Eur. J. Biochem. **268,** 5633–5638 (2001) © FEBS 2001

Isolation and functional analysis of a cDNA encoding a myrcene synthase from holm oak (*Quercus ilex* L.)

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An 859-bp cDNA segment of a terpene synthase gene was amplified by PCR from the evergreen sclerophyllous holm oak (*Quercus ilex* L.) using heterologous primers for conserved regions of terpene synthase genes (*TPS*) in dicotyledonous plants. Based on the sequence of this segment, homologous primers were designed for amplification by RACE-PCR of a cDNA segment carrying the monoterpene synthase gene *myrS*. The gene encodes a protein of 597 amino acids including an N-terminal putative plastid transit peptide. The gene without the segment encoding the transit peptide was cloned by PCR into a bacterial expression vector. Expression in *Escherichia coli* yielded an active monoterpene synthase, which converted geranyl diphosphate (GDP) predominantly into the acyclic monoterpene myrcene and to a very small extent into cyclic monoterpenes. Sequence comparison with previously cloned monoterpene synthases revealed that the myrcene synthase from *Q. ilex* belongs to the *TPSb* subfamily.

Keywords: functional expression; geranyl diphosphate; monoterpene synthases; myrcene synthase; *Quercus ilex*.

Leaves of the evergreen sclerophyllous holm oak (*Quercus ilex* L.) emit large amounts of monoterpenes despite the absence of storage tissue [1–3]. This monoterpene emission is largely associated with photosynthesis, as it is stimulated by light, declines in the dark, and is inhibited in the absence of CO_2 [4]. The physiological function of this emission is still a matter of debate. An actual hypothesis assumes that monoterpenes protect leaves against high-temperature damage [5] by enhancing membrane stability under heat stress. Recently it was shown that the monoterpene emission correlates with the monoterpene synthase activity present in the leaves [6]. Apart from this knowledge of the physiological control of monoterpene emission from *Q. ilex* leaves, information is currently lacking on the monoterpene synthase genes, the number of synthases and their biochemical properties, and the regulation of these genes. Terpene synthases (TPS) participating in secondary terpenoid metabolism have been cloned as cDNAs from a number of species [7], including gymnosperms, members of the Lamiaceae, Solanaceae and the Brassicaceae *Arabidopsis thaliana* L [8]. The *TPS* gene family includes monoterpene (C_{10}), sesquiterpene (C_{15}), and diterpene (C_{20}) synthases. In addition, very recently an isoprene (C_5) synthase gene was isolated from *Populus alba* x *P. tremula* [9] showing strong sequence homology to the *TPS* gene family. According to Bohlmann *et al*. [7], the *TPS* gene family of higher plants can be divided into six subfamilies. However, sequence relatedness is not sufficient to predict the catalytic properties of different gene products [8]. Recombinant expression of related genes and the functional characterization of the active enzymes is necessary to identify new members of the *TPS* gene family. This paper is the first report on a monoterpene synthase gene from the evergreen tree *Q. ilex*. It describes the isolation of a monoterpene synthase gene (*myrS*), the cloning and expression in *Escherichia coli* of the *myrS* cDNA, and the functional characterization of the active enzyme as myrcene synthase.

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Abbreviations: DMADP, dimethylallyl diphosphate; GDP, geranyl diphosphate; *TPS*, terpene synthase genes.

Note: a web page is available at http://www.ifu.fhg.de/

(Received 5 June 2001, revised 4 September 2001, accepted 7 September 2001)

MATERIALS AND METHODS

Chemicals and enzymes

Antibiotics were supplied by Roche Molecular Biochemicals (Mannheim, Germany). All restriction enzymes, ligase and PCR *Taq* polymerase were obtained from Gibco BRL (Berlin, Germany). Buffer reagents and other chemicals were supplied by Merck (Merck Eurolab, Darmstadt, Germany) and were of analytical grade. Geranyl diphosphate (GDP) and dimethylallyl diphosphate (DMADP) were synthesized by the method of Keller & Thompson [10].

