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# A cDNA clone for β-caryophyllene synthase from *Artemisia annua*☆

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**Abstract**

An homology-based cloning strategy yielded a full-length cDNA from *Artemisia annua* that encoded a protein of 60.3 kDa which resembled a sesquiterpene synthase in sequence. Heterologous expression of the gene in *Escherichia coli* provided a soluble recombinant enzyme capable of catalyzing the divalent metal ion-dependent conversion of farnesyl diphosphate to β-caryophyllene, a sesquiterpene olefin found in the essential oil of *A. annua*. In reaction parameters and kinetic properties, β-caryophyllene synthase resembles other sesquiterpene synthases of angiosperms. The β-caryophyllene synthase gene is expressed in most plant tissues during early development, and is induced in mature tissue in response to fungal elicitor thus suggesting a role for β-caryophyllene in plant defense.



*Keywords:* *Artemisia annua*; Asteraceae; Sweet wormwood; Homology-based cDNA cloning; Recombinant protein expression; Sesquiterpene synthase; β-Caryophyllene synthase; Sesquiterpene; β-Caryophyllene; Gene expression; Elicitation

## 1. Introduction

Terpenoid biosynthesis in *Artemisia annua* L. (Asteraceae) has received considerable attention because this plant (sweet wormwood; qinghao in traditional Chinese medicine) is the source of the endoperoxide sesquiterpene lactone antimalarial drug artemisinin (Klayman, 1985). Several probable steps of the pathway have been described (Bouwmeester et al., 1999; Dhingra and Narasu, 2001; Wallaart et al., 1999), and a number of groups have reported the molecular cloning of the sesquiterpene cyclase amorpha-4,11-diene synthase responsible for catalyzing the committed step in the biosynthesis of artemisinin (Chang et al., 2000; Mercke et al., 2000; Wallaart et al., 2001). Searches by us and others have revealed a number of cDNAs from *A. annua* that encode other terpenoid synthases (Jia et al., 1999; Van Geldre et al., 2000), including that for *epi*-cedrol synthase (Hua and Matsuda, 1999; Mercke et al., 1999) which catalyzes a very complex sesquiterpene cyclization from the precursor farnesyl diphosphate. In this paper we describe another sesquiterpene cyclase cDNA from *A. annua* (AF472361) that encodes a β-caryophyllene synthase.

β-Caryophyllene is a common sesquiterpene that is quite widely distributed in plants (Knudsen et al., 1993; Kubo et al., 1996). It possesses anti-inflammatory (Martin et al., 1993; Tambe et al., 1996) and anticarcinogenic (Kubo et al., 1996; Zheng et al., 1992) activities, and this macrocyclic olefin and its derivatives also could play a role in plant defense (Ulubelen et al., 1994; Wadhams et al., 1999). Reported here are the expression patterns of this caryophyllene synthase gene during the development of *A. annua* and in response to wounding and elicitation.

## 2. Results and discussion

### 2.1. Isolation of a β-caryophyllene synthase cDNA

To isolate cDNA clones encoding terpene synthases of *A. annua*, a comparison of angiosperm terpene

☆ The nucleotide sequence reported in this article has been deposited with the GenBank/EMBL Database under accession number AF472361

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synthase sequences was first conducted. This search revealed a consensus peptide sequence, GVYXEP, found only in angiosperm sesquiterpene synthases. Using a primer designed to this sequence and an anchored-PCR strategy with *A. annua* library cDNA as target yielded a 900 bp DNA fragment that showed significant homology to plant sesquiterpene synthases. Subsequent screening of the same cDNA library with this probe led to the isolation of a clone, designated QHS1, which contained an insert of 1902 bp with an apparent open reading frame of 1644 bp. A stop codon immediately upstream of the first ATG codon confirmed the latter as the translation initiation site, from which a molecular mass of 60.3 kD was calculated for the translated protein; this size is consistent with that of other enzymes of this general class (Cane, 1999).

