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RESEARCH ARTICLE

Zingiber zerumbet CYP71BA1 catalyzes the conversion of α-humulene to 8-hydroxy-α-humulene in zerumbone biosynthesis

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Abstract Plant cytochrome P450s are involved in the biosynthesis of various classes of secondary metabolites. To elucidate the biosynthesis of zerumbone, a sesquiterpenoid with multiple potential anticancer properties, a family of P450 genes expressed in rhizomes of *Zingiber zerumbet* Smith, were cloned using a PCR-based cloning strategy. After functional expression in yeast, one of these P450s was found to convert α-humulene into 8-hydroxy-α-humulene, a proposed intermediate of zerumbone biosynthesis. This P450 has been designated CYP71BA1, a new member of the CYP71 family. *CYP71BA1* transcripts were detected almost exclusively in rhizomes and showed a similar expression pattern to *ZSS1* transcripts during rhizome development. Coexpression of a gene cluster encoding four enzymes of the mevalonate pathway with *CYP71BA1* and *ZSS1* in *Escherichia coli* leads to the production of 8-hydroxy-α-humulene in the presence of mevalonate, suggesting the possibility of microbial production of this zerumbone intermediate from a relatively simple carbon source by metabolic engineering.

Keywords P450 · *Zingiber zerumbet* Smith · *CYP71BA1* · 8-hydroxy-α-humulene · Mevalonate pathway · Metabolic engineering

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Abbreviations

ATR2	*Arabidopsis* P450 reductase 2 gene
MK	Mevalonate kinase
PMK	Phosphomevalonate kinase
MPPD	Mevalonate diphosphate decarboxylase
IPPI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl diphosphate
DMAPP	Dimethylallyl diphosphate
CPR	Cytochrome P450 reductase

Introduction

The rhizome oil of the tropical ginger *Zingiber zerumbet* Smith contains a remarkably high content of terpenoids, with a sesquiterpenoid, zerumbone, as the predominant component. Recently, zerumbone has gained considerable significance as a pharmaceutical compound. Zerumbone was found to not only exert anti-inflammatory and anti-HIV effects but also to possess anti-proliferative activities in a wide variety of cancer cell lines, implying its potential application in the treatment of several types of cancer [1–7].

Extraction of terpenoids from natural plant sources is often low-yielding and inefficient due to its limited supply, and chemical synthesis is also difficult and uneconomical. However, synthetic biology and metabolic engineering may provide an alternative approach to commercial-scale production of terpenoids [8–11]. Such a strategy should be based on a full understanding of the biosynthetic pathway for terpenoid formation and on identification of the responsible enzymes and genes.

Our recent study proposed a reasonable zerumbone biosynthetic pathway based on the presence of α-humulene and a trace amount of 8-hydroxy-α-humulene in rhizome oil of *Z. zerumbet*. The first committed step of zerumbone

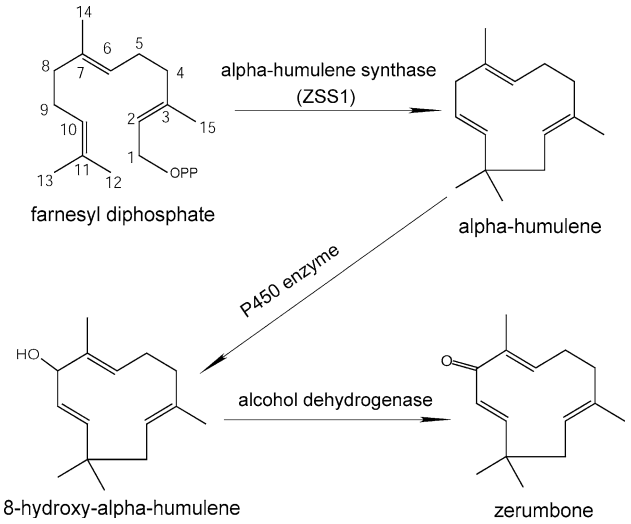

Fig. 1 Proposed pathway for zerumbone biosynthesis in *Z. Zerumbet*

biosynthesis is catalyzed by α-humulene synthase (ZSS1), which cyclizes the universal sesquiterpene precursor farnesyl diphosphate (FPP) to α-humulene to establish the basic skeleton (Fig. 1). This parent olefin is thought to be hydroxylated at the C_8 position by a regiospecific cytochrome P450 enzyme and then oxidized by an alcohol dehydrogenase to generate zerumbone (Fig. 1).

