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ORIGINAL ARTICLE

Molecular cloning and functional characterization of α-humulene synthase, a possible key enzyme of zerumbone biosynthesis in shampoo ginger (*Zingiber zerumbet* Smith)

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Abstract Shampoo ginger (*Zingiber zerumbet* Smith) has a high content and large variety of terpenoids in the essential oil of its rhizome. Here, we report on the isolation of a cDNA clone (*ZSS1*) encoding α-humulene synthase, a possible key enzyme of zerumbone biosynthesis. This clone contains an open reading frame of 1,644 bp and is predicted to encode a protein of 548 amino acids with a calculated molecular mass of 64.5 kDa. The deduced amino acid sequence shows 34–63% identity with known sesquiterpene synthases of other angiosperm species. Based on exon-intron organization, *ZSS1* is classified as the terpene synthase-III (TPS-III) subfamily. When expressed in *Escherichia coli*, the recombinant enzyme catalyzed the formation of a major product, α-humulene (95%) and a minor by-product, β-caryophyllene (5%). Introduction of *ZSS1* and the foreign mevalonate pathway involved in FPP synthesis into *E. coli* results in *in vivo* production of α-humulene. Transcript of *ZSS1* was detected almost exclusively in rhizomes and was up-regulated in both leaves and rhizomes after treatment with methyl jasmonate (MeJA), suggesting its ecological function in shampoo ginger.

Keywords β-Caryophyllene · α-Humulene · Sesquiterpene synthase · Zerumbone biosynthesis (in-vivo production) · *Zingiber*

Abbreviations

DMAPP	Dimethylallyl diphosphate
DXP	Deoxyxylulose 5-phosphate
FPP	Farnesyl diphosphate
GC-MS	Gas chromatography-mass spectrometry
HMGS	Hydroxymethylglutaryl CoA synthase
HMGR	Hydroxymethylglutaryl CoA reductase
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
MK	Mevalonate kinase
MeJA	Methyl jasmonate
MPD	Mevalonate diphosphate decarboxylase
ORF	Open reading frame
PMK	Phosphomevalonate kinase
RACE	Rapid Amplification of cDNA Ends
RT-PCR	Reverse transcription PCR
TPS	Terpene synthase

Introduction

Zingiber zerumbet Smith, a perennial root herb native to Southeast Asia, is most widely known as the "shampoo ginger" for the milky substance in the cone-shaped bracts. The creamy substance has long been used as a natural shampoo and as an ingredient in several commercial shampoos. In addition, the fresh rhizome of shampoo ginger is widely used as a folk medicine for sprains, indigestion, toothaches

The nucleotide sequences reported in this article has been deposited in DDBJ/GenBank/EMBL under accession number(s) AB 247331 and AB263736.

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and other ailments. Because of its versatile properties, shampoo ginger is cultivated in many tropical and subtropical areas around the world.

Recently, shampoo ginger has received considerable attention because of the pharmacological significance of zerumbone. Zerumbone, the most abundant component of rhizome oil, is a unique sesquiterpeoid found exclusively in shampoo ginger. It has been reported that zerumbone possesses striking anti-inflammatory and anti-HIV activities (Ozaki et al. 1991; Dai et al. 1997). In addition, zerumbone has also been shown to inhibit the proliferation of several cancer cell lines via the induction of apoptosis, suggesting its potential use as a promising agent for the treatment of several cancers, including cancers of the colon, skin, liver, breast and leukemia (Murakami et al. 2002, 2004; Kirana et al. 2003; Sharifah-Sakinah et al. 2007; Xian et al. 2007). Recent progress in understanding the anticancer properties of zerumbone and the increasing public interest in health will lead to the demand for large amounts of zerumbone in the future.

Terpenoids are naturally produced in small quantities in plants. Purification from plants leads to poor yields, low purities and high consumption of plant sources. Chemical synthesis of terpenoids is also inherently difficult and expensive due to their structural complexity (Martin et al. 2003a). Nevertheless, rapid advances in modern biotechnology suggest that engineering the metabolic pathway in microbial hosts is a promising approach for commercial production of terpenoids (Kakinuma et al. 2001; Martin et al. 2003a; Yoon et al. 2006). This approach requires a detailed understanding of the biosynthetic pathways, enzyme reactions, and genes encoding these enzymes. The first committed step of zerumbone biosynthesis should be the cyclization of the universal sesquiterpene precursor, farnesyl diphosphate (FPP) by a sesquiterpene synthase to produce an olefinic sesquiterpene (Cane 1999).

In an effort to identify the sesquiterpene synthase involved in zerumbone biosynthesis and use the corresponding cDNA clone for metabolic engineering, we have attempted to isolate sesquiterpene synthase genes from this valuable plant. In this paper, we describe the isolation and functional characterization of α-humulene synthase gene (*ZSS1)* from shampoo ginger. The α-humulene production in *E. coli* by importing *ZSS1* and the exogenous mevalonate pathway was also investigated.