Heterologous expression of this sequence from the pET28 vector in *Escherichia coli* afforded a 60 kD protein in the resulting soluble enzyme fraction, as determined by SDS-PAGE, that was absent in control preparations derived from the empty vector similarly expressed. Evaluation of the soluble enzyme preparation by the standard sesquiterpene synthase assay using [1-$^3$H]farnesyl diphosphate as substrate (Crock et al., 1997; Mercke et al., 1999) revealed the production of both sesquiterpene olefins and oxygenated products. Ten-fold scale-up of the recombinant enzyme preparation allowed more than sufficient conversion of farnesyl diphosphate to the biosynthetic products for capillary GC–MS analysis (Crock et al., 1997; Steele et al., 1998) which revealed the presence of essentially a single product (97% of the olefin fraction) with a spectral library match to that of β-caryophyllene. Subsequent analysis confirmed the biosynthetic olefin to possess a GC retention time and mass spectrum identical to those of an authentic *trans*-β-caryophyllene standard (Fig. 1); the presence of about 2% α-humulene was also confirmed. Similar examination of the oxygenated sesquiterpenoids revealed very low levels of farnesol and nerolidol, resulting from the action of endogenous phosphatases and/or nonenzymatic solvolysis during the course of the assay, and a trace of humulene oxide, likely derived by oxidation of α-humulene by the bacterial host. Only farnesol and nerolidol were produced from farnesyl diphosphate in control assays using extracts from bacteria containing empty vector. Steam distillation of *A. annua* leaves (6-week-old plants) by standard protocol (Gershenzon et al., 2000) yielded an essential oil in which the presence of β-caryophyllene (30%) and α-humulene (2%) was confirmed by similar analytical procedures; this observation is consistent with a previous analysis (Tellez et al., 1999). Thus, a cDNA encoding the β-caryophyllene synthase of *A. annua* had been isolated. The cyclization mechanism of β-caryophyllene synthase (Fig. 2) from *Salvia officinalis* has been described previously (Dehal and Croteau, 1988).

