Characterization of δ-Guaiene Synthases from Cultured Cells of *Aquilaria*, Responsible for the Formation of the Sesquiterpenes in Agarwood\textsuperscript{1}[C][W][OA]

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The resinous portions of *Aquilaria* plants, called agarwood, have been used as medicines and incenses. Agarwood contains a great variety of sesquiterpenes, and a study using cultured cells of *Aquilaria* showed the production of sesquiterpenes (α-guaiene, α-humulene, and δ-guaiene) to be induced by treatment with methyl jasmonate (MJ). In this study, the accumulation and production of sesquiterpenes were quantified. The amounts accumulated and produced reached a maximum at 12 h, and the most abundant product was α-humulene at 6 h and δ-guaiene after 12 h. However, a headspace analysis of the cells revealed that α-humulene is likely to be volatilized; so overall, the most abundant sesquiterpene in the cells was δ-guaiene. A cDNA library from RNA isolated from MJ-treated cells was screened using PCR methodologies to isolate five clones with very similar amino acid sequences. These clones were expressed in *Escherichia coli*, and enzymatic reactions using farnesyl pyrophosphate revealed that three of the clones yielded the same compounds as extracted from MJ-treated cells, the major product being δ-guaiene. These genes and their encoded enzymes are the first sesquiterpene synthases yielding guaiane-type sesquiterpenes as their major products to be reported. Expression of a fourth terpene synthase gene in bacteria resulted in the accumulation of the protein in insoluble forms. Site-directed mutagenesis of the inactive clone and three-dimensional homology modeling suggested that the structure of the N-terminal domain was important in facilitating proper folding of the protein to form a catalytically active structure.

The genera *Aquilaria* and *Gyrinops* of the Thymelaeaceae are large evergreen trees found mainly in Southeast Asia. The resinous portions of their branches and trunks, known as agarwood, have been used in natural medicine as a digestive, sedative, and antiemetic, and also as incense. However, the source of agarwood is facing serious depletion because of uncontrolled collection in forests and the rapid loss of tropical rain forests. Consequently, *Aquilaria* and *Gyrinops* have been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora since 2005 (Ito and Honda, 2008), and the international import and export of their products are under strict control.

The main fragrant compounds of agarwood are sesquiterpenes and phenylethyl chromone derivatives, and a great variety of sesquiterpenes are contained in high-quality agarwood (Varma et al., 1963; Nakanishi et al., 1981; Hashimoto et al., 1985; Ishihara et al., 1993; Yagura et al., 2003). Terpenoids form one of the largest and most diverse groups of natural products, mainly composed of monoterpene (C10), sesquiterpene (C15), diterpene (C20), and triterpene (C30) compounds, and these compounds and their derivatives include valuable natural products used for pharmaceuticals or perfumes. Their diverse cyclic and acyclic structures are known to be synthesized from prenyl diphosphates or squalene by various terpene synthases (TPSs); a few species of substrates are transformed into a number of terpenoids with various structural types. Mechanisms of reaction control and structures of enzymes are studied by many groups trying to clarify the structure-function relationship in TPSs (Steele et al., 1998; Felicetti and Cane, 2004; Deguerry et al., 2006; Landmann et al., 2007; Lee and Chappell, 2008). The resinous portion of agarwood is rich in sesquiterpenes such as guaiene, eudesmane, and their oxidized forms, jinkoh-eremol and agarospirol, both of which are peculiar to agarwood and are known to have sedative and analgesic effects (Okugawa et al., 1996, 2000). Our recent studies on the effects of the fragrance of agarwood on mice by inhalation suggest that these compounds might induce sedative effect (Takemoto et al., 2008) and thus have promise for therapeutic applications. Consequently, *Aquilaria* and *Gyrinops* have promise for therapeutic applications.
of plants producing oleoresins, conifers have been used to study the mechanisms by which the resin forms, including the biosynthesis of terpenoids. It has been reported that both the resin and terpenoids are produced in response to biotic and abiotic stress (Lewinsohn et al., 1991; Bohlmann et al., 1998a; Martin et al., 2002; Fält et al., 2003). The sesquiterpenes found in agarwood are also considered to be produced as phytoalexins under stress, but there are no reports about the biosynthesis of fragrant compounds in agarwood. This is in part because all agarwood-producing plants are timber species that require considerable time to grow, the resinous portions are formed inside the wood, and the cells thought to contain the relevant enzymes are difficult to obtain. Because studies with fresh plants are difficult, we used cell cultures derived from the leaves of *Aquilaria* sp. Treatment with methyl jasmonate (MJ), an elicitor of plant defensive responses, was performed to determine if fragrant-like compounds accumulated, and three species of sesquiterpene (α-guaiene, α-humulene, and δ-guaiene) were found to be induced in the cultured cells (Ito et al., 2005; Okudera and Ito, 2009). Guaiane-type sesquiterpenes are thought to be synthesized via two cyclization reactions, the first constituting a C1-to-C10 cyclization, yielding a macrocyclic germacrene-like intermediate, and the second cyclization event occurring between C2 and C6 to generate the guaiene product (Fig. 1). Although guaiane-type sesquiterpenes are common in nature and a few enzymes described as producing guaianes as secondary reaction products have been described (Steele et al., 1998; Deguerry et al., 2006), an enzyme catalyzing the formation of this class of sesquiterpenes as its dominant reaction product has yet to be described. Guaiane-type structures are unique, being composed of five- and seven-membered rings. Given the unique five- and seven-membered ring systems in guaiane sesquiterpenes, the identification of the corresponding TPSs responsible for their biosynthesis should provide yet another tool for the molecular dissection of TPSs in general and possibly provide another perspective on the evolution and diversity of TPSs found in nature.

