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Isolation and functional characterization of a β-eudesmol synthase, a new sesquiterpene synthase from *Zingiber zerumbet* Smith

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Abstract In this paper, we have identified a new sesquiterpene synthase gene (*ZSS2*) from *Zingiber zerumbet* Smith. Functional expression of *ZSS2* in *Escherichia coli* and in vitro enzyme assay showed that the encoded enzyme catalyzed the formation of β-eudesmol and five additional by-products. Quantitative RT-PCR analysis revealed that *ZSS2* transcript accumulation in rhizomes has strong seasonal variations. To further confirm the enzyme activity of *ZSS2* and to assess the potential for metabolic engineering of β-eudesmol production, we introduced a gene cluster encoding six enzymes of the mevalonate pathway into *E. coli* and coexpressed it with *ZSS2*. When supplemented with mevalonate, the engineered *E. coli* produced a similar sesquiterpene profile to that produced in the in vitro enzyme assay, and the yield of β-eudesmol reached 100 mg/L.

Keywords: Sesquiterpene synthase gene; β-Eudesmol; Seasonal variation; Metabolic engineering; Mevalonate pathway; *Zingiber zerumbet* Smith

1. Introduction

Sesquiterpenes, the C_{15} member of the terpenoid family of natural products, play a variety of ecological roles in higher plants. Some of these compounds exhibit antifeedant and antifungal activities [1,2]. In addition, many sesquiterpenes are volatile compounds that are commonly emitted from flowers and leaves of the plant that function as volatile cues to attract pollinators or parasitic and predatory insects [3–5]. Interestingly, volatile sesquiterpenes have also been found to be synthesized and accumulated in rhizomes and roots or released from these below ground tissues [6,7]. The first committed step of terpene biosynthesis is the cyclization of the prenyl diphosphate precursors to the parent structures of terpenes catalyzed by terpene synthases (TPSs). While a large group of TPS genes and enzymes has been characterized in above ground tissues of angiosperms and gymnosperms [8], only a few TPSs have been identified in plant roots and they are thought to have some important roles in the rhizosphere [9,10].

The rhizome oil of *Zingiber zerumbet* contains a complex mixture of terpenes, with sesquiterpenes predominating. Recent research on *Z. zerumbet* has demonstrated that zerumbone is a potential drug for the treatment of several cancers as well as leukemia [11–14]. Therefore, we have initiated a project devoted to elucidating the biosynthetic pathway of sesquiterpenes in *Z. zerumbet*, cloning the genes encoding the corresponding biosynthetic enzymes and high-level production of valuable sesquiterpene compounds in *Escherichia coli* by metabolic engineering. Our first attempt to investigate sesquiterpene biosynthesis in *Z. zerumbet* has led to the isolation and characterization of α-humulene synthase (GenBank accession numberAB247331), a possible key enzyme in zerumbone biosynthesis [15].

To improve our understanding of the sesquiterpene biosynthesis in rhizomes of *Z. zerumbet* and its possible function in the below ground environment, it is first necessary to isolate the additional members of the sesquiterpene synthase gene family. Because many sesquiterpene synthases catalyze the formation of multiple products from a single substrate [16–18], molecular cloning of cDNAs encoding these key enzymes is also essential for deciphering whether sesquiterpene formation in *Z. zerumbet* requires specialized sesquiterpene synthases for each component or multi-functional synthases are involved. Here, we report the isolation and characterization of a sesquiterpene synthase responsible for the formation of β-eudesmol from rhizomes of *Z. zerumbet*.

Recent studies demonstrated that engineering a mevalonate pathway in *E. coli* holds considerable promise for high-yield production of terpenes [19,20]. Because β-eudesmol is known to have unique effects on the nervous system, including blocking the nerve-evoked contraction and markedly alleviating muscle fasciculation, tremor and convulsion [21,22], we also investigated the potential for β-eudesmol production in metabolically engineered *E. coli*.

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; FPP, farnesyl diphosphate; GC–MS, gas chromatography–mass spectrometry; HMGS, hydroxymethylglutaryl CoA synthase; HMGR, hydroxymethylglutaryl CoA reductase; IPP, isopentenyl pyrophosphate; IPPI, isopentenyl pyrophosphate isomerase; MK, mevalonate kinase; MeJA, methyl jasmonate; MPD, mevalonate diphosphate decarboxylase; PMK, phosphomevalonate kinase; RACE, rapid amplification of cDNA ends; TPS, terpene synthase

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2. Materials and methods

2.1. Plant materials

Z. zerumbet were generously provided by SAKATA Co. Ltd., Japan. Saplings were grown in a horticulture chamber under natural light and environmental conditions. Mid-summer plants approximately 30 cm high were used for cDNA cloning. Rhizomes for analysis of gene expression were harvested in different seasons, immediately frozen in liquid N_2 and then stored at −80 °C for RNA isolation.