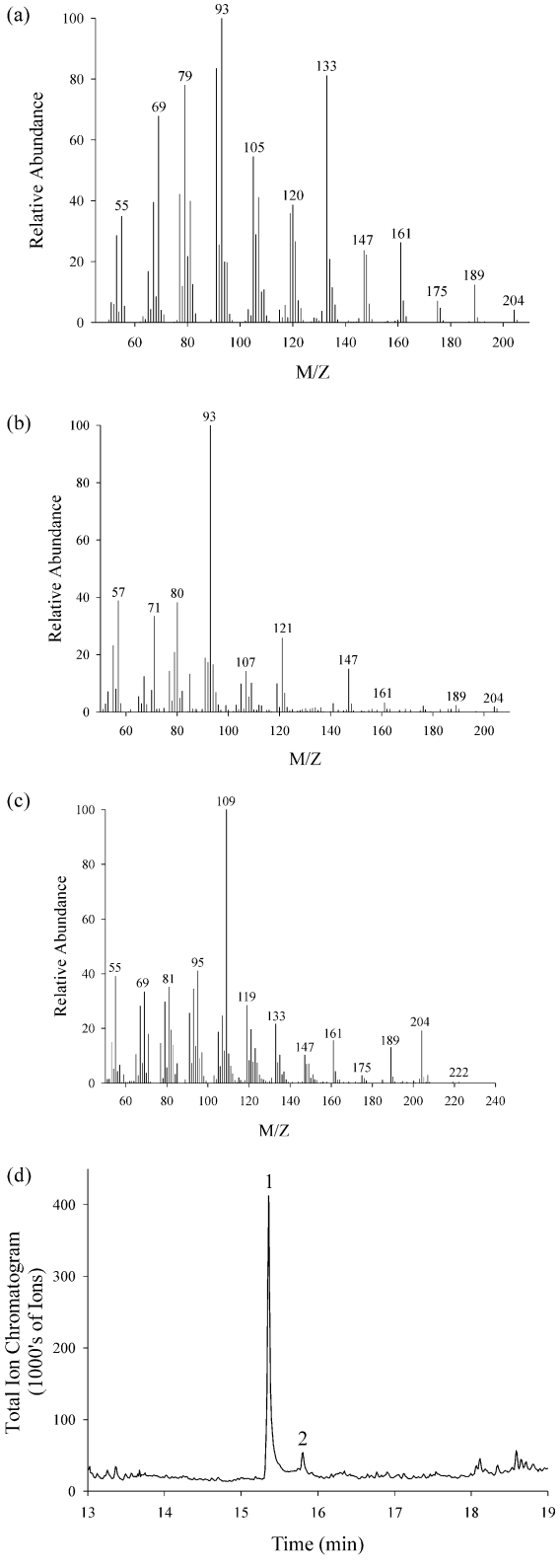


Fig. 1. GC–MS analysis of the products generated from farnesyl diphosphate by the recombinant β-caryophyllene synthase. The spectra illustrated correspond to β-caryophyllene (a), α-humulene (b) and humulene oxide (c); the latter is almost certainly formed by secondary oxidation of α-humulene. The total ion chromatogram (d) is provided to illustrate the relative amounts of β-caryophyllene (peak 1, $R_t = 15.38$ min) and α-humulene (peak 2, $R_t$15.81 min).

The deduced sequence of β-caryophyllene synthase is nearly identical (98.5%) to that of a terpene synthase sequence (*cASC34*; accession no. AJ271793) of undefined function previously reported by Van Geldre et al. (2000), and about 60% identical to the *epi*-cedrol synthase (Hua and Matsuda, 1999; Mercke et al., 1999) and amorpha-4,11-diene synthase (Chang et al., 2000; Mercke et al., 2000; Wallaart et al., 2001) of *A. annua*. The sequence of β-caryophyllene synthase clearly resembles (40–60% identity) those of other sesquiterpene synthases of angiosperm origin, with which it shares a common reaction mechanism (Cane, 1999), and is more distantly related to extant monoterpene synthases and diterpene synthases (Bohlmann et al., 1998). β-Caryophyllene synthase shares less than 30% deduced sequence identity with the monoterpene synthase (3*R*)-linalool synthase of *A. annua* (Jia et al., 1999).

The β-caryophyllene synthase bears the GVYXEP element common to angiosperm sesquiterpene synthases, and the DDXXD divalent metal ion-substrate binding motif that is highly conserved in terpene synthases (Davis and Croteau, 2000). The sequence lacks any N-terminal organelle targeting information, consistent with the assumed cytosolic location of sesquiterpene biosynthesis, and is devoid of the diterpene synthase insertion element; β-caryophyllene synthase (accession no. AF472361) falls within the *Tpsa* subfamily of terpenoid synthases (Bohlmann et al., 1998; Trapp and Croteau, 2001).

### 2.2. Properties of the recombinant enzyme

The recombinant β-caryophyllene synthase was partially purified by anion-exchange chromatography and gel permeation chromatography to remove competing phosphatases (~40% pure by SDS–PAGE and densitometry). Gel permeation chromatography on a calibrated Sephadex S200 column indicated a molecular weight of about 60,000 which, along with the results of SDS–PAGE, confirmed the enzyme to be monomeric.

This synthase exhibited a fairly broad pH optimum, with maximum near pH 7.7, and required a divalent metal ion for catalysis. The preferred metal ion was $Mg^{2+}$ (saturation at 0.5 mM); $Mn^{2+}$ was only half as efficient at a saturating concentration of 0.1 mM. The $K_m$ value with farnesyl diphosphate as substrate was about 1.8 μM, with estimated $k_{cat}$ of 0.04 $s^{-1}$. β-Caryophyllene synthase was not active with geranylgeranyl diphosphate ($C_{20}$) as substrate, and geranyl diphosphate ($C_{10}$) was an inefficient substrate ($K_m > 25$ μM; $k_{cat} \sim 0.004$ $s^{-1}$) which afforded principally limonene (42%), along with terpinolene (30%), β-pinene (16%), γ-terpinene (9%), α-terpinene (3%), and minor amounts of linalool and geraniol as products. Surprisingly, β-caryophyllene synthase readily accepted (+)-chrysanthemyl diphosphate as substrate (with kinetics similar to that of farnesyl diphosphate) and afforded two irregular monoterpenes, yomogi alcohol (63%) and artemisia alcohol (37%), along with solvolysis products. Most of these monoterpenes have been previously reported in the essential oil of *A. annua* (Tellez et al., 1999); however, because of the compartmentalization of monoterpene and sesquiterpene biosynthesis (Bohlmann et al., 1998), it seems unlikely that β-caryophyllene synthase is the source of these monoterpenoid compounds *in planta*. In principal reaction parameters and general properties, β-caryophyllene synthase of *A. annua* resembles β-caryophyllene synthase of *Salvia officinalis* (Dehal and Croteau, 1988), and is typical of the general class of sesquiterpene synthases from angiosperms (Cane, 1999).