**RESULTS**

Quantification of the Sesquiterpenes Produced in the MJ-Treated Cells and Enzymatic Activities of Crude Cell Extracts from MJ-Treated Cells

Our previous study showed that MJ induced the production of three species of sesquiterpenes (α-guaiene, α-humulene, and δ-guaiene; Fig. 1) in cultured cells of *Aquilaria crassna* (Okudera and Ito, 2009). Here, the amount of sesquiterpene produced in response to MJ was examined over a 24-h period. There was a temporary increase in the amount of sesquiterpene in the cells, though little accumulated in untreated control cells (Fig. 2). The total amount of sesquiterpenes accumulating reached a maximum at 12 h then decreased, of which δ-guaiene was the most abundant. Oxidized sesquiterpenes were not detected.
The activities of TPSs in the crude extracts of control and MJ-treated cells were observed over 24 h as for the quantification of sesquiterpenes. The activity levels increased, reached a maximum at 12 h, and then gradually decreased in the extracts of MJ-treated cells, while no activity was detected in the control extracts, similar to the profile of sesquiterpene accumulating (Fig. 2). The most abundant of the products was \( \alpha \)-humulene in the first 6 h, while \( \delta \)-guaiene biosynthesis dominated thereafter. The biosynthesis rate for \( \alpha \)-humulene was greater than \( \alpha \)-guaiene during the entire period. Together, these results suggest that the different sesquiterpenes are synthesized and accumulate at different rates after the MJ treatment.

Analysis of Volatile Compounds in the Headspace of the Culture

The most abundant sesquiterpene accumulated in the MJ-treated cells was \( \delta \)-guaiene, whereas the major product of the crude extracts was \( \alpha \)-humulene at 6 h after MJ treatment. To clarify the reason of this difference, the volatile compounds emitted from MJ-treated cells were analyzed by solid-phase microextraction (SPME)-gas chromatography (GC)-mass spectrometry (MS) using an airtight container, and the major product was revealed to be \( \alpha \)-humulene (13%); \( \alpha \)-humulene, 61%; \( \delta \)-guaiene, 26%). These results confirmed that \( \alpha \)-humulene was the major compound produced by MJ-treated cells, at least during the first 6 h after treatment, but was quickly emitted; therefore, the most abundant compound in the cells was \( \delta \)-guaiene.