2.2. Essential oil extraction

Fresh rhizomes were sliced and extracted overnight with excess pentane at 4 °C. The pentane extract was collected and passed through a column of $MgSO_4$ and silica gel (60 Å, Merck) to remove water and provide the olefin fraction. The oxygenated metabolites were then released from the column by washing with diethyl ether. Both fractions were identified and quantified for monoterpene and sesquiterpene content by GC–MS.

2.3. Isolation of the ZSS2 cDNA and genomic DNA

Total RNA from rhizomes was isolated with a Spectrum Plant Total RNA Kit (Sigma–Aldrich). Three micrograms of RNA was reverse transcribed into cDNA using a SuperScript III First-strand Synthesis Kit (Invitrogen). Two degenerate primers 5′-TTYCGAYTIYTIMGR-MARCAIGG-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 1 μL of cDNA template, 1 μM of each primer, 200 μM of dNTP and 1 U of ExTag polymerase (TaKaRa). Primary reaction (5 μL) served as template in a secondary amplification. The temperature program for PCR was denaturation at 94 °C for 2 min, followed by 4 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 581 bp fragment was cloned into the pGEM-T easy vector (Promega) and sequenced. To isolate the full-length cDNA, the partial sequence was extended toward 5′ and 3′ end by the Smart RACE cDNA Amplification Kit (Clontech) following the manufacturer's protocol. The two gene-specific primers used for RACE-PCR were as follows: 5′-CCCAATAATAACCTTCCA-CAACTCGG-3′ for 5′RACE, 5′-CCGAGTTGTGGAAGGTTATT-ATTG G-3′ for 3′RACE.

Genomic DNA clone for*ZSS2* was amplified from genomic DNA using the forward primer 5′-ATGGAGAAGCAATCACTAAC-3′ and the reverse primer 5′-CTTATTGAAGTAGTCACAAGATTC-3′. Intron junctions were mapped by nucleotide sequence comparison of cDNA and genomic clones.

2.4. Bacterial expression and in vitro enzyme assays of ZSS2

The full-length ORF of *ZSS2* were amplified by PCR using Advantage® HF 2 polymerase (Clontech) with the forward primer 5′-CACCATGGAGAAGCAATCACTAC-3′ and the reverse primer 5′-CTTATTGAAGTAGTCACAAGATTCAAC-3′. The amplified product was cloned into a pET101/DTOPO vector (Invitrogen). The recombinant plasmid pET-*ZSS2* was transformed in *E. coli* TOP10F' cells for sequence characterization and into *E. coli* BL21-CodonPlus (DE3) (Stratagene) for expression.

For functional expression, recombinant *E. coli* cells were grown to $OD_{600} = 0.5–0.6$ at 37 °C in LB medium containing ampicillin (50 g mL^{-1}). Cultures were then induced by addition of 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 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 15000 × *g* for 30 min, the supernatant was collected and purified on a nickel–nitrilotriacetate agarose column (Qiagen). 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 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 (555GBq mol^{-1}, American Radiolabeled Chemicals) overlaid with 0.5 mL of pentane to trap volatile products. After incubation at 30 °C for 3 h, the mixture was extracted with pentane (3 × 1 mL) and the combined extracts were passed through a column of anhydrous $MgSO_4$ and silica gel (60 Å, Merck) to provide the sesquiterpene hydrocarbon fraction free of oxygenated products. To collect the oxygenated products, assay mixtures were subsequently extracted with diethyl ether (3 × 1 mL) and the combined extracts were also passed through the same column. Aliquots of each fraction were taken for liquid scintillation counting to determine activity. To obtain sufficient product for analysis by GC–MS, the enzyme reaction was scaled up to a final volume of 4 mL using 80 μM unlabeled farnesyl diphosphate (FPP) (Echelon Research Laboratories Inc.) as the substrate.