The high similarity between α-humulene and zerumbone in structure and configuration suggests that α-humulene is the most likely candidate for the olefinic sesquiterpene intermediate in the biosynthesis of zerumbone (Fig. 1). To our knowledge, although α-humulene is a common component of many plant essential oils, a sesquiterpene synthase that produces α-humulene as the predominant product has not been reported.

Materials and methods

Plant materials and treatments

Ginger plants (*Zingiber zerumbet* Smith) were generously provided by SAKATA Co., (Kochi, Japan). Saplings were grown in a horticulture chamber under natural light and environmental conditions from April to July 2005. Mid-summer plants ~30 cm high were used in all experiments. For MeJA treatment, saplings were sprayed with 300 μM of MeJA dissolved in 0.1% Tween 20. Control saplings were sprayed with 0.1% Tween 20. For mechanical wounding, leaves were cut with scissors and stems were cut to a depth of 2–3 mm with razor. Wounding proceeded from the apex to the base with each wound spaced approximately 2 cm apart. Leaves, stems and rhizomes were quickly harvested at 0, 4, 8, 24, 48 and 72 h after treatment, immediately frozen in liquid N_2 and then stored at −80°C for RNA isolation until use.

Isolation of the *ZSS1* cDNA sequence

Total RNA from rhizomes for RT-PCR was isolated with a Spectrum Plant Total RNA Kit (Sigma–Aldrich, CA, USA). Three micrograms of RNA was reverse transcribed into cDNA in a 20-μl reaction with poly (dT) priming using a SuperScript III First-strand Synthesis Kit (Invitrogen). Two degenerate primers 5′-AYCWYTTYGAIRAIGA-RAT-3′ (forward) and 5′-TAIGHRTCAWAIRTRTCRTC-3′ (reverse) were used for RT-PCR. Two-rounds of PCR were performed each in a total volume of 50 μl containing a 1 μl cDNA template, 1 μM of each primer, 200 μM each of dNTP and 1U of ExTag polymerase (TaKaRa). Five microliters of the completed primary reaction served as template in a secondary amplification. The temperature program for PCR was denaturation at 94°C for 2 min, followed by four cycles of 94°C for 30 s, 35°C for 1 min 30 s, 72°C for 1 min, 30 cycles of 94°C for 30 s, 40°C for 1 min 30 s, 72°C for 1 min and the final elongation at 72°C for 3 min. The resulting purified 670 bp fragment was cloned into the pGEM-T easy vector (Promega) and sequenced. The partial sequence was extended toward 5′ end and 3′ end by the Smart RACE cDNA Amplification Kit (BD Biosciences Clontech) following the manufacture's protocol. The two gene-specific primers used for RACE-PCR were as follows: 5′-GCATCTCCATTCAAGGTCGGCAA-3′ for 5′RACE, 5′-GAGCAGCCTTTAGCAATAGAGGTGTC-3′ for 3′RACE.

Genomic DNA was isolated from leaves with the Plant Genomic Isolation Mini Kit (Qiagen). Genomic DNA clone for *ZSS1* was amplified from genomic DNA by PCR using the forward primer 5′-ATGGAGAGGCAGTCGATGGCC CTTG-3′ and reverse primer 5′-AATAAGAAAGGATTC AACAAATATGAGAG-3′ designed against the full-length