Construction of cDNA Library and Sequencing

Because our findings suggested that unique TPSs might be selectively activated in the MJ-treated cells, a cDNA library was constructed from the cells to isolate the genes responsible for the sesquiterpene biosynthesis. Two hundred seventy-four clones randomly selected from the cDNA library were sequenced and their function deduced using the BLASTP algorithm. One clone, AcL154, shared 40% identity in amino acid sequence with the germacrene D synthase from *Vitis vinifera* (GenBank accession no. AY561842; Lücker et al., 2004). AcL154 contains an open reading frame of 1641 bp encoding a protein of 547 amino acids harboring motifs highly conserved among TPSs, such as the RPX-W motif (Bohlmann et al., 1998b) at the N terminus and the DDxxD motif (Starks et al., 1997; Whittington et al., 2002) known to be a divalent metal ion substrate-binding site (Fig. 3). However, AcL154 does not have the transit peptide usually found in monoterpene synthases at the N terminus nor sequences specific to diterpene synthases (Bohlmann et al., 1998b), consistent with it encoding for a sesquiterpene synthase.

Isolation and Functional Expression of Sesquiterpene Synthase from Cultured Cells of *Aquilaria*

Although AcL154 harbors start and stop codons at appropriate locations, it has an extra stop codon (bp 334 approximately 336 from the 5’ end). Therefore, a pair of primers was designed according to the sequence of AcL154 and used for PCR with cDNA derived from the MJ-treated cells of *Aquilaria* as a template. Four clones (AcC1–AcC4) of the same length as AcL154 were obtained, and their sequences turned out to be almost the same as the AcL154 sequence, with a difference of 17 to 19 amino acids (Fig. 3).

In an attempt to express and functionally characterize AcL154 as well as AcC1 to AcC4, the stop codon of AcL154 was substituted with that for Trp, which is the conserved residue at this position among AcC1 to

![Figure 2. Time course of sesquiterpene content and sesquiterpene synthase activities in cultured cells after treatment with MJ. Columns are for sesquiterpene content, and line graphs show sesquiterpene synthase activities. Data are the means ± SE of duplicate or triplicate analyses.](image-url)
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Then, AcC1 to AcC4 and AcL154' were expressed in *Escherichia coli* [BL21 (DE3) Codon Plus-RIL] for functional characterization. Heterologously expressed proteins were visualized by SDS-PAGE (data not shown), but the AcC1 protein was detected only in the insoluble fraction, and even attempts to be in the soluble fraction with various species of *E. coli* were unsuccessful. All clones except AcC1 were functionally characterized.

The enzyme assays were performed by using farnesyl pyrophosphate (FPP) as a substrate, and the reaction products were analyzed by GC-MS. All clones except AcC1 yielded the same compounds (α-guaiene, α-humulene, and δ-guaiene), which are those accumulated in the MJ-treated cells, and the major product was δ-guaiene (Fig. 4; Table I). The assays that did not include silica in the workup confirmed that these cyclases did not generate sesquiterpene alcohols. These results suggest that these TPSs are expressed in the MJ-treated cells and contribute to the biosynthesis of particular sesquiterpenes that accumulate.

Kinetic characterization of these clones revealed that AcC2 and AcC4 showed similar kinetic profile, but \( K_m \) values of AcC3 and AcL154 were one-sixth and one-seventh, and the catalytic efficiencies (\( k_{cat}/K_m \)) were one-thirteenth and one-seventieth of those of AcC4, respectively (Table I). However, \( K_m \) values of these clones were within the range of those previously reported for sesquiterpene synthases (0.1–10 \( \mu \)M; Cane, 1999).

Confirmation of Induction of TPS Gene Expression by MJ Treatment in Cultured Cells

A quantitative reverse transcription (RT)-PCR method was employed to measure the transcript levels for the TPS genes in MJ-treated cells (Fig. 5). Similar to the results above, the transcript levels for TPSs were elevated by MJ treatment, reached a maximum at 12 h, and then decreased.