Plant cytochrome P450s are one of the most important classes of enzymes in terpenoid biosynthesis and can participate in a wide range of metabolic reactions, such as hydroxylation, epoxidation, and dehydrogenation. For example, the biosynthesis of taxol, a diterpenoid having multiple anticancer properties, consists of approximately 20 steps, at least nine of which are thought to be cytochrome P450-mediated oxygenations [13]. Sesquiterpenes represent the largest subfamily of natural terpenoids, and P450s are assumed to play a major role in their biosynthetic pathways. However, because the substrates of P450s are difficult to predict and often are not commercially available, only a limited number of sesquiterpene-modifying P450s have been characterized [14–16].

To confirm our hypothesis concerning the biosynthesis of zerumbone and to further improve our understanding of the sesquiterpene metabolism in *Z. zerumbet*, we have begun to isolate P450 genes from rhizomes using a homology-based cloning strategy. As a result of this effort, we have identified a cDNA clone *CYP71BA1* encoding a P450 hydroxylase catalyzing the conversion of α-humulene to 8-hydroxy-α-humulene, a proposed zerumbone intermediate. Because our efforts focus on the large-scale production of zerumbone by genetic engineering, the in vivo production of 8-hydroxy-α-humulene in *E. coli* by importing a gene cluster encoding four enzymes of the mevalonate pathway, CYP71BA1 and its upstream enzyme, α-humulene synthase was also investigated.

Materials and methods

Plant materials and chemicals

8-hydroxy-α-humulene was obtained from NARD Institute, Ltd., α-humulene and other reagents were purchased from Sigma-Aldrich. Ginger plants (*Z. zerumbet* Smith) were generously provided by SAKATA Co. (Kochi, Japan).

cDNA cloning

PCR was performed using two degenerate primers matching conserved sequence domains of various plant P450 monooxygenases: 5′-AARGARACIHTIMGIHTICAY-CC-3′ (forward) and 5′-CANATYCTICKICCISHICCRAAIGG-3′ (reverse). The PCR conditions was adjusted as follows: denaturation at 94°C for 2 min, followed by four cycles of 94°C for 30 s, 48°C for 1 min, 72°C for 30 s, 35 cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 30 s and a final extension at 72°C for 3 min. The PCR product was purified and inserted into a pGEM-T vector (Promega). After sequencing, several possible candidate clones (based on the abundance) were selected to isolate the full-length cDNA using the Smart RACE cDNA Amplification Kit (BD Biosciences Clontech) following the manufacturer's instructions. The specific primers for isolating complete *CYP71BA1* were 5′-CAGAGATGAGAAGTATTGGGG CTCC-3′ and 5′-CTTGAAGCTCTCTGCATCGGAG-3′.

Phylogenetic analysis

Deduced amino acids of selected P450s were aligned using the neighbor-joining algorithm with ClustalW (http://align.genome.jp/). The phylogenetic tree was visualized using TreeView software.

Expression in yeast and functional characterization

The expression vector pYeDP60 [17] and the *Saccharomyces cerevisiae* WAT11 strain containing a chromosomally integrated *Arabidopsis* NADPH-dependent P450 reductase [18] were employed for heterologous expression of *CYP71BA1*. A *Bam*HI site upstream of the start codon and a S*ac*I site downstream of the stop codon were introduced into the full-length cDNA by PCR using the primers 5′-CGGGATCCATGGAAGCTATTTC-CCTCTTCTCCC CTTT-3′ (forward) and 5′-CGCGAGCTCCTATGGCAG AGGAATT-ATTAGCTTGG-3′ (reverse). After digestion with the restriction enzymes, the DNA fragment was inserted into pYeDP60. The verified pYeDP60-*CYP71BA1* construct was then transformed into WAT11 by the lithium chloride method. Transformants were cultured by a high-density procedure and broken mechanically according to Pompon

et al. [17]. Microsomes were harvested by ultracentrifugation at 100,000 × *g* for 90 min and then resuspended and stored in TE buffer containing an additional 20% (v/v) glycerol. Enzyme reactions were conducted in 4 ml of Tris buffer (50 mM, pH 7.4) containing 1 mM EDTA (pH 8.0), 2 mM DTT, 0.1% BSA, 1 mM NADPH, 5 μM flavin adenine dinucleotide (FAD), 5 μM flavin mono-nucleotide (FMN), 5 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, and 300 μg of microsomes. The reaction was initiated by adding substrate α-humulene to the final concentration of 300 μM, incubated at 30°C for 3 h, and stopped by chilling on ice. The reaction mixture was extracted three times with diethyl ether, the extract was passed through a column of anhydrous $MgSO_4$ and concentrated to approximately 100 μl with nitrogen stream. The entire sample was subjected to GC-MS analysis as previously described [12].