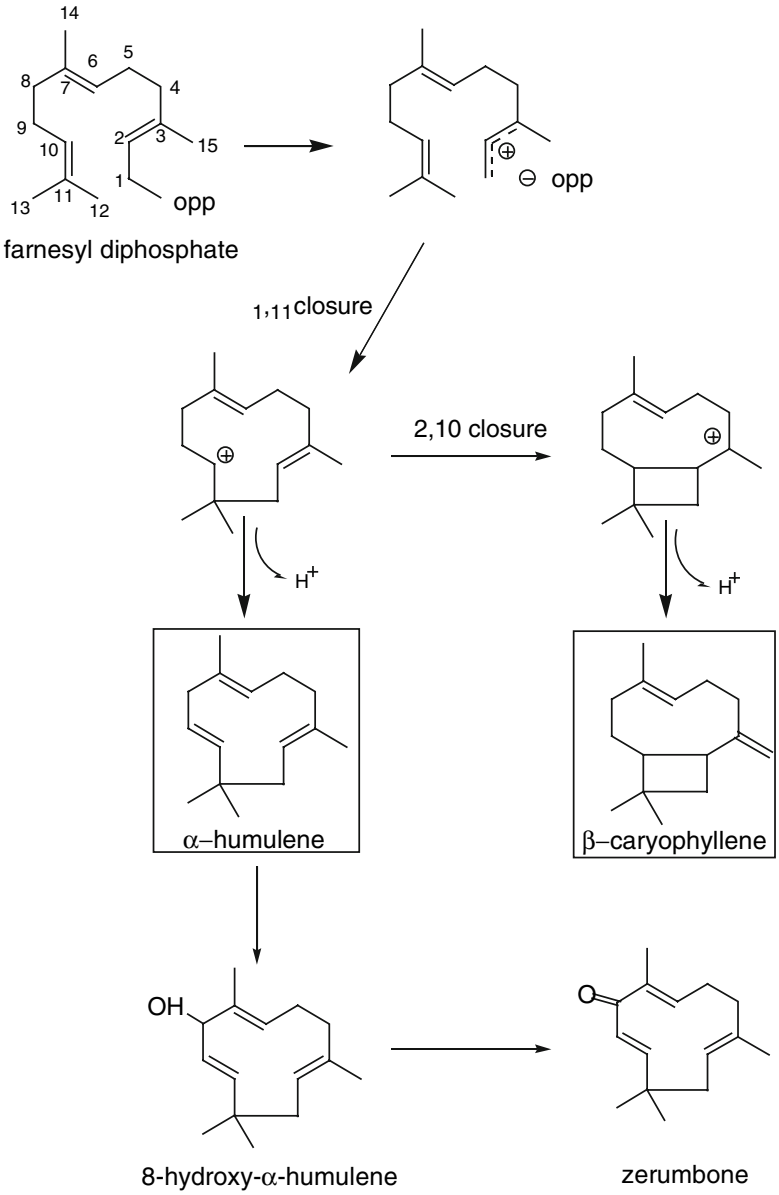

Fig. 1 Proposed mechanism for α-humulene and zerumbone biosynthesis in shampoo ginger. Products of ZSS1 were enclosed in boxes. OPP denotes the diphosphate moiety

cDNA sequences. Intron junctions were mapped by nucleotide sequence comparison of cDNA and genomic clones.

Bacterial expression and in vitro enzyme assays of *ZSS1*

The full-length ORF of *ZSS1* were amplified by PCR using Advantage® HF 2 polymerase (Clontech) with the forward primer 5′-CACCATGGAGAGGCAGTCGATGGC-3′ and reverse primer 5′-AATAAGAAAGGATTCAACAAATA TGAGAG-3′. The amplified product was cloned into the pET101/DTOPO directional expression vector (Invitrogen). The recombinant plasmid pET-*ZSS1* was transformed into *E. coli* TOP10F' cells for sequence characterization and into *E. coli* BL21-CodonPlus (DE3) (Stratagene) for expression.

Recombinant *E. coli* cells were grown to OD_{600} = 0.5–0.6 at 37°C in 50 ml LB medium containing ampicillin (50 μg ml^{-1}). Cultures were then induced by addition of isopropyl-1-thio-*β*-D-galactopyranoside (IPTG) to a final concentration of 1 mM and grown for another 20 h at 18°C. The cells were collected by centrifugation and disrupted by a 10 × 10 s treatment with a sonicator (Branson W185 D) in chilled extraction buffer (50 mM MOPSO, pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 10% [v/v] glycerol). Following centrifugation at 15,000*g* for 30 min, the supernatant was collected and subjected to purification on a nickel–nitrilotriacetate agarose column (Qiagen) according to manufacturer's instructions. The protein eluate was further desalted into assay buffer (10 mM MOPSO, pH 7.0, 1 mM dithiothreitol, and 10% [v/v] glycerol) by passage

through a Econopac 10DG column (Bio-Rad), and the resulting enzyme eluate was used for enzyme assay.

Each assay was done in a volume of 1-ml with 900 µl of enzyme extract, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM $NaWO_4$, 0.1 mM NaF and 20 µM [1-^{3}H]-(E, E)-FPP (555 GBq mol^{-1}, American Radiolabeled Chemicals) overlaid with 0.5 ml of pentane to trap volatile products in a Teflon-sealed, screw-capped glass test tube. After incubation at 30°C for 3 h, the mixture was extracted with pentane three times and the combined extracts were passed through a column of anhydrous $MgSO_4$ and silica gel (0.04–0.063 nm, Merck) to provide the sesquiterpene hydrocarbon fraction free of oxygenated products. To collect the oxygenated products, assay mixtures were subsequently extracted by diethyl ether three times and the combined extracts were also passed through the same $MgSO_4$–silica gel column. Aliquots of each fraction were taken for liquid scintillation counting to determine activity. To obtain sufficient product for GC-MS analysis, the enzyme reaction was scaled up to a final volume of 4 ml with 80 µM unlabeled FPP (Echelon Research Laboratories Inc.) as the substrate. After incubation overnight, the reaction mixture was extracted with pentane (3 × 1 ml), filtered through a column of $MgSO_4$–silica gel and concentrated to a minimum volume for GC-MS analysis.

Construction of plasmids for the mevalonate pathway and α-humulene production in *E. coli*

The genes encoding HMGS, HMGR, MK, PMK, MPD and IPPI were isolated by PCR from chromosomal DNA of *Streptomyces* sp. CL190 (Takagi et al. 2000). PCR products were spliced together and inserted into the NdeI-EcoRV sites of vector pACYC184 to construct the plasmid pAC-Mev (data not shown). *E. coli* BL21(DE3) was co-transformed with pET-*ZSS1* and pAC-Mev. The metabolically modified *E. coli* was grown in LB medium containing ampicillin (100 µg/ml, Wako) and chloramphenicol (30 µg/ml, WaKo) at 37°C with shaking until OD_{600} reached 0.5. After supplementation with 1mM of IPTG, 0.5 mg/ml of D-mevalonolactone (Tokyo Kasei) and 20% (v/v) dodecane, cultures were grown for another 48 h at 25°C with shaking. The dodecane phase was then collected after centrifugation and directly subjected to GC-MS analysis.