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**Figure 3.** Alignment of deduced amino acid sequences of sesquiterpene synthases from cultured cells of *A. crassna*. Shading indicates levels of sequence conservation (100%, black on white; over 50%, white on black; under 50%, black on gray). Triangles indicate residues mutated in this study. The conserved RPxW and DDxxD motifs are underlined.
Mutagenesis of AcC1

AcC1 expression, unsuccessful in E. coli, was carried out in an in vitro system to investigate whether the clone had TPS activities. The protein of AcC1 was visualized on a membrane by western blotting to confirm successful expression in vitro system (data not shown) and was subjected to an enzymatic reaction to reveal that it did not have any TPS activity with FPP or geranyl pyrophosphate as a substrate (Fig. 6). AcC1, which alone was inactive among the four clones, was mutated differently from active clones at five amino acids, 110Y, 144W, 241V, 296P, and 337H (Fig. 3). These five were replaced with the corresponding amino acids in the active clones so as to investigate their individual contributions to the enzymatic activity. Possible single, double, triple, quadruple, and quintuple mutants of AcC1 were constructed, and Table II shows the quadruple and quintuple mutants among them. Expression of all mutants and enzymatic reactions revealed that only M42 and M50 had activity for sesquiterpene synthesis to almost the same product ratio as AcC2 to AcC4 and almost the same catalytic efficiencies ($k_{cat}/K_m$) as AcC3 (Fig. 6; Table II). These

| Table 1. Products of sesquiterpene synthases expressed in E. coli |
|----------------------|-------------------|-------------------|-----------------|-----------------|-----------------|
| Clone Name | Total Products | Kinetic Parameters |
| Alpha-Guaiene | Beta-Humulene | Delta-Guaiene | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
| AcC2 | 18.1 | 0.7 | 81.2 | 4.99 x 10^-3 | 2.71 | 1.836 |
| AcC3 | 44.6 | 1.7 | 53.7 | 9.72 x 10^-5 | 0.51 | 0.189 |
| AcC4 | 20.9 | 0.9 | 78.2 | 7.34 x 10^-3 | 3.05 | 2.404 |
| AcL154' | 20.8 | 0.7 | 78.5 | 1.58 x 10^-5 | 0.45 | 0.035 |

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Figure 4. GC-MS profiles. A, Total ion chromatogram of the products formed by sesquiterpene synthases (AcC2) with FPP as a substrate. B, Mass spectra of the sesquiterpenes and their authentic standards. GC-MS profiles of control assay are provided in Supplemental Figure S1.
results suggest that the mutations Y110S, V241E, P296S, and H337Y are important to the activity for sesquiterpene synthesis.

Comparison of AcC1 and AcC2 in 3D Homology Models

X-ray crystallography revealed the structure of 5-epi-aristolochene synthase from Nicotiana tabacum (Starks et al., 1997), trichodiene synthase from Fusarium sporotrichoides (Rynkiewicz et al., 2001), pentalenene synthase from Streptomyces UC5319 (Lesburg et al., 1997), and aristolochene synthase from Penicillium roqueforti (Caruthers et al., 2000). These vegetative sesquiterpene synthases share almost the same three-dimensional structure despite the absence of any significant sequence similarity (Greenhagen and Chappell, 2001). The locations of four amino acids necessary for restoration of the enzymatic activities were examined in models of AcC1 and AcC2 created using Deep View/Swiss-PDBViewer and SWISS-MODEL based on the structure of 5-epi-aristolochene synthase from N. tabacum (Protein Data Bank ID code 5EAT; Fig. 7). The 5-epi-aristolochene synthase is made up of α-helices and short connecting loops and turns and is separated into two structural domains, an NH2 terminus domain (helices 1–8) and a COOH terminus domain (helices A–K; Starks et al., 1997). The putative three-dimensional structures of AcC1 and AcC2 showed that all of the four amino acids existed on the α-helices. These models suggested that the hydrogen bonds of 241E (in helix A) and 269Y (in helix C), and 337Y (in helix E), 322D (in loop D1 to D2), and 317R (in helix D1) were formed by the mutations V241E and H337Y, respectively. On the other hand, the CH–π bond, the weak hydrogen bond of 110Y and 106L in the same α-helix, disappeared when the mutation Y110S was present. The distortion of the α-helix caused by the disappearance of the hydrogen bond, or the change in space between α-helices caused by the replacement of a bulky Tyr with a nonbulky Ser would explain the structural changes at the N terminus of AcC1. Furthermore, it has been reported that Pro is rarely found in α-helices because it distorts their structure (Madison, 1977). It was considered that the mutation P296S (in helix D) resolved the distortion of the α-helix and led to a recovery of the enzymatic activity. The DDxxD motif was found in Helix D, whose structure was suggested to play a major role in the enzymatic activity.