2.5. Determination of ZSS2 gene expression by RT-PCR and quantitative real-time RT-PCR

Total RNA isolated from leaves, stems and rhizomes were subjected to RT-PCR analysis using *ZSS2*-specific primers: forward 5′-CAC-TCATGCAAAGGAGTCCAC-3′ and reverse 5′-CTGTAATGGT-TGTCTCTAATAGTCC-3′. The two primers used for amplification of ubiquitin were: forward 5′-CACAAGAAGGTGAAGCTCGC-3′ and reverse 5′-GCCTTCTGGTTGTAGACGTAGG-3′.

For real-time PCR analysis, 1 μg of total RNA was reverse transcribed with the First-strand Synthesis Supermix for qRT-PCR (Invitrogen). Relative quantification PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems) on an ABI 7300 cycler with ubiquitin as a reference. Samples collected on June 12 served as a calibrator for comparative analysis. Specific primers used for PCR amplification of respective genes were as follows: *ZSS2* (forward, 5′-GAATCTTGTGACTACTTCATAAG-3′and reverse 5′-CTGTA-ATGGTTGTCTCTAATAGTCC-3′), *ubiqutin* (forward, 5′-AAG-GAGTGCCCCAACGCCGAGTG-3′and reverse, 5′-GCCTTCTG-GTTGTAGACGTAGGTGAG-3′).

2.6. β-Eudesmol production in metabolically engineered E. coli

The plasmid pAC-Mev for expression of the mevalonate pathway gene cluster was constructed as follows. The tac promoter (Ptac) and rrnB terminator (TrrnB) were amplified from pTTQ18 by PCR and were moved to the EagI–SalI and HindIII–ClaI sites of pACYC184, respectively. The genes encoding hydroxy-methylglutaryl CoA synthase (HMGS); hydroxymethylglutaryl CoA reductase (HMGR); mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (MPD) and isopentenyl pyrophosphate isomerase (IPPI) were isolated from *Streptomyces* sp. strain CL190 and kindly provided by Dr. T. Kuzuyama [23]. The individual genes were spliced together and inserted between Ptac and TrrnB to create pAC-Mev. *E. coli* BL21(DE3) was co-transformed with pET-*ZSS2* and pAC-Mev. The metabolically modified *E. coli* was grown in terrific

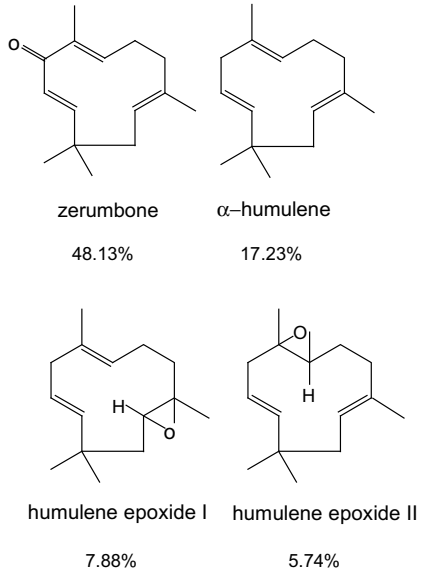

Fig. 1. Chemical structures of the major sesquiterpenes of the rhizome oil of *Zingiber zerumbet*. The percentage contribution to the total terpene fraction of rhizome oil is indicated.

broth containing ampicillin (100 μg/mL) and chloramphenicol (30 μg/mL) at 37 °C with shaking until OD_{600} reached 0.5. After supplementation with 1 mM 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 extracted and subjected to GC–MS analysis.