Expression analysis

Total RNA was extracted from *Z. zerumbet* as previously described [8]. Possible traces of DNA were removed using Ambion's Turbo DNA-free™ kit. Then, 1 μg of RNA was reverse transcribed into cDNA using Superscript III first-strand synthesis Supermix for qRT-PCR (Invitrogen). For RT-PCR analysis of gene expression in different tissues, two primer pairs 5′-CGGAACTACTCCTATTTGC-3′ (forward), 5′-TAGCTTCGTCA-AGTATCATT-3′ (reverse) for *ZSS1* and 5′-TGATAAAGAGATCATCAAGG-3′ (forward) 5′-GATTAATGACAGGTGTTTGAGCA-3′ (reverse) for *CYP71BA1* were used to amplify 415-bp and 553-bp fragments, respectively. Quantitative PCR was performed using a Thermal Cycler Dice Real Time System and a SYBR Premix Ex Taq™ II kit (Takara) according to the manufacturer's instructions. The quantitative PCR reaction consisted of 10 μl of master mix (Takara), 2 μl of primers (0.4 μM), and 1 μl of cDNA in a final volume of 20 μl. The specific primer pairs used for amplification of *ZSS1* and *CYP71BA1* transcripts were 5′-GCAAGTGATGGTGG AGCAAAA-3′, 5′-CCGAGCTACTTGTGTTGGACGT-3′ and 5′-AGCGTGCATAAGCAACAAGTAC-3′, 5′-TGAG TTCGGGCGAGTTGGAG-3′, respectively. The amplification of the endogenous reference gene (ubiquitin) was carried out using the following primers: 5′-AAGGAG TGCCCCAACGCCGAGTG-3′ and 5′-GCCTTCTGGT TGTAGACGTAG-GTGAG-3′. Each sample was analyzed in triplicate, and the results represent normalized mean values and SD.

Plasmid construction

A plasmid for in vivo production of 8-hydroxy-α-humulene was constructed as described elsewhere (Harada et al. unpublished observation). In brief, the two genes encoding HMG-CoA reductase and HMG-CoA synthase were removed from the plasmid pAC-Mev, which contains the genes encoding six enzymes of the mevalonate pathway [15], generating pAC-Mv. This modification was performed by digesting pAC-Mev with *Sac*I and *Eco*RV and ligating into a DNA fragment of the IPP isomerase gene. Then, a DNA fragment containing multiple cloning sites prepared by annealing two complementary single-stranded oligonucleotides was digested with *Eco*RV and *Hind*III and inserted into the corresponding site of pAC-Mv. The *ATR2* gene encoding an *Arabidopsis* P450 reductase was subsequently ligated into the *Eco*RV and *Mfe*I sites of pAC-Mv, resulting in pAC-ATR2. Finally, the *ZSS1* gene amplified from pUC-*ZSS1* [19] was digested with *Eco*RI and *Xba*I and ligated into the *Mfe*I and *Spe*I sites of pAC-ATR2, thereby generating pAC-MvATR2Hum. To generate plasmid pET-ZzP450, the full-length open reading frame (ORF) of *CYP71BA1* was amplified by PCR using primers 5′-CACCATGGAAGCTATTTCCCTCTTC-3′ and 5′-CTA TGGCAGAGGAAT-TATTAGCTTG-3′. The amplified product was directly cloned into the pET101/DTOPO expression vector (Invitrogen).

In vivo production of 8-hydroxy-α-humulene

E. coli BL21(DE3) was co-transformed with pET-ZzP450 and pAC-MvATR2Hum. The metabolically modified *E. coli* was grown in 3 ml of LB broth containing ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) at 30°C for 16 h with shaking. Then, 50 μl of the medium was diluted into 5 ml of Terrific broth containing 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 0.5 mg/ml D-mevalonolactone, 80 μg/ml 5-aminolevulinic acid, and 0.1 mM $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, followed by incubation at 30°C until OD_{600} reached 0.8. Cultures were then induced by addition of isopropyl-*β*-D-thio-galactopyranoside (IPTG) to a final concentration of 0.1 mM and incubated for an additional 72 h at 18°C with shaking. After incubation, 1 ml of saturated NaCl and 2 ml of ethyl acetate were added to the cultures and the ethyl acetate phase was extracted, concentrated and then subjected to GC-MS analysis.