Bacterial strains, plasmids and plant material

The derivatives of pCR2.1 (Invitrogen, BV, Groningen, the Netherlands), pCRII-TOPO (Invitrogen BV) and pQE30 (Qiagen, Hilden, Germany) were amplified in *E. coli* TG1 [11], INVαF′ (Invitrogen BV), TOP10F′ (Invitrogen BV). Two-year-old saplings of *Q. ilex* L. were obtained in December 1997 from the nursery Coccetti, Lisanza di Sesto Calende, Italy. The saplings were planted in plastic pipes (55 cm in length and 15 cm in diameter) containing commercially available garden soil, and further cultivated in a greenhouse during the following vegetation periods. Eight-week-old leaves were tested for monoterpene synthase activity [12] in spring 1999 before isolation of total mRNA.

Isolation of total RNA and cDNA synthesis

Total RNA was prepared from 8-week-old leaves of *Q. ilex* as described by Kiefer *et al*.[13]. The fraction of mRNA was isolated from total RNA by a batch purification procedure using the Oligotex mRNA mini kit (Qiagen). Single-stranded cDNA was synthesized using Superscript II Reverse Transcriptase (Life Technologies, Karlsruhe, Germany).

DNA sequencing

Cycle sequencing dideoxy chain-termination reactions with Big Dye Terminators (PE Applied Biosystems, Weiterstadt, Germany) were performed for both cDNA strands of all DNA segments investigated, using universal forward and backward primers (Gibco BRL) or sequence-specific oligonucleotides. The sequences were analysed by using an ABI PRISM-System 310 (PE Applied Biosystems).

Amplification of a terpene synthase fragment of *Q. ilex*

Oligonucleotides [Roth, Karlsruhe, Germany; *TPS*-for, 5′-TTCCGI(GC)TICTI(AC)GI(GC)A(AG)CA(CT)GG-3′; *TPS*-rev, 5′-GTGCCATAGACATC(AG)TA(AGT)AT(AG)TC-3′] were designed for conserved regions of known monoterpene synthase genes from other dicotyledonous plants and used for PCR with template single-stranded cDNA from *Q. ilex*. PCR (thermocycler: Personal cycler, Biometra, Göttingen, Germany) was performed in a volume of 50 μL containing 1 U *Taq* polymerase (Gibco–BRL), 1 × PCR buffer (Gibco–BRL), 2.0 mM $MgCl_2$, dNTPs each 0.2 mM, oligonucleotide each 1 μM, and 10 ng template cDNA (PCR conditions: 1 min at 94 °C; 30 s touch-down from 52 °C to 44 °C in 1 °C steps followed by 30 cycles at 52 °C; 1 min at 72 °C). An amplicon was ligated into pCR2.1 (Invitrogen BV) and transformed into *E. coli* TG1. Plasmid DNA was prepared from single transformants and sequenced. An 859-bp insert sequence was identified and used for the design of homogeneous oligonucleotides used in RACE-PCR.