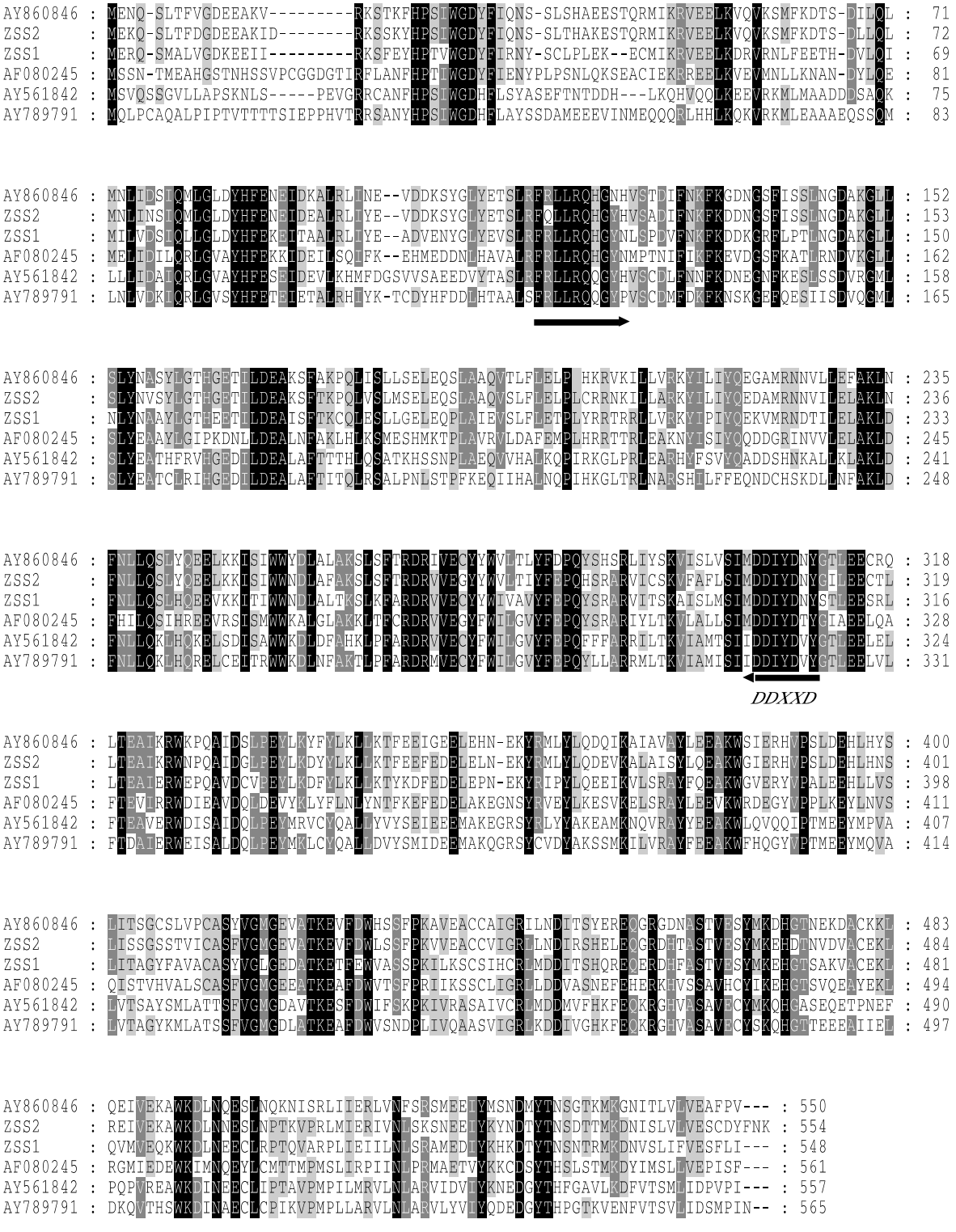

Fig. 2. Comparison of the deduced amino acid sequence of *ZSS2* with other terpene synthase sequences of the highest similarity. GenBank accession number AY860846, *Elaeis oleifera* sesquiterpene synthase; *ZSS1* GenBank accession number AB247331, *Zingiber zerumbet* α-humulene synthase; GenBank accession number AF080245, *Z. officinale* germacrene D synthase; GenBank accession number AY561842, *Vitis vinifera* terpene synthase; GenBank accession number AY789791, *Actinidia deliciosa* germacrene-D synthase. Amino acid residues conserved in all the genes are shaded in black. Amino acid residues conserved in four or five genes are shaded in gray. Regions selected to design degenerate primers are indicated with arrows. The universally conserved DDXXD motif [8] is indicated as well.

2.7. GC–MS analysis

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 L) 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