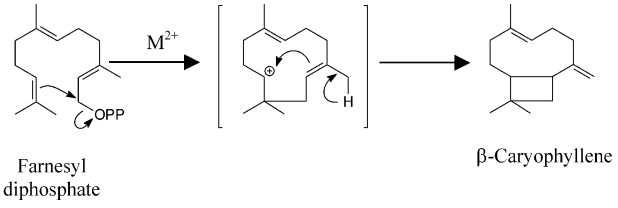


Fig. 2. Cyclization of farnesyl diphosphate to β-caryophyllene by metal ion-dependent ionization with ring closure to the macrocyclic humulyl carbocation (in brackets), followed by formation of the cyclobutane ring and deprotonation. α-Humulene is formed by direct deprotonation of the carbocationic intermediate.

### 2.3. Expression and induction patterns

To determine the specificity of β-caryophyllene synthase expression, total RNA was isolated from several tissues of 6-week-old seedlings, as well as from inflorescences of mature plants, and was analyzed by RT-PCR. Synthase transcripts were observed in most tissues, except roots (Fig. 3). In leaves, caryophyllene transcripts were readily detectable, but expression levels were seemingly much lower in petioles. Transcripts were observed at a particularly high level in stem cortext and epidermis, and were also detected in the stem stele (Fig. 3). By contrast, message for the monoterpene synthase, linalool synthase, could not be detected in the stele, consistent with prior observation (Jia et al., 1999). Compared with the abundance of β-caryophyllene synthase transcripts in inflorescences, monoterpene synthase transcript abundance was very low (data not shown).

To assess temporal expression patterns, 4-, 6- and 8-week-old seedlings were investigated by similar means. These results showed that the level of β-caryophyllene synthase transcripts in the respective tissues decreased gradually with time (Fig. 4). In 8-week-old seedlings, these transcripts were present at only low levels in stem and were barely detected in leaves, indicating substantial diminution of expression with development. In contrast to the present results, Van Geldre et al. (2000)

observed that *cASC34* (β-caryophyllene synthase) transcripts were readily detectable in leaves late in development. This minor difference in developmental expression pattern may result from chemotypic variation in *A. annua* (Ahmad and Misra, 1994).

Wounding of basal leaves of 8 week-old plants, in which the level of transcription of caryophyllene synthase was low, resulted in observable gene activation. During the 3-day interval following abrasive wounding, caryophyllene transcripts accumulated to a higher level on the first day after wounding, then decreased sharply on the second and third day, yet to a level still somewhat higher than before wounding (Fig. 5). By contrast to caryophyllene synthase, for which induction was rapid but transient, the transcriptional induction of the monoterpene synthase linalool synthase was substantial and of longer duration (Fig. 5).

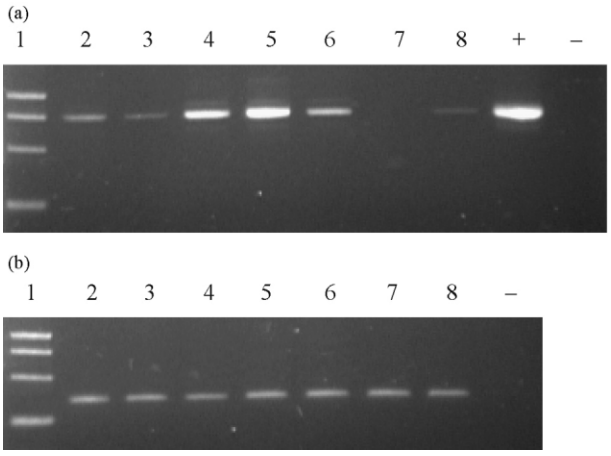


Fig. 3. RT-PCR analysis of β-caryophyllene synthase transcripts in tissues of *A. annua*. (a) amplification with β-caryophyllene synthase-specific primers (750 bp); (b) amplification with ubiquitin-specific primers (300 bp) as internal standard. For both panels, the lanes are: 1000, 750, 500 and 250 bp markers (1); RNA from 6-week-old seedling basal leaves (2), petioles (3), whole stem (4), stem epidermis and cortex (5), stem stele (6), root (7), and inflorescences of mature plants (8); the plus sign denotes the positive control with β-caryophyllene synthase plasmid DNA; the minus sign denotes the negative control without template.