Results

Isolation of the cDNA *CYP71BA1*

Since our previous study showed that *ZSS1* was predominantly expressed in rhizomes, we began to clone the P450 genes using total RNA extracted from rhizomes. A pair of degenerate primers designed from two highly conserved domains, including the heme-binding motif of known plant P450 enzymes was used to isolate partial cDNA fragments

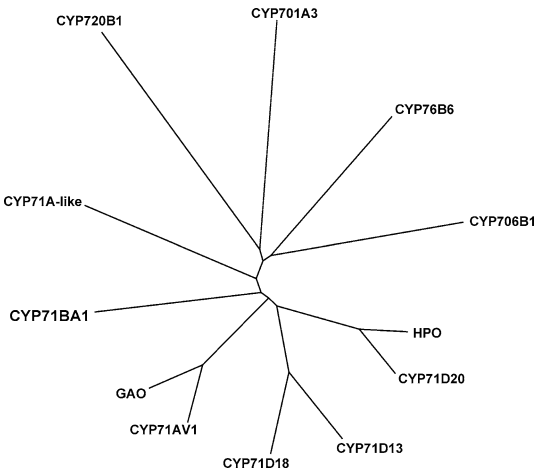

Fig. 2 Phylogenetic tree of *Z. zerumbet* CYP71BA1 and related functionally characterized terpene-modifying P450s. CYP706B1, *G. arboreum* (+)-δ-cadinene-8-hydroxylase (AF332974); CYP76B6, *C. roseus* geraniol 10-hydroxylase (AJ251269); CYP71A-like, *Mentha x piperita* (+)-menthofuran synthase (AF346833); CYP71AV1, *A. annua* amorpha-4,11-diene monooxygenase (DQ315671); HPO, *H. muticus* premnaspirodiene oxygenase (EF569601); CYP71D20, *N. tabacum* 5-epiaristolochene-1,3-dihydroxylase (AF368376); CYP71D13, *Mentha x piperita* (−)-limonene-3-hydroxylase (AY281027); CYP71D18, *Mentha spicata* (−)-limonene-6-hydroxylase (AF124815); GAO, *B. spinosa* germacrene A oxidase (GU256647); CYP720B1, *Pinus taeda* abietadienol/abietadienal oxidase (AY779537); CYP701A3, *A. thaliana* ent-kaurene oxidase (AF047719)

using RT-PCR. The resulting fragments with the expected length of approximately 250 bp were cloned. After random sequencing, these cDNA fragments were shown to represent a family of related cytochrome P450 genes. Specific primer pairs were subsequently designed based on the sequence information of the partial fragments to isolate the full-length cDNAs. A combination of 5′ and 3′ RACE-PCR yielded five complete cDNA clones, one of which contains a reading frame encoding a protein of 513 amino acid residues with a calculated molecular mass of 57.8 kDa and p*I* of 8.24. A BLAST search demonstrated that the deduced amino acid sequences of this P450 have several characteristic motifs of eukaryotic P450s, including the N-terminal membrane-anchoring domain [20], the oxygen-binding domain [21], and the highly conserved heme-binding motif [22]. This P450 was placed into the CYP71 family (Fig. 2) and was designated *CYP71BA1* according to the Nelson nomenclature based on the sequence identity with other family members (DDBJ Accession Number: AB331234). *CYP71BA1* was found to be most similar to three sesquiterpene oxygenases, premnaspirodiene oxygenase from *Hyoscyamus muticus* [23], amorpha-4, 11-diene hydroxylase from *Artemisia annua* [16] and germacrene A oxidase from *Barnadesia spinosa* [24] (41–43% identity).