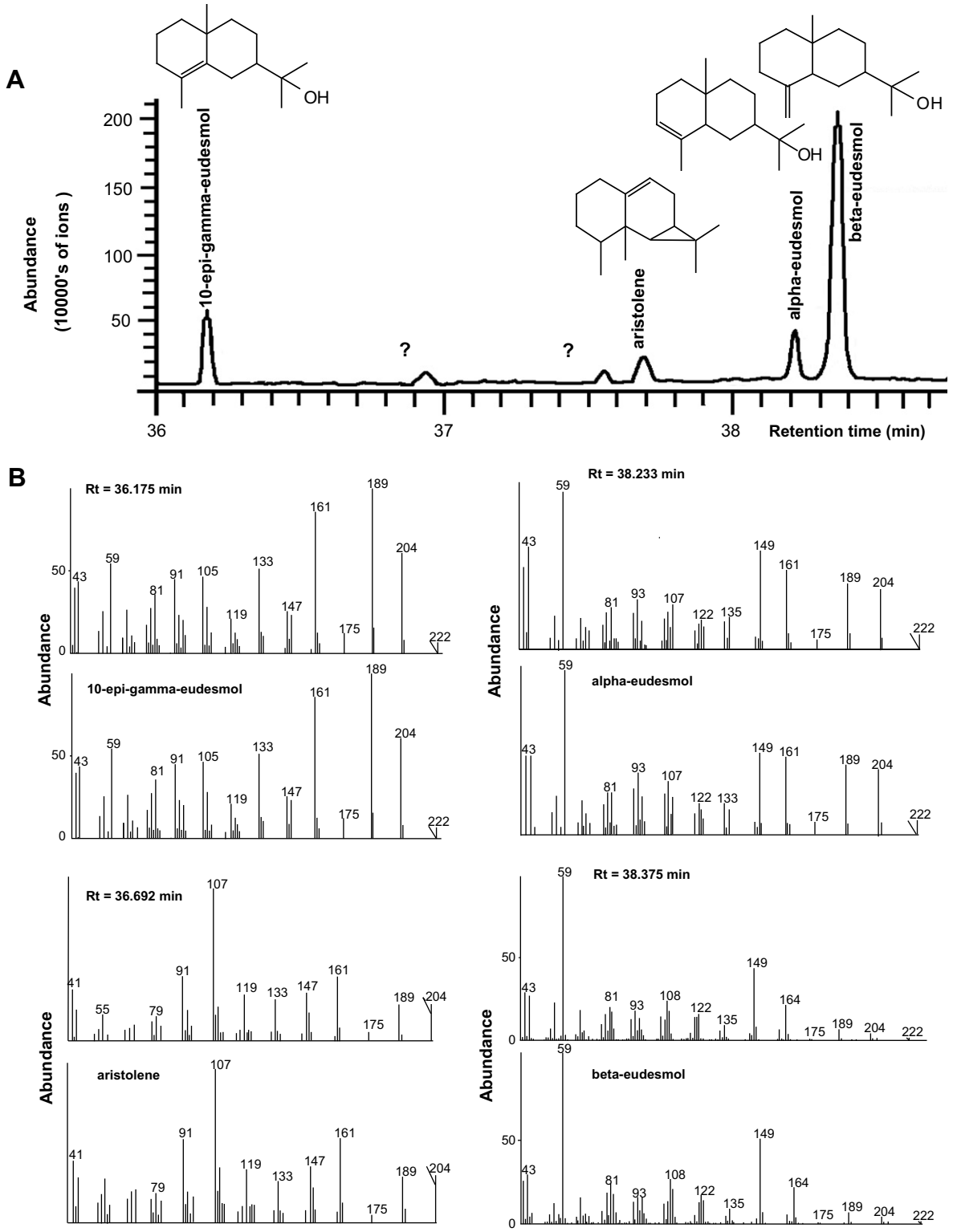

Fig. 3. GC–MS profiles of the sesquiterpene products generated in vitro by the recombinant ZSS2. (A) Gas chromatographic separation of products, the question marks represent unidentified products. (B) Mass spectra of the products (top) and the authentic standard (β-eudesmol) or the best library match (below).

1.8 mL min^{-1}. Mass spectra were measured with the mass range of *m/z* 40–400, at an electron voltage of 70 eV, and interface temperature of 230 °C.

3. Results

3.1. Sesquiterpenoid composition of the rhizome oil

The essential oil from rhizomes of *Z. zerumbet* has been subjected to extensive chemical and pharmaceutical investigations because the rhizomes are known to possess medicinal properties and are used as traditional medicine for indigestion, severe sprains, toothache and other ailments. The combination of GC and GC/MS analysis of the rhizome oil revealed the presence of over 16 sesquiterpeniods comprising 85.81% of the oil, with the remaining 24.19% of monoterpenoids. The oil was characterized by the presence of zerumbone (48.13%), α-humulene (17.23%), humulene epoxide I (7.88%) and humulene epoxide II (5.74%), accounting for 92% of the total sesquiterpenoid fraction (Fig. 1). β-Caryophyllene (1.15%), caryophyllene oxide (3.18%), β-eudesmol (0.21%) and other minor sesquiterpenoid components were also identified.