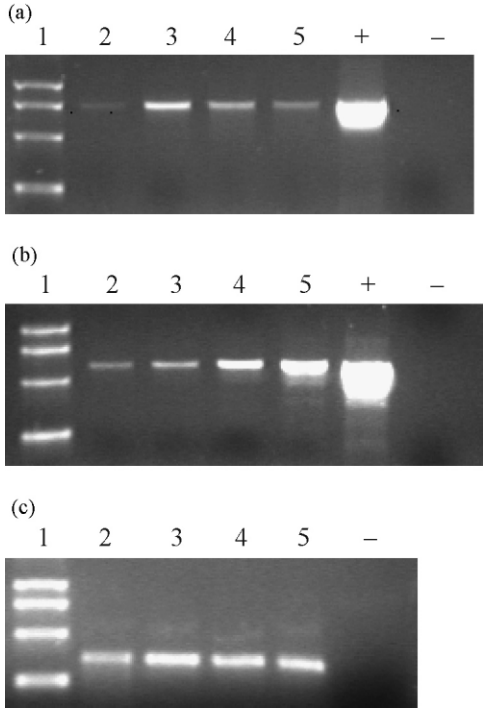


Fig. 5. RT-PCR analysis of β-caryophyllene synthase transcripts and linalool synthase transcripts in wounded basal leaves of 8-week-old *A. annua* seedlings. (a) amplification with β-caryophyllene synthase-specific primers (750 bp); (b) amplification with linalool synthase-specific primers (560 bp); (c) amplification with ubiquitin-specific primers (300 bp). For all panels, the lanes are: 1000, 750, 500 and 250 bp markers (1); RNA from leaves of non-wounded controls (2), and from wounded leaves after 1 day (3), 2 days (4) and 3 days (5) post-treatment; the plus sign denotes the positive control with β-caryophyllene synthase (a) or linalool synthase (b) plasmid DNA; the minus sign denotes the negative control without template.

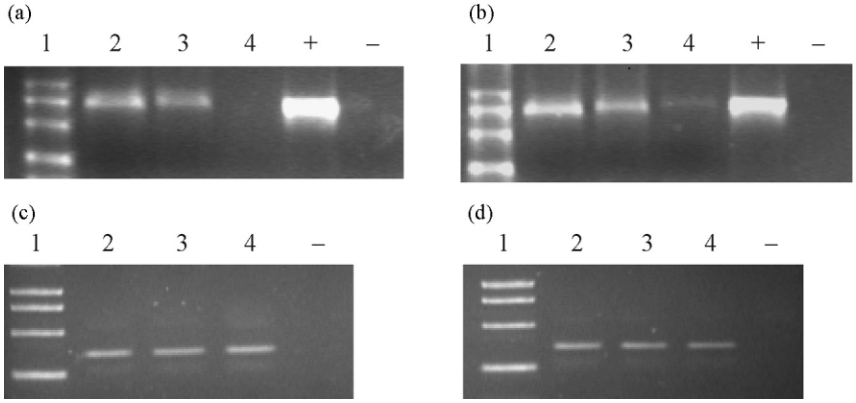


Fig. 4. RT-PCR analysis of β-caryophyllene synthase transcripts in 4-, 6- and 8-week-old *A. annua* seedlings. (a and b) amplification with β-caryophyllene synthase-specific primers (750 bp); (c and d) amplification with ubiquitin-specific primers (300 bp) as internal standard. Total RNA was isolated from basal leaves (a and c) and whole stem (b and d). For all panels, the lanes are: 1000, 750, 500 and 250 bp markers (1); RNA from 4-week-old (2), 6-week-old (3), and 8-week-old (4) seedlings; the plus sign denotes the positive control with β-caryophyllene synthase plasmid DNA; the minus sign denotes the negative control without template.

