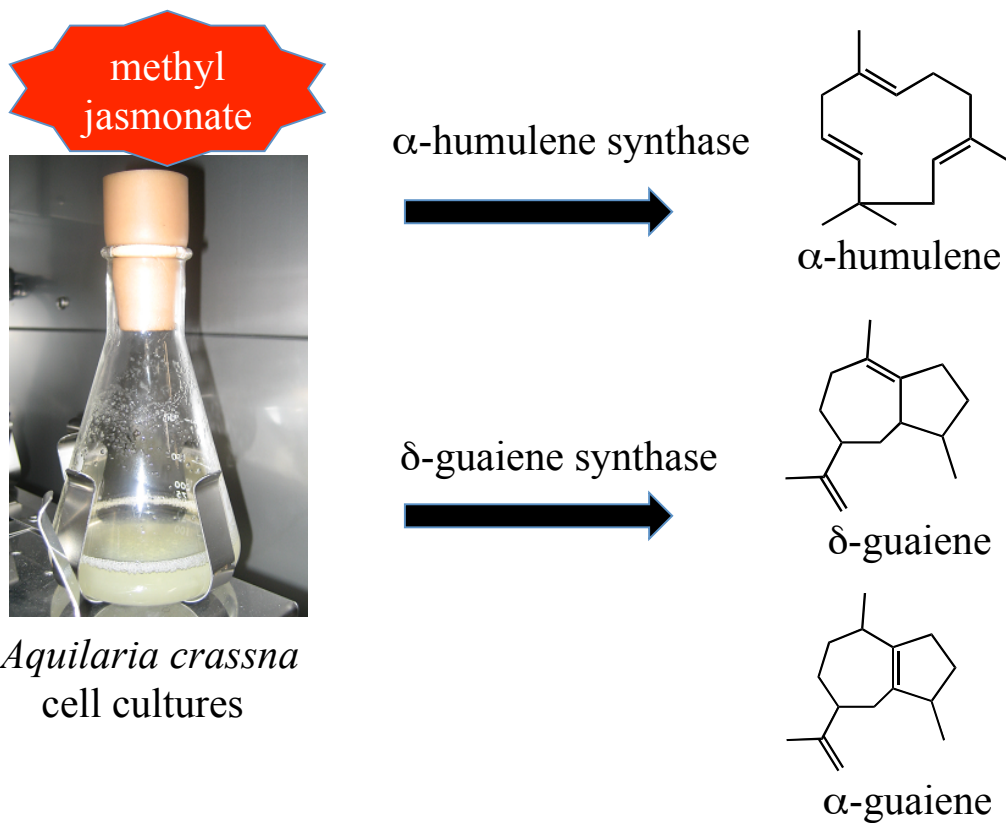
4 Takemoto, H., Ito, M., Shiraki, T., Yagura, T., Honda, G., 2008. Sedative effects of
5 vapor inhalation of agarwood oil and spikenard extract and identification of their active
6 components. *J. Nat. Med.*, 62, 41-46.

7 Varma, K.R., Maheshwari, M.L., Bhattacharyya, S.C., 1963. The constitution of
8 agarospirol, a sesquiterpenoid with a new skeleton. *Tetrahedron* 21, 1079-1090.

9 Whittington, D.A., Wise, M.L., Urbansky, M., Coates, R.M., Croteau, R.B.,
10 Christianson, D.W., 2002. Bornyl diphosphate synthase: structure and strategy for
11 carbocation manipulation by a terpenoid cyclase. *Proc. Natl. Acad. Sci. USA* 99,
12 15375-15380.

13 Yahyaa, M., Tholl, D., Cormier, G., Jensen, R., Simon, P.W., Ibdah, M., 2015.
14 Identification and characterization of terpene synthases potentially involved in the
15 formation of volatile terpenes in carrot (*Daucus carota* L.) roots. *J. Agric. Food Chem.*
16 63, 4870-4878.

17 Yu, F., Okamoto, S., Nakasone, K., Adachi, K., Matsuda, S., Harada, H., Misawa, N.,
18 Utsumi, R., 2008. Molecular cloning and functional characterization of α -humulene
19 synthase, a possible key enzyme of zerumbone biosynthesis in shampoo ginger
20 (*Zingiber zerumbet* Smith). *Planta* 227, 1291-1299.



Graphical abstract

α -Humulene synthases were isolated from *Aquilaria crassna* cells. They and previously cloned δ -guaiene synthases are responsible for the production of the sesquiterpenes induced by MJ treatment.