RACE-PCR and amplification of full-length cDNA

Double-stranded cDNA synthesis and ligation of specific cDNA adaptors were performed with the Marathon cDNA Amplification kit (Clontech, Heidelberg, Germany). 5′ cDNA ends were amplified by PCR using the homologous primer Qi-5′ (5′-AAGTGTGGCTTGAACTATGTTGAAATC-3′) in combination with the adaptor-specific primer. For amplification of 3′ cDNA ends, the primer Qi-3′ (5′-TCCTTGGAGCTCCCACTGCATTGGAGG-3′) was combined with the respective adaptor primer. PCRs were performed in volumes of 50 μL containing 1 μL Advantage-2-Polymerase mix (Clontech), 5 μL 10 × PCR buffer (Clontech), 3.5 mM $MgCl_2$, dNTPs each 0.2 mM, oligonucleotide primer each 0.2 μM, and 50 ng double-stranded cDNA. 5′-RACE-PCR conditions were: 30 s at 94 °C; 30 s touch-down from 68 °C to 47 °C in 1 °C steps followed by 30 cycles at 52 °C; 4 min at 72 °C. 3′-RACE-PCR conditions were: 30 s at 94 °C; 30 s touch-down from 52 °C to 37 °C in 1 °C steps followed by 30 cycles at 50 °C; 3 min at 72 °C. The resulting PCR products were verified on agarose gels, ligated into pCR2.1 (Invitrogen BV), transformed into *E. coli* TG1 (5′-RACE) and *E. coli* INVαF′ (3′-RACE), and sequenced. Based on the sequence information primers for the 5′ end (Qi-5′end, 5′-ACAAAAACCATAGTAAGCAACTAAGC-3′) and 3′ end (Qi-3′end, 5′-AGTAGAATTGTTAAGTTAAAATTTTCATATATGC-3′) were designed and used for the amplification of full-length cDNA. PCRs were performed in a volume of 50 μL containing 1 μL Advantage-2-Polymerase mix (Clontech), 5 μL 10 × PCR buffer (Clontech), 3.5 mM $MgCl_2$, dNTPs each 0.2 mM, oligonucleotide primer each 0.2 μM, and 50 ng double-stranded cDNA, employing a constant temperature program (30 s 94 °C, 30 s 48 °C, and 4 min 72 °C) with 30 cycles. The amplicon (*myrS*) was ligated into pCR2.1 (Invitrogen BV), transformed into *E. coli* TG1, and sequenced. The EMBL accession number of the complete *myrS* cDNA containing an ORF of 1874 bp is AJ304839.

Construction of an expression fusion

Computational analysis showed that the peptide sequence of MyrS preceding the pair of arginines (A47, A48) was similar to that of plastid transit peptides of other monoterpene synthases (Fig. 1). Based on this information, restriction sites of *Bam*HI and *Pst*I for subcloning *myrS* without this putative plastid transit peptide were introduced by PCR. Fragments were amplified by PCR using the primers *Bam*HI-for (5′-CCCGGATCCTTGCGGAGATCAGCAAATTACC-3′) and *Pst*I-rev (5′-GCACCTGCAGATATAAATAATTAATATAATGTC-3′). PCR was performed in a volume of 50 μL containing 1 U *Taq* polymerase (Gibco–BRL), 1 × PCR buffer (Gibco–BRL), 2.0 mM $MgCl_2$, dNTPs each 0.2 mM, oligonucleotide 1 μM each, and 10 ng template cDNA (PCR program: 30 s at 92 °C, 30 s at 50 °C, and 3 min at 72 °C) with 36 cycles. The amplicon was ligated into pCRII-TOPO (Invitrogen BV) and transformed

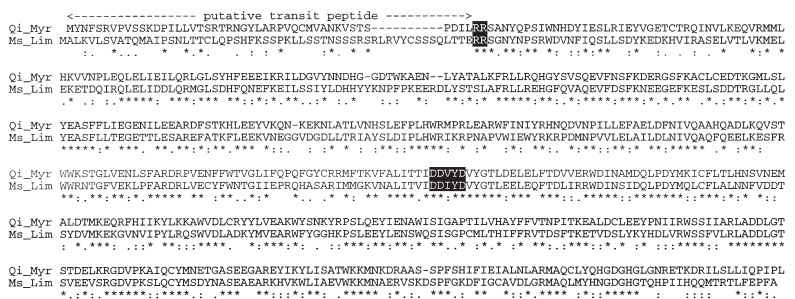

Fig. 1. Sequence alignment of the deduced amino-acid sequence of the isolated terpene synthase gene from *Q. ilex* (Qi_ Myr) and the limonene synthase Ms_Lim (SwissProt Database, accession no. Q40322) from *M. spicata*. The alignment was calculated by the program CLUSTALW 1.8. The conserved Mg^{2+}-binding motif DDXXD and the two conserved arginine residues after the putative transit peptide are marked by inversion (asterisks mark similar amino acids in both sequences; dots mark allowed amino acid exchanges without changing biochemical properties of the proteins).

into *E. coli* INVαF′. After verification of the sequence, the *Bam*HI–*Pst*I fragment of the gene was subcloned into pQE30 resulting in a vector habouring the gene 5′ fused to the His-encoding region downstream of an inducible *lac* promoter.