DISCUSSION

In this article, we reported the first cloning and functional characterization of δ-guaiene synthases from cell cultures of Aquilaria. The enzymes that catalyze the formation of sesquiterpenes via germacrene A, such as valencene synthase (Sharon-Asa et al., 2003), vetispiradiene synthase (Back and Chappell, 1995), and β-selinene synthase (Iijima et al., 2004), have been cloned, and their reactions are proposed to involve eudesmane cations formed by closure of C2 and C7 of germacrene A. However, guaiane-type sesquiterpenes have a unique adjoining five- and seven-membered ring, which is thought to be formed by closure of C2 and C6 of germacrene A (Fig. 1). The TPSs from Aquilaria cells produced α-guaiene, α-humulene, and δ-guaiene simultaneously, which indicates that the cyclization mechanisms for these sesquiterpenes are closely related to each other. Most of the genes encoding sesquiterpene synthases have been isolated by homology-based PCR approaches based on their similarity to known gene sequences; however, the TPSs cloned in this study were derived from the screening of a cDNA library and shared only 40% identity in amino acid sequence with other sesquiterpene synthases. Moreover, although these TPSs...
had almost the same amino acid sequences, they had various ranges of $K_m$ and $k_{cat}$ values. These results suggest that these TPSs may provide some information for the molecular dissection of TPSs in general.

Of the three species of sesquiterpenes produced by the enzymatic reaction of cloned enzymes, $\alpha$-huminene is known as a feeding deterrent to insects and pathogens (direct defense; Suga et al., 1993) and as a volatile attracting natural enemies of herbivores (indirect defense; Mattiacci et al., 2001; Rodriguez-Soana et al., 2003). However, no reports have referred to the role of guaiane-type sesquiterpenes in plant metabolism. Analysis of volatile compounds in the headspace of the culture and the data in Figure 2 revealed that $\alpha$-huminene was emitted more quickly than $\delta$-guaiene from the cells, which suggests that $\delta$-guaiene has some different biological roles from those of $\alpha$-huminene.

The major product was $\delta$-guaiene in the enzymatic reactions using active enzymes (AcC2, AcC3, and AcC4) expressed in E. coli but $\alpha$-huminene in the assays using crude enzymes extracted from the cells cultured for 6 h after MJ treatment. These results suggested that in the MJ-treated cells, there existed other enzymes that produced $\alpha$-huminene as a main product in addition to the cloned enzymes that produced $\delta$-guaiene as a main product.