Product identification

GC-MS analysis was performed on a Shimadzu QP5050A GC/MS system with a DB-WAX column (0.25 mm × 0.25 mm × 30 m, J&W Scientific). Split injections (1 µl) were made at a ratio of 22:1 with an injector temperature of 250°C. The instrument was programmed from an initial temperature of 40°C (3 min hold) and increased at a rate of 3°C min^{-1} until 80°C, 5°C min^{-1} until 180°C and 10°C min^{-1} until 240°C (5 min hold). Helium was used at a constant flow of 1.8 ml min^{-1}. Mass spectra were measured with the mass range of *m/z* 40–400, the electron voltage of 70 eV, and the interface temperature of 230°C.

Determination of gene expression among different tissues

Total RNA for RT-PCR from leaves was isolated with the RNAqueous®-Micro Kit (Ambion). Total RNA from stems and rhizomes was isolated with Spectrum™ Plant Total RNA Kit (Sigma–Aldrich). For RT-PCR analysis of *ZSS1* expression in different tissues, a fragment of ~725 bp was amplified with the following primers: forward 5′-GGTG AACTTGAGCAGCCTTTAGCAAT-3′ and reverse 5′-GT CTCTTTTGTTGCATCTTCTCCCAA-3′. The two primers used for PCR amplification of ubiquitin were forward 5′-AAGGAGTGCCCCAACGCCGAGTG-3′ and reverse 5′-GCCTTCTGGTTGTAGACGTAGGTGAG-3′. PCRs for *ZSS1* were performed for 30 cycles with an annealing temperature of 68°C. For ubiquitin, reactions were carried out in separate tubes for 25 cycles with the same cycling conditions as that of *ZSS1*.

Quantitative real-time RT-PCR

cDNA was synthesized from 1 µg of total RNA from leaves and rhizomes with the SuperScript III First-strand Synthesis Supermix for qRT-PCR (Invitrogen) according to manufacturer's instructions. Relative quantification PCR was carried out using SYBR® Green PCR Master Mix (Applied Biosystems) with the ubiquitin as endogenous control. Untreated control samples served as calibrator, the basis for comparative results. Five replicates of each sample and endogenous control were amplified in separate wells. The specific primer pairs 5′-GCAAGTGATGGTGGAGCAAA A-3′ (forward) and 5′-CCGA GCTACTTGTGTTGGACG T-3′ (reverse) for *ZSS1* and 5′-AAGGAGTGCCCCAACG CCGAGTG-3′ (forward) and 5′-GCCTTCTGGTTGTA GACGTAGGTGAG-3′ (reverse) for ubiqitin were used to amplify 70 and 107 bp products, respectively. Thermal cycling was performed in an ABI 7300 Sequence Detection system and the cycling condition was as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 67°C for 40 s. The relative expression ratios were quantified by Δ ΔCt method (Livak 1997).

Results

Cloning of the *ZSS1* cDNA

To clone the gene encoding sesquiterpene synthases of shampoo ginger, a pair of degenerate primers were