Purification of a myrcene synthase from transformed *E. coli*

Purification of the His_6-tagged protein was performed according to the QIAexpress Type IV protocol (Qiagen), except that the *E. coli* cells were disrupted by a French pressure cell press (SLM Instruments, Inc., Urbana, IL, USA) at 140 MPa and 0–4 °C as described [14]. After affinity chromatography, 2.5 mL of eluate was desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) with monoterpene synthase assay buffer [12], resulting in a final volume of 3.5 mL. Protein concentration was determined by the method of Bradford [15] with BSA as a standard. All fractions were analysed by SDS/PAGE on a precast 10% Tris/glycine gel (Novex, Frankfurt, Germany) at 120 V for 2 h. The gels were fixed for 1 h (8.5% phosphoric acid in 20% methanol), stained with a colloidal Coomassie-staining solution (Roti blue; Roth), and washed with 25% methanol.

Assay of monoterpene synthase

The purified enzyme preparations were assayed for monoterpene synthase activity as described previously [12]. Compounds were identified by comparison of retention times and co-chromatography with authentic standards. To test for the formation of isoprene from the appropriate C_5 substrate DMADP, enzyme assays were performed as previously described for isoprene synthase [16]. Heat-denatured extracts served as controls, yielding no detectable isoprene or monoterpene product.

RESULTS AND DISCUSSION

Amplification of a putative terpene synthase cDNA segment from *Q. ilex*

As a first step towards the identification of terpene synthase genes from *Q. ilex*, heterologous oligonucleotide primers (*TPS*-for and *TPS*-rev 2.7) for conserved sequence motifs of previously cloned monoterpene synthases from the *TPS* gene subfamily *TPSb* were designed. When this study started, genes of the *TPSb* group had been described only from members of the Lamiaceae [7]. At the time of RNA isolation, the leaves contained monoterpene synthase activities of 7.24 ± 0.89 μkat·(kg protein)$^{-1}$ for α-pinene, 8.55 ± 1.03 μkat·(kg protein)$^{-1}$ for sabinene, 5.02 ± 0.65 μkat·(kg protein)$^{-1}$ for β-pinene, 1.30 ± 0.14 μkat·(kg protein)$^{-1}$ for myrcene, and 2.28 ± 0.39 μkat·(kg protein)$^{-1}$ for limonene (for GC analysis of the products see Fig. 4A). Using cDNA derived from this RNA preparation as template for PCRs in combination with the oligonucleotides *TPS*-for and *TPS*-rev, an 859-bp DNA segment was amplified. Sequence alignment of this putative terpene synthase cDNA segment with previously cloned monoterpene synthase genes revealed strong similarities to 1,8-cineole synthase from *Salvia officinalis* L. (O81191; 58%; overlapping region 387 bp), linalool synthase from *Artemisia annua* L. (Q9SSPN1; 57%; overlapping region 327 bp), and (–)-4*S*-limonene synthase from *Mentha spicata* L. (Q40322; 63%; overlapping region 192 bp).

Isolation of a full-length terpene synthase cDNA by RACE-PCR

Based on the sequence of the putative *Q. ilex* terpene synthase cDNA segment, new primers were designed directed upstream of the 5′ end (primer Qi-5′) and downstream of the 3′ end (primer Qi-3′) of the coding segment. Using either the

Table 1. Distance matrix of the myrcene synthase of *Q. ilex* and other monoterpene, isoprene and sesquiterpene synthases of dicotyledons. The distance matrix was calculated by the Phylib program PROTDIST version 3.5c using the Dayhoff PAM matrix. The deduced amino-acid sequences are from: Qi_ Myr myrcene synthase of *Q. ilex* (this work), At_Lim limonene synthase of *Arabidopsis thaliana* L. (accession no. AB028607), Pf_Lim limonene synthase of *Perilla fruticans* L. (accession no. 004806), Ms_Lim limonene synthase of *Mentha spicata* L. (accession no. L13459), So_Cin cineol synthase of *Salvia officinalis* L. (accession no. O81191), At_MyO myrene/ocimene synthase of *Arabidopsis thaliana* L. (accession no. AAG09310), Pa_Iso isoprene synthase of *Populus alba x tremula* (accession no. Q9AR86), Ga_DeC delta-cadinene synthase of *Gyssopium arboreum* L. (accession no. Q39760), Nt_EpA epi-aristolochene synthase of *Nicotiana tabaccum* L. (accession No. Q40577), St_Vet vetispiratene synthase of *Solanum tuberosum* L. (accession no. Q9XJ32), Mp_Far farnesene synthase of *Mentha x piperita* (accession no. O48935).