3.2. Similarity-based cloning of ZSS2, a sesquiterpene synthase gene from rhizomes

To isolate genes encoding sesquiterpene synthases of *Z. zerumbet*, degenerate primers based on two conserved amino acid domains, present in most angiosperm sesquiterpene synthases, were employed to isolate partial cDNA fragments by reverse transcription PCR (RT-PCR) (Fig. 2). A predominant fragment with the expected size of 581 bp was cloned and sequenced. A full-length cDNA, named as *ZSS2*, was subsequently acquired by a combination of 5′ Rapid Amplification of cDNA Ends (5′RACE) and 3′RACE. The *ZSS2* cDNA contains a putative ORF of 1662 bp that encodes 554 amino acids with a predicted molecular mass of 64.4 kDa and a PI of 5.09 (GenBank accession number AB247334) (Fig. 2). The deduced amino acid sequence of *ZSS2* was found to be homologous to sesquiterpene synthases of angiosperms, with the highest level of similarity (82%) to α-humulene synthase from the same species, followed by germacrene D synthase (68% similarity) from *Z. officinale* [17], and a sesquiterpene synthase (62% similarity) from *Elaeis oleifera* sesquiterpene synthase [24] (Fig. 2).

PCR amplification of genomic DNA yielded a fragment of 3232 bp (GenBank accession number AB263738). Comparison of the *ZSS2*genomic DNA and cDNA sequences revealed six introns of 79–693 bp and seven exons of 126–373 bp, similar to several previously reported angiosperm TPSs. *ZSS2* was then classified as Class-III TPS family [25].

3.3. Recombinant ZSS2 enzyme expressed in E. coli converts FPP to β-eudesmol as the principal product in vitro

For functional identification of *ZSS2*, the partially purified recombinant protein was incubated with [1-^{3}H] FPP in assay buffer and the activity was analyzed by scintillation counting. The recombinant enzyme proved to be active with FPP. After scaling up the reaction, products were identified by gas chromatography–mass spectrometry (GC–MS). The major product was identified as β-eudesmol, which accounted for 62.6% of the total products. Several minor products including 10-epi-γ-eudesmol (16.8%), α-eudesmol (10%), aristolene (5.6%) and two unknown products (5%) were also detected (Fig. 3). A control extract prepared from *E. coli* BL21 (DE3) transformed with pET101/DTOPO without the *ZSS2* insert did not produce any product. Because β-eudesmol is the major product, this enzyme was then designated β-eudesmol synthase.

3.4. ZSS2 transcript accumulations were strongest in rhizomes in summer and exhibited strong seasonal variations

To determine the tissue-specific expression of *ZSS2* in summer plants, RT-PCR analysis was performed with RNA isolated from different tissues. The result showed that *ZSS2* is expressed strongest in rhizomes and much less in stems, but not in leaves (Fig. 4A). To investigate the temporal expression pattern, we also monitored *ZSS2* transcript levels during rhizome development by quantitative RT-PCR. As shown in Fig. 4B, *ZSS2* transcript was detected in all the samples examined. However, transcript levels increased rapidly in summer, peaked in early August and declined significantly in fall and winter.

3.5. Engineering a mevalonate pathway in E. coli results in β-eudesmol production in vivo

Previous studies showed that engineering a heterologous mevalonate-dependent pathway in *E. coli* is superior to engineering *E. coli* native 1-deoxy-D-xylulose-5-phosphate (DXP) pathway for large-scale terpene production [19]. To further confirm the contribution of ZSS2 to the sesquiterpene profile and to assess the potential for metabolic engineering of β-eudesmol production in *E. coli*, a gene cluster pAC-Mev encoding six enzymes of the mevalonate pathway: HMGS, HMGR, MK, PMK, MPD and IPPI was introduced into *E. coli* (Fig. 5). The recombinant *E. coli* harboring pET-*ZSS2* was co-transformed with pAC-Mev and incubated with exogenous mevalonate. GC–MS analysis of the dodecane phase in the