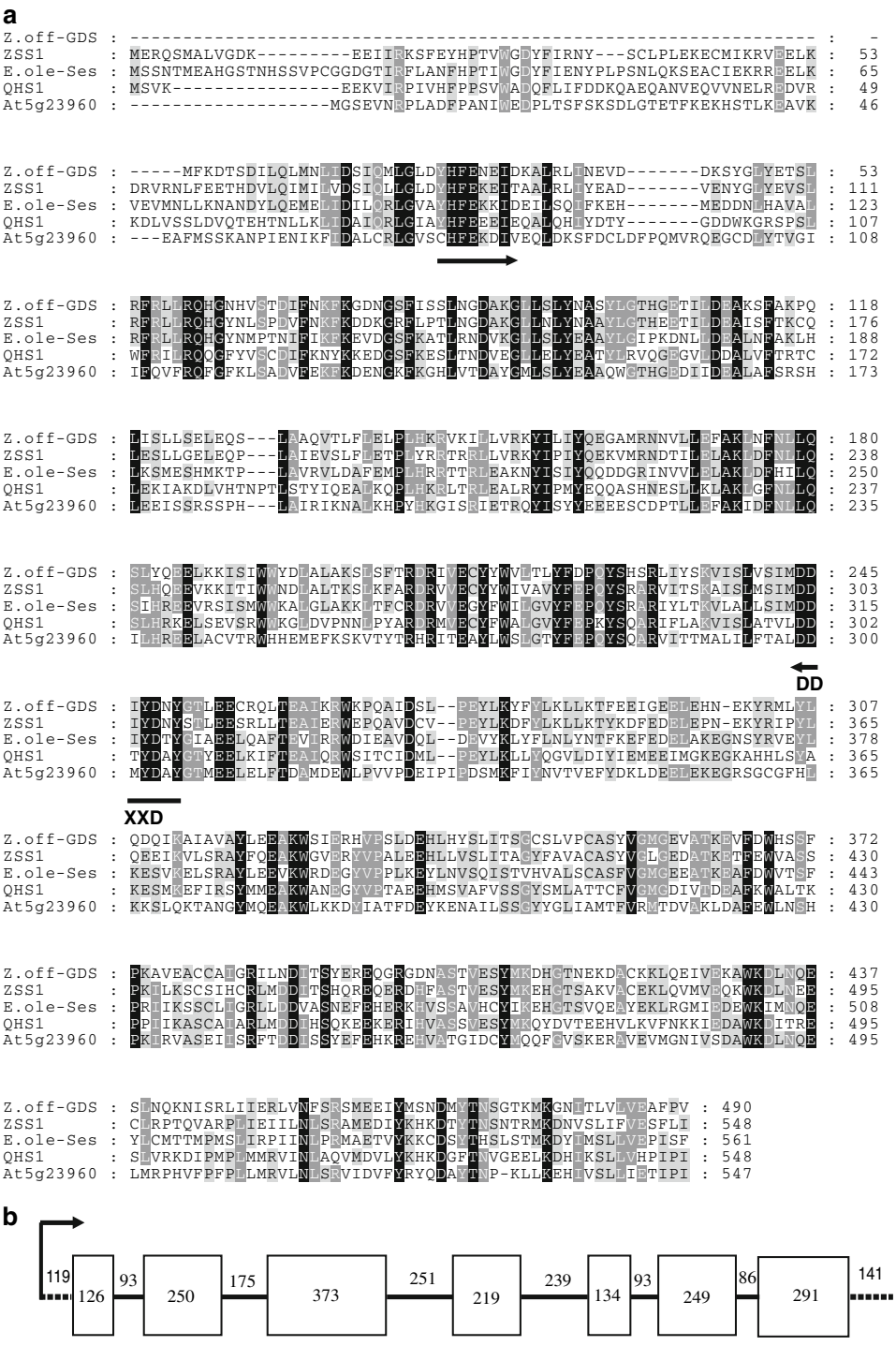

Fig. 2 **a** Comparison of deduced amino acid sequence of *ZSS1* with that of sesquiterpene synthases of selected angiosperm species. Z.off-GDS, *Zingiber officinale* germacrene D synthase (AY860846); E.ole-Ses, *Elaeis oleifera* sesquiterpene synthase (AF080245); QHS1, *Artemisia annua* β-caryophyllene synthase (AF472361); At5g23960, *Arabidopsis thaliana* β-caryophyllene/α-humulene synthase (AF472361). Amino acid residues conserved in all the genes are shaded in black. Amino acid residues conserved in five, six or seven genes are shaded in gray. Regions selected to design degenerate primers are indicated by arrows. The universally conserved DDXXD motif is indicated. **b** *ZSS1* gene structure. Exons are represented by open boxes with introns shown as thick lines. The nucleotide numbers in each exon are shown in the boxes. Horizontal arrow refers to the transcription initiation site identified by S1 nuclease assay (unpublished data). Dashed lines indicate the 5′-Untranslated Region (UTR) and 3′-UTR

designed based on two conserved amino acid domains of several known sesquiterpene synthases from closely related angiosperm species (Fig. 2a). This primer pair was employed for reverse transcription PCR (RT-PCR) using total RNA extracted from rhizomes. A predominant fragment with the expected length of approximately 700 bp was obtained. The 5′ and 3′ ends of the coding sequence were subsequently obtained by 5′ Rapid Amplification of cDNA Ends (5′ RACE-PCR) and 3′ RACE-PCR, respectively. The full-length cDNA, designated as *ZSS1*, contains a putative ORF of 1,644 bp that encodes 548 amino acids with a predicted molecular mass of 64.5 kDa and a PI of 5.25, similar to previously characterized sesquiterpene synthases from other species. A sequence similarity search of the GenBank database demonstrated that *ZSS1* was most closely related to the germacrene D synthase from *Zingiber officinale* with 63% identity. Significant homology was also observed with sesquiterpene synthases from other angio-

sperm species, with sequence identities ranging from 50% with *Elaeis oleifera* sesquiterpene synthase to 34% with *Arabidopsis thaliana* β-caryophyllene/α-humulene synthase (Chen et al. 2003) (Fig. 2a). Based on amino acid sequence and phylogenetic analysis (data not shown), *ZSS1* belongs to the *TPSa* subfamily, a group of sesquiterpene and diterpene synthases from angiosperms (Bohlmann et al. 1998). The apparent lack of an encoded plastidial transit peptide is consistent with the assumed cytosolic biosynthesis location of most of sesquiterpenes (Lichtenthaler 1999).