Recombinant CYP71BA1 catalyzed in vitro production of 8-hydroxy-α-humulene

For functional identification of *CYP71BA1,* microsomal proteins prepared from yeast cells expressing *CYP71BA1* were incubated with α-humulene in the presence of NADPH. As shown in Fig. 3, GC–MS analysis revealed a single product peak that was identified as 8-hydroxy-α-humulene by comparison of the retention time as well as the mass spectrum with those of an authentic standard, whereas, no product was detected when a microsomal preparation from the control yeast carrying the empty vector was used. Moreover, no enzyme activity was observed in the absence of NADPH in the reaction mixture (data not shown). Thus, we concluded that *CYP71BA1* encodes an enzyme displaying α-humulene-8-hydroxylase activity, although the yields of 8-hydroxy-α-humulene in yeast were not so high, which is probably due to the poor solubility of α-humulene in the aqueous reaction solution.

Transcripts of *CYP71BA1* showed a similar expression pattern to that of *ZSS1*

To examine the expression levels of *CYP71BA1* transcripts in different tissues, RT-PCR analysis was performed with RNA isolated from leaves, stems and rhizomes. As shown in Fig. 4, CYP71BA1 is expressed most abundantly in rhizomes and much less in leaves, but not in stems. This expression pattern is identical to that of ZSS1 and is consistent with the predominant accumulation of zerumbone in rhizomes (Fig. 4).

Since the rhizome of *Z. zerumbet* grows fast from June until December, we also investigated the transcript accumulation of *ZSS1* and *CYP71BA1* in *Z. zerumbet* during rhizome development using quantitative RT-PCR (2007) (Fig. 4b, c). The results showed that transcripts of both genes increased very rapidly from June 12 to June 28, a period of strong temperature increase in 2007. Transcript levels remained high throughout the summer and autumn, but declined significantly in December.

Introduction of the mevalonate pathway genes and *Z. zerumbet ZSS1* and *CYP71BA1* into *E. coli* leads to the production of 8-hydroxy-α-humulene

Our previous studies have shown that introducing mevalonate pathway genes into *E. coli* and coexpressing it with *ZSS1* efficiently increased α-humulene production [12]. To assess the potential for metabolic engineering of zerumbone production in *E. coli*, we constructed the plasmid pAC-MvATR2Hum that contains a gene cluster responsible for the mevalonate pathway, *Z. zerumbet ZSS1* encoding α-humulene synthase and an *Arabidopsis* P450 reductase 2