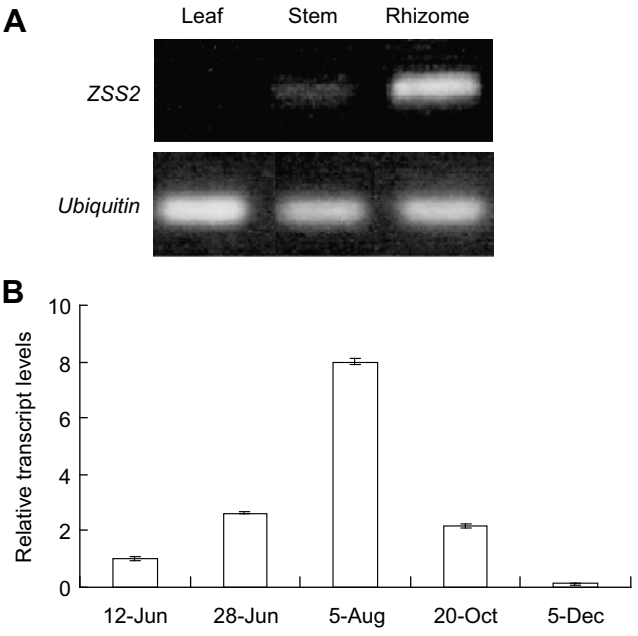

Fig. 4. Tissue (A) and temporal (B) levels of the ZSS2 transcript determined by RT-PCR. (A) *ZSS2* gene expression in leaves, stems and rhizomes. (B) Seasonal variations of *ZSS2* transcript accumulations in Rhizomes. Values are means of three replicates. Error bars indicate S.D. The experiment was repeated once.

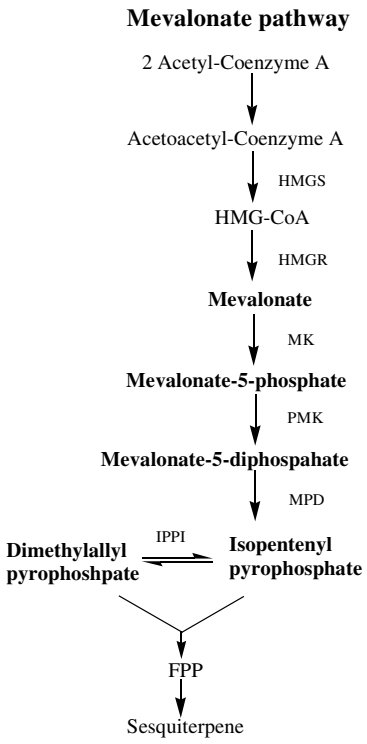

Fig. 5. The mevalonate pathway of sesquiterpene biosynthesis. Six enzymes (HMGS, HMGR, MK, MPK, MDD and IPPI) used in this study are listed.

engineered strains observed a similar product profile to that produced in the in vitro assay, with β-eudesmol accounting for 72.4% of the total products, followed by 10-epi-γ-eudesmol (11.2%), α-eudesmol (7.1%) and aristolene (6.1%) (Fig. 6). No sesquiterpene products were detected in the control strains co-transformed with pET and pAC-Mev. The in vivo production of β-eudesmol reached 100 mg/L, indicating that *ZSS2* was functionally expressed in metabolically engineered *E. coli*.

4. Discussion

In this paper, we provide the first cloning and functional characterization of a cDNA (*ZSS2*) encoding β-eudesmol synthase from natural sources. β-Eudesmol synthase is a typical plant sesquiterpene synthase in terms of amino acid sequence, gene architecture and multi-functional nature.

It is interesting to note that despite the apparently high level of *ZSS2* gene expression in rhizomes of *Z. Zerumbet*, no β-eudesmol and other products of ZSS2 were detected in this tissue, except for a trace amount of γ-eudesmol (0.21%). The high content of sesquiterpenes in rhizomes implies that β-eudesmol biosynthesis is not limited by the substrate (FPP) supply. The lack of accumulation of β-eudesmol and other products may also be due to their further conversion to other derivatives. However, despite thorough analysis, eudesmane-type metabolites have not been found in rhizomes. Therefore, it is highly possible that β-eudesmol and other products formed in rhizomes of *Z. zerumbet*are released as volatiles to the rhizosphere.