Intro-exon organization of the genomic *ZSS1*

PCR amplification of genomic DNA using a pair of primers based on the *ZSS1* cDNA sequence generated a 2,582-bp fragment. Comparison of the *ZSS1* genomic DNA and cDNA sequences revealed seven exons and six introns (Fig. 2b). Thus, *ZSS1* belongs to the Class-III TPS subfamily, which contains 6 introns and 7 exons and represents the only angiosperm monoterpene, sesquiterpene and diterpene synthases involved in secondary metabolism (Trapp and Croteau 2001).

Heterologous expression and functional identification

To confirm that *ZSS1* encodes an enzyme with sesquiterpene synthase activity, the complete *ZSS1* coding region was cloned into vector pET101 and transformed into BL-21DE3 (RIL) *E. coli* cells for expression. The His-tag purified recombinant protein was incubated with [1-^{3}H] FPP and [1-^{3}H] GPP in assay buffer as previously reported for TPSs (Schnee et al. 2002; Chen et al. 2003), and the activity was analyzed by scintillation counting. The recombinant enzyme proved to be active with FPP but not with GPP. After scaling up the reaction with unlabeled FPP as the substrate, compounds produced in the reaction were analyzed by gas chromatography-mass spectrometry (GC-MS). The recombinant enzyme generated two sesquiterpene products identified as α-humulene (95%) and β-caryophyllene (5%) by comparison of retention times and mass spectra to authentic standards (Fig. 3). Because α-humulene is the major product, this enzyme was then designated α-humulene synthase.

In vivo production of α-humulene in metabolically engineered *E. coli*

It is known that *E. coli* uses the deoxyxylulose 5-phosphate (DXP) pathway for isoprenoid synthesis. To investigate the possibility of α-humulene production in engineered *E. coli* host and further confirm that *ZSS1* encodes α-humulene synthase, we constructed the plasmid pAC-Mev by introducing a gene cluster responsible for the mevalonate pathway into vector pACYC184. The gene cluster encodes

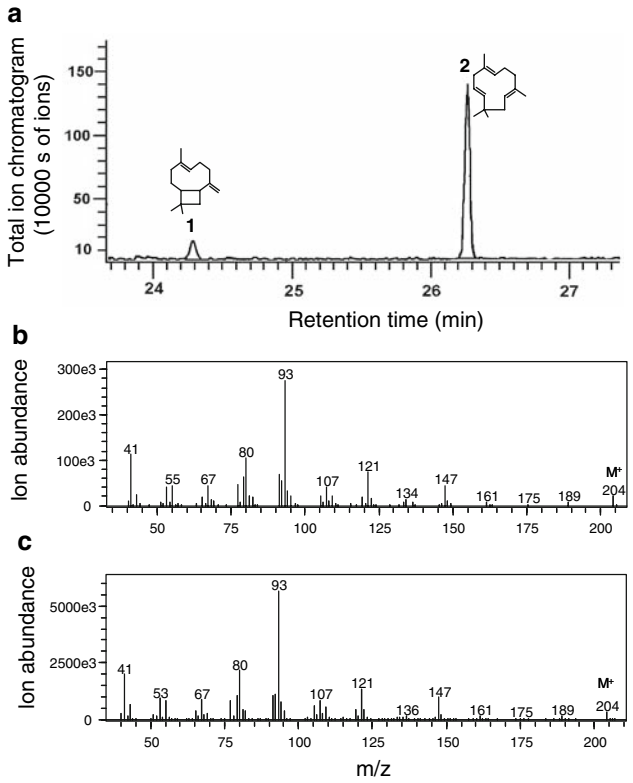

Fig. 3 GC-MS analysis of sesquiterpene products generated by recombinant *ZSS1* enzyme activity in vitro. **a** Total ion chromatogram of the products formed by the recombinant ZSS1. The major product (*peak 2*) and a minor compound (*peak 1*) were identified as α-humulene and β-caryophyllene, respectively. **b** Mass spectrum of α-humulene (*peak 2*) produced by the incubation of FPP with purified recombinant ZSS1. **c** Mass spectrum of authentic α-humulene. M^+ represents the molecular ion of α-humulene

six enzymes, hydroxymethylglutaryl CoA synthase (HMGS), hydroxymethylglutaryl CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (MPD) and isopentenyl diphosphate isomerase (IPPI) that converts mevalonate into FPP precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). *E. coli* BL21(DE3) harboring pET-*ZSS1* was co-transformed with pAC-Mev and cultivated in terrific broth in the presence of exogenous mevalonate. GC-MS analysis of the culture extract revealed in vivo production of α-humulene, supporting that *ZSS1* encodes α-humulene synthase and was functionally expressed in metabolically engineered *E. coli* (Fig. 4).