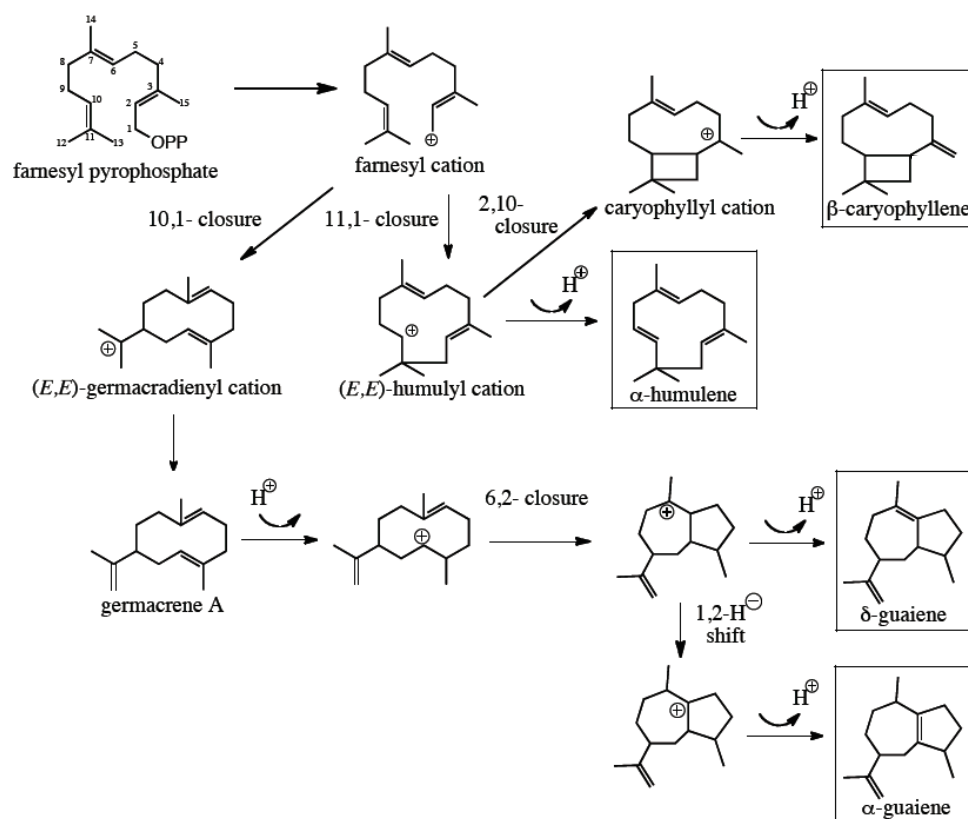


Fig. 1 Putative biosynthetic pathways for sesquiterpenes found in agarwood and cell suspension cultures



Fig. 2. Alignment of deduced amino acid sequences of sesquiterpene synthases from cultured cells of *A. crassna*. AcC2 is d-guaiene synthase (GU083697) cloned in our previous study. Shading indicates levels of sequence conservation (100%: black on white, over 50%: black on grey, under 50%: black on white). The conserved RPx₈W and DDxxD motifs are underlined. The WWK(E/D/S)(L/F)DF motif which is used for designing the degenerated primer is boxed.