Extracts of the phytopathogenic fungus *Verticillium dahliae* often serve as non-specific elicitors of oxidative burst and phytoalexin biosynthesis in plant cells (Davis et al., 1992). When intact basal leaves of 8-week-old *A. annua* seedlings were treated with such an elicitor preparation, transcription levels of caryophyllene synthase dramatically increased, with a maximum on the second day post-elicitation; at the same time, the transcription levels of linalool synthase apparently decreased (Fig. 6). Thus, 'fungal' elicitation seemingly resulted in simultaneous induction of the sesquiterpene synthase and suppression of transcription of the monoterpene synthase. These preliminary results, indicating differential expression of the monoterpene synthase and sesquiterpene synthase genes after elicitation, in addition to their differential induction patterns by wounding, suggest that *A. annua* may rely on terpenoid-based defenses, and that independent signaling pathways may operate in regulating the induced biosynthesis of monoterpenes in response to insect attack (wounding) and the induced biosynthesis of sesquiterpenes in response to fungal infection (elicitation).

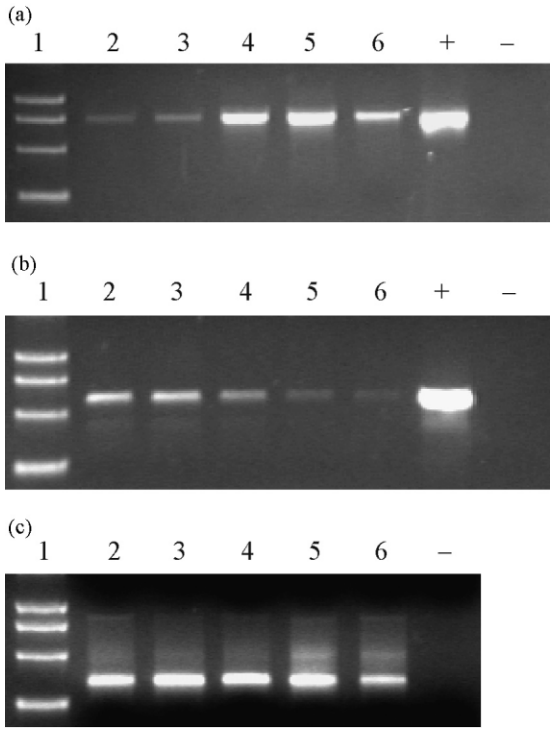


Fig. 6. RT-PCR analysis of β-caryophyllene synthase transcripts and linalool synthase transcripts in elicitor-treated basal leaves of 8 week-old *A. annua* seedlings. (a) amplification with *β*-caryophyllene synthase-specific primers (750 bp); (b) amplification with linalool synthase-specific primers (560 bp); (c) amplification with ubiquitin-specific primers (300 bp). For all panels, the lanes are: 1000, 750, 500 and 250 bp markers (1); RNA from leaves of non-elicited controls (2), sterile water-treated controls (3), and from elicitor-treated leaves after 1 day (4), 2 days (5) and 3 days (6) post-treatment; the plus sign denotes the positive control with β-caryophyllene synthase (a) or linalool synthase (b) plasmid DNA; the minus sign denotes the negative control without template.

## 3. Experimental

### 3.1. cDNA isolation

*A. annua* was grown as described previously (Jia et al., 1999) and plants from 4 to 8 weeks old were used for RT-PCR. A PCR-based strategy was employed to isolate sesquiterpene synthase cDNA fragments using a primer designed by comparing extant terpene synthases, from which a peptide sequence GVPFEP was found to occur only in angiosperm sesquiterpene synthases. Thus, degenerate reverse primer tGVY [5′-GG(TC)TC(AG)TA(AG)AA(AGCT)AC(AGCT)CC-3] and vector-specific primer T3 (Stratagene) were used to amplify sequences from the previously described *A. annua* λ-ZAP cDNA library (Jia et al., 1999). The products were reamplified with previously described reverse primer 9315 (Chen et al., 1995) and another vector-specific primer TP1 [5′-CTGCAGGTTCGACACTAGTGG-3′]. The resulting amplicon of about 900 bp was subcloned and sequenced, and shown to encode a DNA fragment bearing 45.5% identity to that of δ-cadinene synthase from *Gossypium arboreum* (Chen et al., 1995). Specific forward primer P320 [5′-ATGGTGATGACTGGAAAGGC-3′] and reverse primer M760 [5′-TGGAAACTTCGCTAAGCTCC-3′] were employed to generate a 446 bp fragment used as a labeled probe to screen the cDNA library as previously outlined (Jia et al., 1999). Following five rounds of purification, the clone designated QHS1 (AF472361) was obtained and fully sequenced.