Of the five clones isolated in this study, one clone (AcC1) had no TPS activity, and another (AcL154) harbored an extra stop codon, which suggested that the two might be pseudogenes. In fact, the mutant AcL154', which was made by replacing the stop codon in AcL154 with Trp, had little catalytic efficiencies ($k_{cat}/K_m = 0.035$). However, the five clones had very similar amino acid sequences and produced the same products at very similar rates in enzymatic reactions performed using heterologously expressed proteins. Furthermore, phylogenetic analysis showed that the evolutionary distances among the five were quite small compared to those of TPSs from other plants cloned with multiple copies (data not shown). The cells used in this study were cultured in media containing 2,4-dichlorophenoxyacetic acid, which is suggested to maintain plant cells in an undifferentiated state and promote cell division (Dixon, 1985). Mutations are known to occur during DNA replication or as a result of damage caused by radiation and

Table II. Scheme of the mutants of AcC1 and their TPS activities

| Clone Name | Mutation Sites | Total Products | Kinetic Parameters |
|------------|----------------|----------------|-------------------|
|            | $\alpha$-Guaiene | $\alpha$-Humulene | $\delta$-Guaiene | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
| M41        | Y110S          | W144R          | V241E            | P296S      | H337Y | – | – | – | – | – | – |
| M42        | X              | X              | X                | X          | –     | 34.1 | 1.3 | 64.6 | 1.25 x 10^{-3} | 6.85 | 0.182 |
| M43        | X              | X              | X                | X          | –     | –     | – | – | – | – | – |
| M44        | X              | X              | X                | X          | –     | –     | – | – | – | – | – |
| M45        | X              | X              | X                | X          | –     | –     | – | – | – | – | – |
| M50        | X              | X              | X                | X          | –     | 31.1 | 1.5 | 67.4 | 5.86 x 10^{-4} | 4.29 | 0.137 |

$^a$The amount of the products is under the detection limit.

Figure 7. Three-dimensional homology model of AcC1 based on the structure of 5-epi-aristolochene synthase from N. tabacum. The N-terminal domain is shown in blue, and the C-terminal domain is shown in orange. The insets show the mutated residues. [See online article for color version of this figure.]
mutagenic chemicals (Bertram, 2000); thus, the differences in amino acid sequence among the five clones are likely to be derived from the accumulation of mutations that occurred during DNA replication. This is one possibility for the explanation of existing similar multiple genes, and this can be evaluated by looking at the corresponding genes isolated from intact plants.

The heterologously expressed AcC1 in *E. coli* could not be found in the soluble fraction, and the clone was expressed by the in vitro system but was lacking in TPS activity. These results show that there are differences in protein structures between AcC1 and the other clones with TPS activity. This idea is supported by the finding that the mutants M42 and M50, both of which were active mutants of AcC1, were found their proteins in the soluble fraction in the *E. coli* expression system (data not shown). The DDxxD motif located at the C terminus has already been reported to play a major role in the enzymatic activity; however, mutagenesis and homology modeling of AcC1 and AcC2 showed that the space between and structures within α-helices was also important to the enzymatic activities. In recent years, evidence has emerged that this domain acts as a scaffold facilitating proper folding of the catalytically active C-terminal domain (Köllner et al., 2004), which may be supported by the results of this study. Our findings suggested that the structure and orientation between and within α-helices at the N terminus, especially around helix 4 where 110S is located, are important in facilitating proper folding of the protein to form a catalytically active structure.

**MATERIALS AND METHODS**

**Cell Cultures and MJ Treatment**

The methods used to prepare cultures of cells from *Aquilaria crassa* leaves were as described previously (Okudera and Ito, 2009). Cell suspension cultures were incubated with reciprocal shaking at 25°C in Murashige and Skoog medium containing 3% (w/v) Suc, 10 M M 6-benzyladenine and subcultured in fresh medium every 2 weeks. Cells cultured for 5 d after the inoculation were used. MJ (Sigma-Aldrich) was dissolved in DMSO to a concentration of 300 μM, and added to the culture at a final concentration of 0.1 mM.

**Extraction of Terpenes from MJ-Treated Cells**

The cell cultures were harvested by filtration (control), 6, 12, and 24 h after MJ treatment. The cells and filtrate were extracted with 30 and 20 mL of pentane containing 48 ng mL⁻¹ of limonene as an internal standard, respectively. The cells and medium were extracted over 14 h with constant shaking at 25°C. The samples were concentrated to approximately 10 mL under nitrogen before analysis by GC-flame ionization detection (FID). For the assays without silica preparation were also performed, and sesquiterpene alcohols were not detected. The dry weights of extracted cells were determined after 48 h at 65°C. The assays with silica preparation were also performed, and sesquiterpene alcohols were not detected.