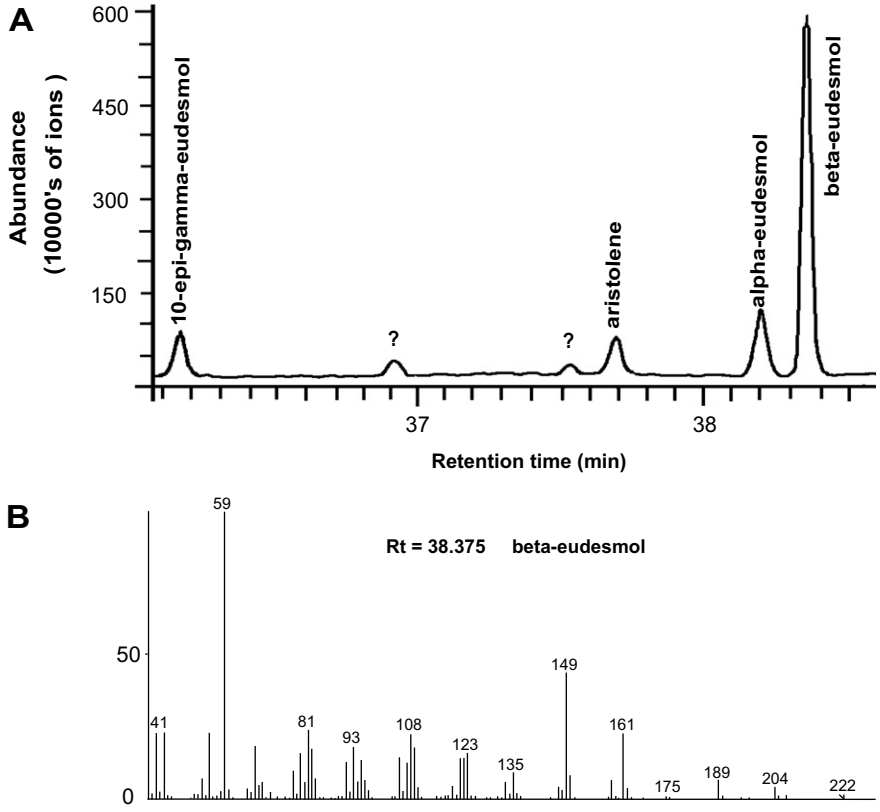

Fig. 6. Identification of the dedocane-extractable sesquiterpene products produced in metabolically engineered *E. coli*. (A) Total ion chromatogram of the products produced from *E. coli* co-transformed with pAC-Mev and pET-*ZSS2*. (B) Mass spectrum of the major peak corresponding to β-eudesmol.

In aerial plant organs, many sesquiterpenes are mainly emitted during mid-summer, and the emission rates are highly correlated with ambient temperature [26,27]. In addition, the activities and gene expression levels of some TPSs were also found to be strongly temperature dependent [28,29]. Because *Z. zerumbet* grows fast from June until December, the obvious seasonal fluctuations of *ZSS2* transcript accumulations in rhizomes should also be temperature-regulated but not development-dependent (Fig. 4B). This finding raises intriguing questions about the biological roles of volatile terpenes in below ground environment. Several terpenes have been reported to be released from plant roots and their emission is considered to be critical for their biological significance [7,9]. For example, maize roots release β-caryophyllene in response to beetle attack as a volatile signal to attract predatory nematodes [7]. β-eudesmol is found to be an active compound responsible for resistance of plants to ant attack [30,31], and also has antifungal activity [32,33]. The remarkable high transcript accumulation of *ZSS2* in early August suggests that β-eudesmol may serve as a defensive agent against active summer pathogens or insects. However, it is difficult to assess the specific roles of β-eudesmol in this plant without further investigation.

Besides its potential roles in plant defense, β-eudesmol is also known to have various beneficial effects on human health and is considered to be a lead compound for treating epileptic seizures [34], angiogenic diseases [35] and dementia [36]. Because insufficient supply of terpene precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) by the native DXP pathway is one of the largest obstacle to high-yield production of terpenes in *E. coli*, we introduced a heterologous mevalonate pathway that can efficiently convert mevalonate to IPP and DMAPP into *E. coli*. The metabolically engineered *E. coli* produced a similar sesquiterpene profile to that observed in the in vitro assay in the presence of mevalonate, confirming that *ZSS2* encodes β-eudesmol synthase. The relatively high-level production of β-eudesmol (100 mg/L) in *E. coli* from an inexpensive carbon source (mevalonate) suggests that large-scale production of β-eudesmol without being affected by plant cultivation is feasible. Our efforts are underway to optimize the expression of the mevalonate pathway and maximize the sesquiterpene production.

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