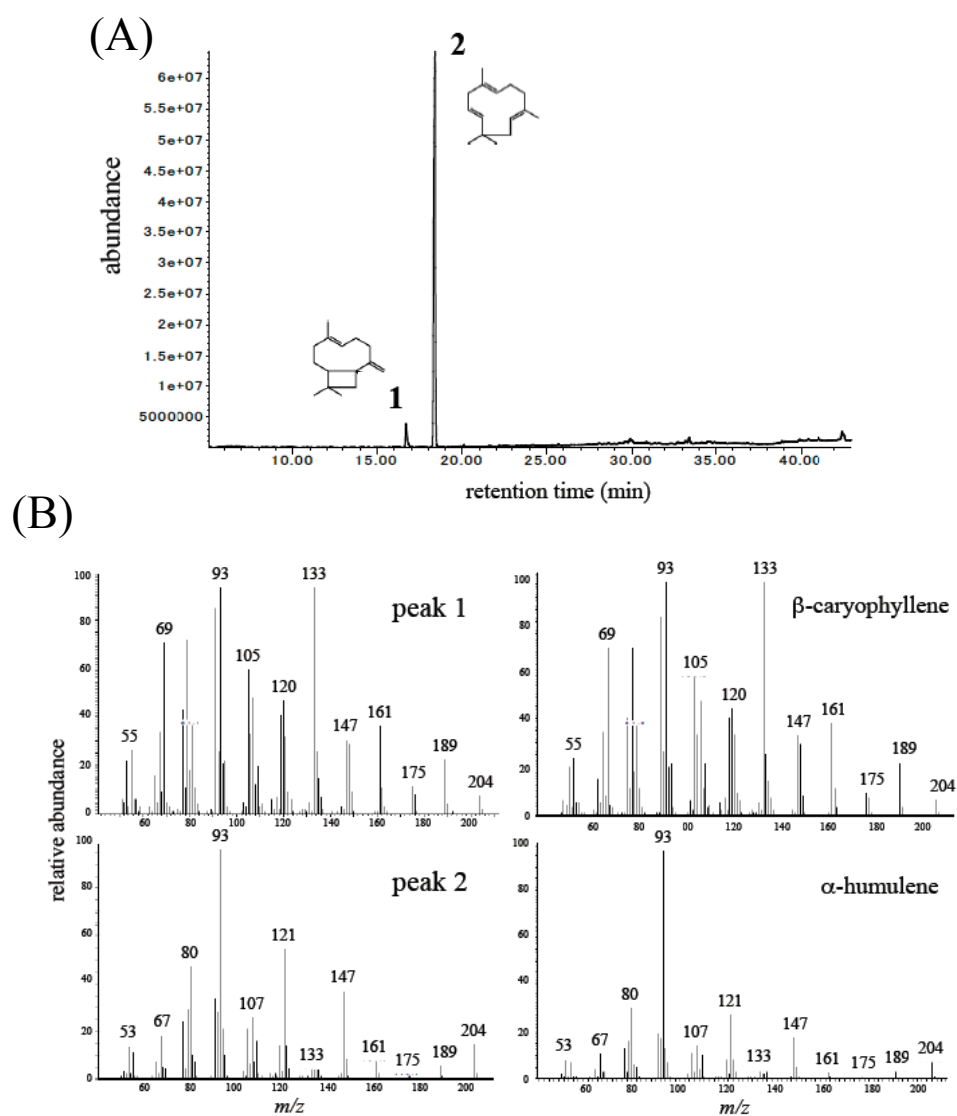


Fig. 3. GC-MS profiles. (A) Total ion chromatogram of the products formed by sesquiterpene synthases (AcHS1) with FPP as a substrate. (B) Mass spectra of the sesquiterpenes and their authentic standards.

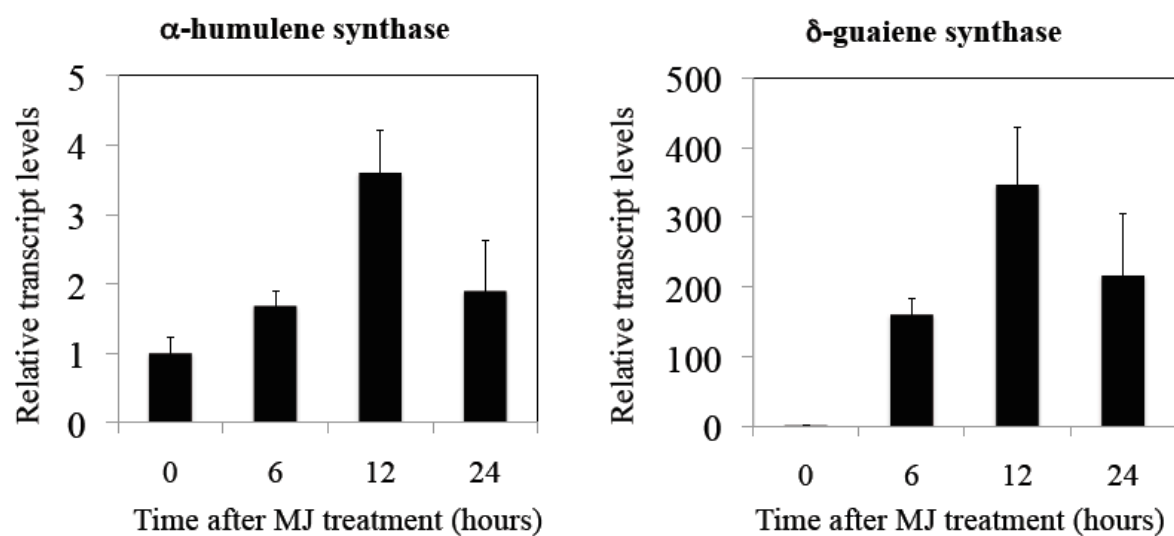


Fig. 4. Real-time PCR mRNA expression analysis of α -humulene synthase gene (A) and δ -guaiene synthase gene (B) in MJ-treated cultured cells of *A. crassna*. For both genes, each transcript level in the control cells was set as 1. Error bars indicate SD of three biological replicates and three technical replicates.