**Isolation of RNA and Construction of a cDNA Library**

Total RNA was extracted from 1 g of the cultured cells incubated for 6 or 10 h with MJ. mRNA was purified using a TaKaRa mRNA purification kit from total RNA extracted with an RNeasy plant mini kit (Qiagen). Two micrograms of the mRNA was employed to construct a cDNA library using the SuperScript Plasmid System (Invitrogen) following the manufacturer’s protocol.

**Sequence Analysis**

Two hundred seventy-eight clones of the cDNA library were randomly sequenced; plasmids extracted using the Mini plus Plasmid DNA Extraction System (Viogene) were sequenced at Bionatrix Co. Ltd. The deduced amino acid sequences of the clones were compared with GenBank data using BLASTP search algorithms of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

**Extraction of Enzymes from MJ-Treated Cells and Enzyme Assays**

Using a mortar and pestle, 2.5 g of the MJ-treated cells was ground to a fine powder in liquid nitrogen and combined with 5 mL of extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 100 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone, and 1% (w/v) polyvinylpyrrolidone. The extract was vortexed and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was filtered through filter paper, divided into 1-mL aliquots, and kept at −80°C. Extracts were thawed only once before the enzyme assay. The total protein concentration of each extract was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as the standard.

Enzymatic activity was assessed with 1 mL of extract with the addition of 87 μM FPP (Sigma-Aldrich). All assays were done in duplicate, with 1 mL of pentane overlaid to collect volatiles and incubation at 30°C for 4 h. After the addition of 1.6 μL mL⁻¹ of limonene as an internal standard, the pentane overlay was removed and filtered through a Pasteur pipette filled with 0.2 g of silica gel overlaid with MgSO₄. Each assay mixture was extracted with an additional 1 mL of pentane twice, and these fractions were also passed through the same column and pooled with the initial eluent. Subsequently, the column was washed with 1 mL of pentane twice, and the total eluent was concentrated to approximately 10 μL under nitrogen and then analyzed immediately by GC-FID and SPME-GC-MS. The assays without silica preparation were also performed, and sesquiterpene alcohols were not detected.

**Collection of Volatiles**

After the administration of MJ, the cells were transferred to flasks with a silicon cap and incubated for 6 h with reciprocal shaking. The SPME fiber was inserted into each flask and left for 1 min, and immediately GC-MS was conducted.

**Analysis of Sesquiterpenes**

For quantification, sesquiterpenes were analyzed with a G-5000 GC system (Hitachi) equipped with a FID fitted with a TC-WAX column (0.25 mm × 0.25 μm × 60 m; GL Science). The flow rate was 40 mL H₂ min⁻¹ and 0.8 mL He min⁻¹, and the FID was operated at 250°C. Split injections (1 μL) were made at a ratio of 5:1 with an injector temperature of 250°C. The GC was programmed with an initial oven temperature of 50°C, increasing at a rate of 3°C min⁻¹ until 110°C, and then a ramp of 5°C min⁻¹ until 240°C (10-min hold). The identification of terpenes was based on retention times with authentic standards, and concentrations of sesquiterpenes were calculated by comparing the integrated peak area with that of the internal standard limonene.

The program had an initial oven temperature of 80°C, a ramp of 5°C min⁻¹ until 220°C (10-min hold), and then a ramp of 10°C min⁻¹ until 240°C (3-min hold). The identification of terpenes was based on a comparison of retention times and mass spectra with authentic standards.