Tissue-specific expression and induction pattern

To determine the expression levels of *ZSS1* mRNAs among different shampoo ginger tissues, RT-PCR analysis was performed with RNA isolated from leaves, stems and rhizomes. The *ZSS1*-specific primers were designed to span

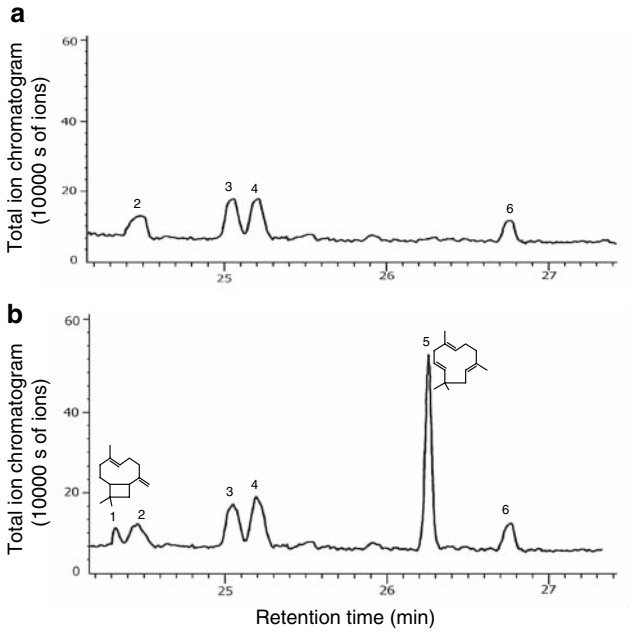

Fig. 4 Identification of α-humulene production in metabolically engineered *E. coli*. **a** Total ion chromatogram of the products obtained from the control *E. coli* carrying pAC-Mev and the empty vector pET101. **b** Total ion chromatogram of the products produced from engineered *E. coli* harboring pAC-Mev and pET-*ZSS1*. Peaks 1 and 5 were identified as *β*-caryophyllene and α-humulene, respectively

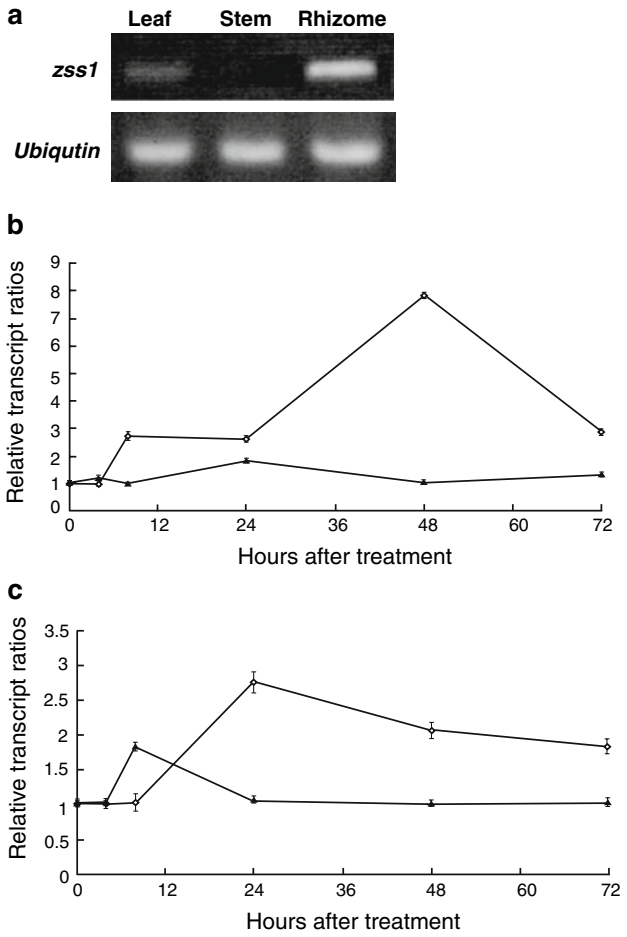

Fig. 5 Transcript expression analysis of *ZSS1*. **a** Tissue-specific expression of *ZSS1*. Total RNA was isolated from leaves, stems and rhizomes and used for RT-PCR analysis. *Ubiquitin* served as an endogenous control. **b, c** Time course analysis of relative transcription levels of *ZSS1* by real-time RT-PCR. Data represent the means of five replicates of relative transcripts in leaves (**b**) and rhizomes (**c**) after mechanical wounding (*filled triangle*) and MeJA treatment (*open diamond*). Relative transcription level of untreated control samples at 0 h is 1. *Error bars* denote standard errors

two introns of the *ZSS1* genomic sequences to prevent potential amplification of contaminating genomic DNA. The partial cDNA sequence of a ubiquitin was used as a reference for cDNA template loading. As shown in Fig. 5a, the expression of *ZSS1* was present in leaves and rhizomes but absent in stems. There was an apparent higher level of *ZSS1* expression in rhizomes than in leaves. The differential expression of *ZSS1* in these tissues is consistent with the higher content of α-humulene in rhizome oil (14.4%) than in leaf oil (2.0%) (Chane et al. 2003).

To further investigate the inducible expression pattern in leaf and rhizome tissues, we performed quantitative PCR to compare the transcription levels of *ZSS1* in MeJA- and wounding-treated saplings with that of untreated control saplings over 72 h. As shown in Fig. 5b, the transcription level in leaves was strongly induced, reaching peak (about eightfold) at 48 h after MeJA treatment (Fig. 5b). In rhizomes, a maximum of only a two to threefold transcription level was induced in MeJA-treated samples at 24 h (Fig. 5c). In contrast, the transcription levels were only marginally affected by the mechanical wounding in leaves and rhizomes (Fig. 5b,c).