### 3.2. cDNA expression

Full length QHS1 was amplified by PCR using *Pfu* DNA polymerase (Promega) with vector-specific reverse primer T7 (Stratagene) and forward primer QHSB1 [5′-CGGATCCCATGTCTGTTAAAG-3′] designed to introduce a 5′-*Bam*HI site. Following digestion with *Bam*HI and *Xho*I, the sequence verified DNA was ligated into similarly digested pET28b (yielding pETQHS1) and transformed into *E. coli* BL21(DE3). Cells harboring pETQHS1 or the empty vector as control were cultured in LB medium as described previously (Jia et al., 1999; Mercke et al., 1999) except that kanamycin was used for selection. These bacterial cultures were readily scaled from 50 to 500 ml.

### 3.3. Enzyme isolation and characterization

Enzyme isolation procedures and methods for partial purification by anion-exchange chromatography have been described (Mercke et al., 1999). Each preparation was monitored by assay and by SDS–PAGE. The standard assay for sesquiterpene synthases was employed (Mercke et al., 1999) in which the conversion of

[1-$^3$H]farnesyl diphosphate (10 μM, 125 Ci/mol), at pH 7.0 in the presence of 10 mM $MgCl_2$ and 20 μM $MnCl_2$, to sesquiterpene olefins and alcohols is determined by pentane extraction and silica gel separation, with scintillation counting of the product classes. Scale-up of the preparation allowed generation of sufficient product for identification by established GC–MS procedures (Crock et al., 1997; Steele et al., 1998). Established protocols for determination of size, divalent metal ion requirement, pH optimum, kinetic constants, and substrate selectivity have also been described elsewhere (Crock et al., 1997; Mercke et al., 1999). Authentic β-caryophyllene (97%) was from Fluka.

### 3.4. RNA analysis

Total RNA was isolated from the various tissues by the phenol-SDS method followed by purification with the RNeasy Plant Total RNA kit (Qiagen). For RT-PCR analysis of QHS1 transcripts, 1 μg of total isolated RNA was added to 20 μl of reverse transcriptase reaction mixture (Promega) for first strand cDNA synthesis. PCR was performed on 1 μl aliquots of the reverse transcription product with forward primer P800 [5′-TCCCAAACAATCTACCTTATG-3′] and reverse primer M1500 [5′-TCGGAATATCTTTACGCACAA-3′] using 28 cycles of fragment amplification for transcript analysis. For comparative purposes, transcripts for the *A. annua* monoterpene synthase linalool synthase (QH5) (Jia et al., 1999) were analyzed by similar means using forward primer QH5P2 [5′-ATACATAAA-GAAAGCATGGGC-3] and reverse primer QH5PR1 [5′-ATGTGAACCCGTGGAACTATC-3′]. Ubiquitin transcripts provided the internal control for PCR analysis using forward primer UB1 [5′-ATGCA(AG)-AT(TCA)TT(TC)GTNAA-3′] and reverse primer UB2 [5′-TGNCC(GA)CA(CT)TT(TC)TT(TC)TT-3′] based upon the *Arabidopsis* ubiquitin sequence (Callis et al., 1990).

Basal leaves, petioles, stems (epidermis, cortex and stele) and roots from 6-week-old seedlings, and the inflorescences from mature plants, were analyzed. Plants that were 4, 6 and 8 weeks old were analyzed in parallel for comparison of the temporal expression patterns in basal leaves and stem. Eight-week-old seedlings were wounded by a described protocol (Lewinsohn et al., 1992). The elicitor preparation was obtained from the phytopathogenic fungus *V. dahliae* as previously described (Heinstein, 1985; Liu et al., 1999). This preparation, at a final con1centration of 1μg sucrose equivalent/ml in sterilized dd water, was used to paint unwounded leaves with a pen brush. Total RNA was isolated from wounded and elicitor-treated (unwounded) leaves of individual plants over a 3-day time course, and RT–PCR was carried out as before. All experiments (from treatment and extraction, to RT-PCR) were repeated three times (three individual plants) with similar results.

## Acknowledgements

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