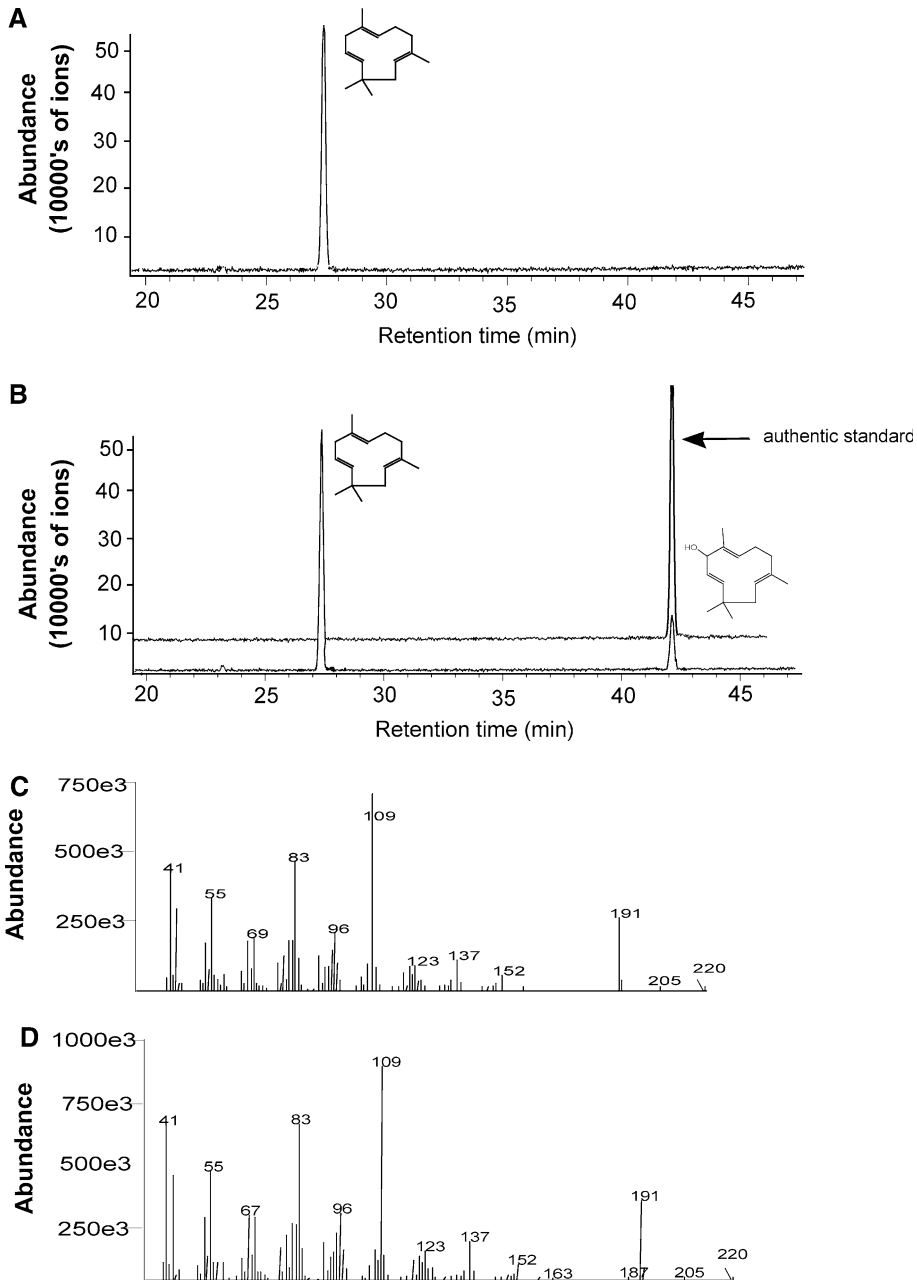

Fig. 3 GC-MS analysis of products formed in in vitro enzyme assays. **a** Chromatogram of products obtained from assay with yeast microsomes harboring empty expression vector (pYeDP60). **b** Chromatogram of products generated by incubation of α-humulene with yeast microsomes harboring CYP71BA1 and a chromatogram of authentic 8-hydroxy-α-humulene. **c** Mass spectrum of product formed by incubation of α-humulene with yeast microsomes expressing CYP71BA1. **d** Mass spectrum of authentic 8-hydroxy-α-humulene

gene (*ATR2*). The gene cluster encodes four enzymes, mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (MPPD), and isopentenyl diphosphate isomerase (IPPI), that convert mevalonate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Fig. 5). *E. coli* BL21(DE3) carrying pAC-MvATR2Hum was co-transformed with pET-*CYP71BA1* and cultured in broth medium with a supplement of mevalonate. GC-MS analysis of the ethyl acetate extract revealed the in vivo production of 8-hydroxy-α-humulene (Fig. 6), confirming that *CYP71BA1* encodes α-humulene-8-hydroxylase and was functionally expressed in the metabolically engineered *E. coli*. Although the yields are not so high (2.972 µg/ml culture medium), it provides the possibility of producing 8-hydroxy-α-humulene in *E. coli* by metabolic engineering.

Discussion

P450s are very abundant in plant genomes and are known to have a wide spectrum of sequence similarity, which makes the identification of the specific gene is rather challenging. Since we have recently demonstrated that the α-humulene synthase gene (*ZSS1*) is almost exclusively expressed in rhizomes of *Z. zerumbet* [12], we assumed that

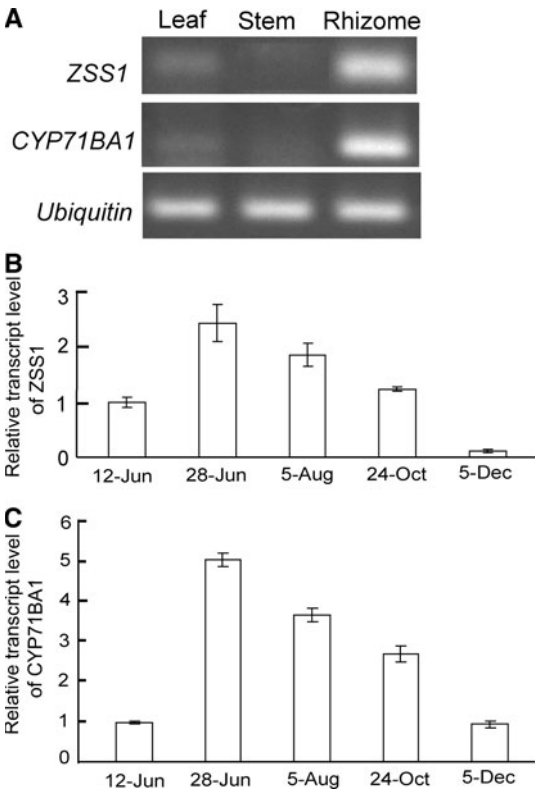

Fig. 4 Transcript expression analysis of *CYP71BA1* and *ZSS1*. **a** RT-PCR analysis of ZSS1 and CYP71BA1 transcripts in leaves, stems and rhizomes. **b** Temporal expression of *ZSS1* and *CYP71BA1* during rhizome development (2007)