Discussion

We provide a first isolation and functional characterization of a terpene synthase gene from shampoo ginger. This gene, designated as *ZSS1*, encodes an enzyme that catalyzes the formation of α-humulene and a trace amount of *β*-caryophyllene. The proposed reaction scheme of (SS((from FPP is shown in Fig. 1. The enzymatic formation of sesquiterpenes begins with the ionization of trans,trans-farnesyl diphosphate to the corresponding transoid allylic cation-diphosphate anion (Cane 1990). Subsequent electrophilic attack on the distal double bond provides the formation of a humulyl cation. The humulyl cation can either form α-humulene directly by deprotonation from C-9 or further cyclize to generate *β*-caryophyllene by electrophilic attack on the 2, 3 double bond followed by deprotonation.

Although α-humulene is one of the most common sesquiterpenes in the plant kingdom, sesquiterpene synthases capable of producing this compound have only been

described from two plant species *A. annua* (QHS1) (Cai et al. 2002) and *A.thaliana* (At5g23960) (Chen et al. 2003), and both of them produce α-humulene as a minor product. The deduced protein sequence of *ZSS1* showed considerably lower homology with that of *QHS1* and *At5g23960* (39 and 34% identities, respectively) compared to germacrene D synthase from *Z. officinale* (Picaud et al. 2006) and a putative sesquiterpene synthase from *E. oleifera* (Cha and Shah 2000) (63 and 50% identities, respectively) (Fig. 2a). This finding supports the notion that in closely related plants, TPSs catalyzing different products may be more similar to each other than TPSs catalyzing the same products in distantly related plant species, suggesting the convergent evolution is common in the plant TPS family (Mau and West 1994).

Recent studies demonstrated that metabolic engineering of a mevalonate pathway in *E. coli* significantly increased the terpene production (Kakinuma et al. 2001; Martin et al. 2003a). To maximize the supply of IPP and DMAPP, a key determinant of terpene biosynthesis, we have engineered *E. coli* by introducing a mevalonate pathway which can produce IPP and DMAPP efficiently from the exogenous mevalonate (Yoon et al. 2006). Compared with extractions from plant sources or strategies that require expensive substrate, α-humulene production from simple carbon source (mevalonate) in engineered *E. coli* suggests that steady, consistent supply of α-humulene at a relatively low cost is possible, although optimization of the mevalonate concentration and substrate pool of IPP and DMAPP is required for large-scale production. Because α-humulene is the most likely intermediate in zerumbone biosynthesis, this result may represent the first essential step toward zerumbone production in *E. coli*.

Increasing evidence suggests that treatment with MeJA and mechanical wounding can simulate herbivory or pathogen invasion to induce enzyme activities, expression of terpene synthases and terpene emissions or accumulations (Steele et al. 1998; Martin et al. 2002,2003b; Miller et al. 2005). Because the expression of terpene synthases is often regulated on the level of transcription (Yin et al. 1997), we investigated the transcription levels of *ZSS1* in response to MeJA treatment and mechanical damage. It was found that MeJA treatment triggered an elevated and transient increase of *ZSS1* transcripts in both leaves and rhizomes, suggesting that *ZSS1* is likely to play an ecological role in shampoo ginger. Curiously, MeJA treatment did not induce α-humulene accumulation in rhizomes (data not shown). This result is in disagreement with findings in conifers where MeJA triggered the accumulation of α-humulene and other terpene compounds (Martin et al. 2003b). It is possible that mechanisms such as the regulation of substrate availability, in addition to induced TPS transcripts, also contribute to the control of terpenoid production, as was previously concluded from other studies (Aharoni et al. 2003; Dudareva et al. 2003). Because rhizomes have an exceptionally high content of humulene-derived sesquiterpenes (zerumbone, humulene epoxide I and humulene epoxide II), the lack of increase in α-humulene accumulation may also due to its conversion to other humulene derivatives. We also can not rule out the possibility that induced α-humulene did not accumulate in rhizomes but released to the belowground environment.

α-Humulene can function as feeding deterrents or repellents to repel insects or pathogens in direct defense (Suga et al. 1993; Takayuki 1994), or as volatile signals to attract natural enemies of herbivores in indirect defense (Mattiacci et al. 2001; Rodriguez-Saona et al. 2003). α-Humulene is almost exclusively accumulated and synthesized in the rhizome of shampoo ginger, suggesting that *ZSS1* may play a role in defense system in the belowground environment.

That MeJA treatment led to much less increase in *ZSS1* transcription level in rhizomes than in leaves may be due to the high constitutively expression level of rhizomes. The observation that single mechanical damage did not induce the *ZSS1* transcription is likely to reflect that cutting leaves with scissors may not be a good method to mimic insect or pathogen invasion. It is possible that the application of herbivore oral secretions or volicitin to the site of the mechanical damage is required to trigger the transcription induction of *ZSS1* (Schnee et al. 2002).

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