**Characterization of Sesquiterpene Synthases from Agarwood**

The methods used to prepare cultures of cells from *Aquilaria crassa* leaves were as described previously (Okudera and Ito, 2009). Cell suspension cultures were incubated with reciprocal shaking at 25°C in Murashige and Skoog medium containing 3% (w/v) Suc, 2,4-dichlorophenoxyacetic acid, and 10⁻⁸ M 6-benzyladenine and subcultured in fresh medium every 2 weeks. Cells cultured for 5 d after the inoculation were used. MJ (Sigma-Aldrich) was dissolved in DMSO to a concentration of 300 μM, and added to the culture at a final concentration of 0.1 mM.
Cloning of the Putative Sesquiterpene Synthases

The sequence of the clone named AcL154 was very similar to that of the germacrene D synthase isolated from *Vitis vinifera* (GenBank accession no. AY561842). A pair of forward (5'-ATGTCCTCGGCAAACACTAGGTCT-GCCTCC-3') and reverse (5'-GATTTCAATAGGCTACGCAAAAG- CAGC-3') primers were used for PCR to generate the fragment for expression. Each reaction mixture contained 2 µL of 10× reaction buffer (TaKaRa), 0.2 µM of each deoxynucleoslic acid triphosphate, 0.5 units of ExTaq polymerase (TaKaRa), 0.2 µM of the forward and reverse primers, 0.2 µL of DMSO, and 0.5 µL of template cDNA prepared from total RNA isolated from MJ-treated cells using ReverTra Ace (Toyobo) in a final volume of 20 µL. The thermal cycling conditions for PCR performed with a Program Temp Control System (Astec) 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, and 72°C for 1 min 40 s. Finally, the reaction mixture was heated at 72°C for 3 min. Following gel purification, the PCR products were ligated into the expression vector pCR T7/CT-TOPO (Invitrogen) and introduced into competent *Escherichia coli* TOP10 Fβ cells (Invitrogen).

Heterologous Expression of Sesquiterpene Synthases in *E. coli* and Enzyme Assays

Full-length cDNAs cloned into expression vectors were transformed into BL21 (DE3) Codon plus-RIL (Stratagene). Cultures initiated from single colonies were incubated in TB medium and cultured at 37°C until the optical density at 600 nm became 0.6 to 0.7 before the administration of isopropylthio-β-D-galactoside at 0.5 mM, and then the cultures were incubated for 22 h at 16°C with shaking at 220 rpm. The cells were separated from the medium by centrifugation at 10,000g for 10 min at 4°C. The cells were resuspended in sonication buffer (100 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 mM EDTA, and 5 mM DTT) and sonicated five times for 30 s. Clear lysate of the sonicated sample was collected by centrifugation at 10,000g for 10 min at 4°C. The lysate was loaded on a Ni-NTA Spin column (Qiagen) to isolate the His-tagged recombinant protein. Protein concentrations were determined by the Bradford assay (Bio-Rad) using bovine serum albumin as the standard. Bacterial pellets and lysates, and purified enzyme samples were examined on SDS-PAGE gels to visualize the proteins synthesized in vitro. The transcend biotin-Lysyl-tRNA (Promega) was added to the translation reaction, and the biotinylated Lys residues were incorporated into nascent proteins during translation. After SDS-PAGE and electroblotting to a nitrocellulose membrane, the biotinylated proteins were visualized by binding streptavidin alkaline phosphatase, followed by colorimetric detection (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate).

Western Blotting

The transcend Colorimetric Translation Detection System was used to visualize the proteins synthesized in vitro. The transcend biotin-L-tyrosine (Promega) was added to the translation reaction, and the biotinylated Lys residues were incorporated into nascent proteins during translation. After SDS-PAGE and electroblotting to a nitrocellulose membrane, the biotinylated proteins were visualized by binding streptavidin alkaline phosphatase, followed by colorimetric detection (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate)

Molecular Modeling

Deep View/3D-PDBView (http://spdbv.vital-it.ch/) and SWISS-MODEL were used to develop three-dimensional models of AcC1 and AcC2 based on the structure of the 5-epi-aristolochene synthase from *Nicotiana tabacum* containing the substrate analog farnesyl hydroxyporphionate (Protein Data Bank ID code 5EAT).

Supplemental Data

The following materials are available in the online version of this article.

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