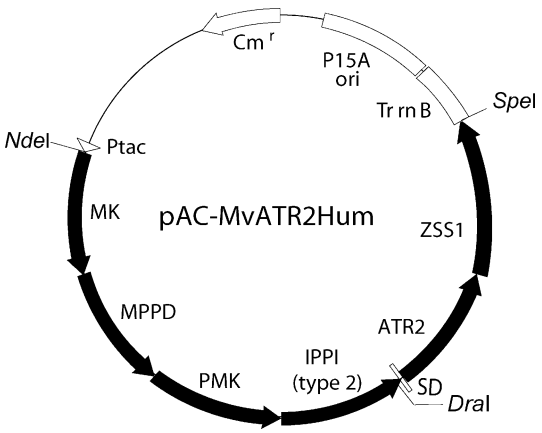

Fig. 5 Schematic structure of plasmid pAC-MvATR2Hum. *MK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MPPD* mevalonate diphosphate decarboxylase, *IPPI* isopentenyl diphosphate isomerase, *ATR2 Arabidopsis* P450 reductase 2 gene, *SD* Shine-Dalgarno sequence

genes encoding other enzymes of the zerumbone biosynthetic pathway are also actively transcribed in rhizomes. By using a homology-based approach, we have successfully cloned a new cytochrome P450 cDNA, *CYP71BA1* encoding α-humulene-8-hydroxylase, which supports our hypothesis that hydroxylation of α-humulene at C_8 is a critical step in the formation of zerumbone. In fact, we have also identified a short-chain dehydrogenase/reductase responsible for the last step of zerumbone biosynthesis (data not shown). With a high similarity of its amino acid sequence to other plant cytochrome P450s, CYP71BA1 represents a new member of the CYP71 family. Phylogenetic analysis demonstrated its close relationship to several sesquiterpene-modifying P450s including *A. annua* amorpha-4, 11-diene hydroxylase [16], *H. muticus* premnaspirodiene oxygenase, *B. spinosa* germacrene A oxidase [24] and *Nicotiana tabacum* 5-epi-aristolochene-1, 3-dihydroxylase [14]. (>39% identity), whereas the evolutionary distance from other similar terpene hydroxylases, such as *Gossypium arboreum* (+)-δ-cadinene-8-hydroxylase (27% identity) and *Catharanthus roseus* geraniol 10-hydroxylase (30% identity) is unusually large.

The pharmacological significance of zerumbone and the importance of CYP71BA1 in zerumbone biosynthesis prompt us to further examine the de novo 8-hydroxy-α-humulene production in metabolically engineered microbes. Our previous study showed that engineering a foreign partial exogenous mevalonate pathway in *E. coli* to supplement endogenous IPP supply, significantly enhanced α-humulene production [12]. We therefore introduced a mevalonate lower pathway into *E. coli* and coexpressed it with ZSS1, CYP71BA1, and a cytochrome P450 reductase redox partner (CPR). The engineered *E. coli* produced a measurable amount of 8-hydroxy-α-humulene when mevalonate was supplied as the substrate, suggesting the potential for production of zerumbone intermediate from simple carbon sources by metabolic engineering. However, the relatively low levels of 8-hydroxy-α-humulene compared to α-humulene indicates that a substantial bottleneck occurred during the transformation from α-humulene to 8-hydroxy-α-humulene, which was possibly due in large part to the poor capability of the bacterial host to efficiently transfer the hydrophobic α-humulene from the cytosol to the membranous hydroxylation sites. The recent success of high-level production of 8-hydroxy-cadinene and artemisinic acid in metabolically engineered *E. coli* using plant-derived CYP706B1 and CYP71AV1 by Keasling et al. [25] implies that optimization of the expression system, including modification of the N-terminal transmembrane domain, replacement of *Arabidopsis* CPR with the CPR isolated from native plant *Z. zerumbet*, and substitution of the expression vector or host, may allow us to produce the high titers of 8-hydroxy-α-humulene.