	Qi_ Myr	At_Lim	Pf_Lim	Ms_Lim	So_Cin	So_Sab	At_MyO	Pa_Iso	Ga_DeC	Nt_EpA	St_Vet	Mp_Far
Qi_ Myr	0											
At_Lim	0.7627	0										
Pf_Lim	0.7876	0.9946	0									
Ms_Lim	0.7903	1.0278	0.3866	0								
So_Cin	0.7931	0.9216	0.6063	0.6178	0							
So_Sab	0.8084	1.0107	0.6671	0.7042	0.6745	0						
At_MyO	0.8188	0.4201	0.9717	0.9952	0.9742	1.0636	0					
Pa_Iso	0.8569	1.0713	1.0305	1.0148	1.0771	1.1116	1.1382	0				
Ga_DeC	1.1868	1.4685	1.6094	1.4990	1.4310	1.5442	1.5033	1.4840	0			
Nt_EpA	1.2165	1.5222	1.5721	1.5152	1.5142	1.5446	1.4881	1.4070	0.8172	0		
St_Vet	1.2227	1.4939	1.5809	1.5052	1.4811	1.5837	1.5044	1.4297	0.8258	0.2397	0	
Mp_Far	1.5451	1.7438	1.9778	1.8516	1.8196	1.9223	1.8208	1.6639	1.2375	1.0473	1.1168	0

RACE-adaptor-specific primer in combination with the Qi-5′ primer or in combination with the Qi-3′ primer, a complete 5′ segment (867 bp) and 3′ segment (1282 bp) of a putative terpene synthase gene could be amplified by PCR with RACE-adaptor-ligated cDNA of *Q. ilex*. Subsequently a new primer pair was designed specifically for the 5′ end (Qi-5′end) and 3′ end (Qi-3′ end) of the cDNA that allowed amplification of the complete terpene synthase gene from the original full-length cDNA.

Sequencing of the isolated cDNA and comparison with other terpene synthases

Sequencing of both strands of the cloned full-length cDNA resulted in a 1874-bp segment with one ORF of 1794 bp with an ATG start codon at position 33 and a TGA stop codon at position 1824. Comparison of the deduced 597-residue sequence with the Swiss protein database revealed greatest similarity to terpene synthases. Highest identity (48%, Fig. 1) of the polypeptide was obtained with the 4*S*-limonene synthase precursor from *M. spicata* [17]. As in other monoterpene synthases, two typical arginine residues (A47, A48, Fig. 1) are present in the *Q. ilex* sequence preceded by an N-terminal presumptive transit peptide, indicating that the protein is transported into plastids [18]. The putative Mg^{2+}-binding motif DDXXD [7] typical of monoterpene synthases is well conserved in the deduced amino-acid sequence of *Q. ilex* (Fig. 1). In a distance matrix (Table 1), the polypeptide sequence from *Q. ilex* (*myrS*) was mostly related to limonene synthases and other terpene synthases from other dicotyledons, whereas the distance to sesquiterpene synthases was higher. A similar degree of relationship was found with a phylogenetic tree calculated for C_5–C_{15} terpene synthases of dicotyledons (Fig. 2). In this tree, the deduced polypeptide sequence of *Q. ilex* grouped together with monoterpene synthases, in which the isoprene synthase from poplar [9] is also located. This group of terpene synthases has been previously designated as the terpene synthase group *TPSb* [7]. These similarities suggest that the DNA sequence of *Q. ilex* encodes a monoterpene synthase rather than a sesquiterpene synthase. However, as stated by other authors [8], the catalytic function of a terpene synthase gene product cannot be predicted on the basis of the degree of relationship in a distance matrix or the location in the phylogenetic tree.