In different tissues and growing seasons, the expression pattern of CYP71BA1 is similar to that of ZSS1, implying that these two enzymes may be regulated in a coordinated manner. Coordinated regulation of P450 enzymes and their upstream terpene synthases has also been found in cottons

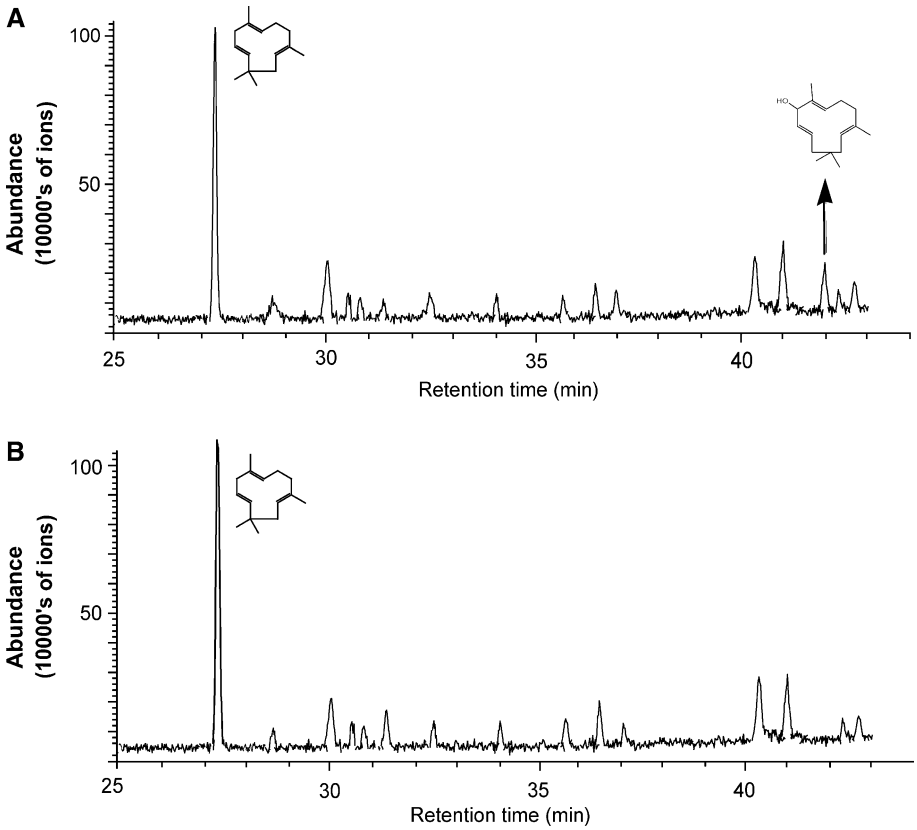

Fig. 6 Identification of in vivo 8-hydroxy-α-humulene production in metabolically engineered *E. coli*. **a** Chromatogram of the products obtained from the control *E. coli* containing pAC-MvATR2Hum and the empty vector pET101. **b** Chromatogram of the products formed from engineered *E. coli* harboring pAC-MvATR2Hum and pET-CYP71BA

and loblolly pines [15, 26]. The variations of *ZSS1* and *CYP71BA1* transcript accumulation over the growing season raises the question of which factors activate the expression of these two genes during rhizome development. The relatively high-level transcripts in summer suggest that gene expression may be temperature- or light-dependent. However, to clarify the expression regulation of these two genes, laboratory studies under controlled conditions are needed.

It is worth mentioning that besides zerumbone, the rhizome oil of *Z. zerumbet* contains two more α-humulene derivatives, α-humulene epoxide I and α-humulene epoxide II [27], demonstrating the presence of the side-routes that divert pathway flux away from zerumbone biosynthesis. Moreover, besides their importance as proposed intermediates in the biosynthesis of zerumbone, α-humulene and 8-hydroxy-α-humulene may also play some physiological roles in *Z. zerumbet*. Accordingly, elucidation of the entire picture of the zerumbone biosynthetic pathway and the competing side routes, together with a comprehensive understanding of the regulatory mechanism of the corresponding key enzymes, will allow us to manipulate zerumbone production in plants by genetic engineering.

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