Functional analysis of the purified terpene synthase

As the peptide sequence of the isolated gene preceding the pair of arginines (A47, A48) was similar to those of plastid transit peptides of other monoterpene synthases and as the truncation of monoterpene synthases upstream of the two arginines resulted in ‘pseudo-mature’ fully active enzymes [19,20], the *myrS* sequence was cloned without this transit peptide-coding part into an expression vector. The ‘pseudo-mature’ enzyme MyrS was purified under native conditions from *E. coli* containing pQE30-*myrS*. A total of 3.3 mg protein was obtained from 1.74 g wet weight. The band of the overexpressed MyrS was already visible in the cleared cell lysate (Fig. 3, lane CL). Analysis of the eluate after two washing steps on SDS/PAGE detected three bands. One of the more intense bands was the expected size of MyrS (64.3 kDa) deduced from the amino-acid sequence (Fig. 3).

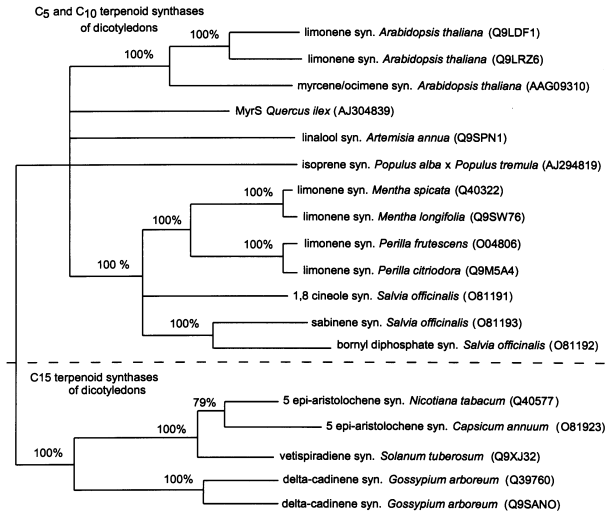

Fig. 2. Phylogenetic tree of isoprene, monoterpene and sesquiterpene synthases of dicotyledons. The tree was calculated on the basis of a CLUSTALW 1.8 alignment by the program PAUP 3.0 using parsimony, a bootstrap 70% majority rule, and mid point rooting. The percentages in the tree are the bootstrap values for the branches. The EMBL or SwissProt Database accession numbers of the corresponding genes or the deduced amino-acid sequences are given in parentheses.

Extracts of induced *E. coli* TG1(pQE30-*myrS*) before and after purification on Ni/nitrilotriacetate/agarose (Fig. 3) were assayed for monoterpene synthase and isoprene synthase activity using the corresponding substrates GDP and DMADP under optimal conditions described for *Q. ilex* leaf extracts [12] and *Quercus robur* [16], respectively. Enzymatic production of monoterpenes was observed only using GDP (Fig. 4 and Table 2). With DMADP at levels up

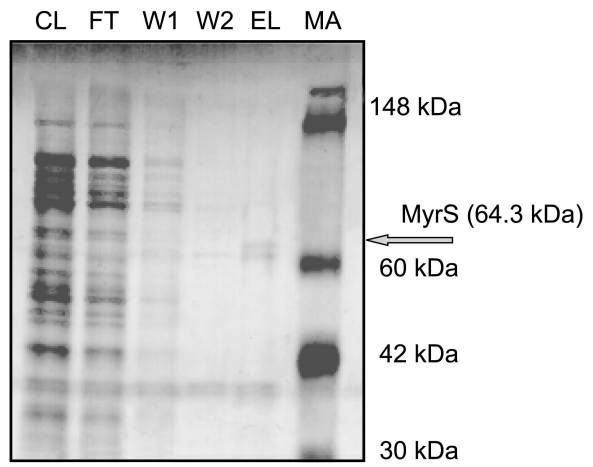

Fig. 3. Purification of His_6-tagged MyrS analyzed by SDS/PAGE (10% polyacrylamide gel, Coomassie staining). Lanes: CL, cleared cell lysate; FT, flow-through of the lysate from a Ni/nitrilotriacetate/agarose column; W1, first washing step; W2, second washing step; EL, eluate; MA, multicolored protein standard (Novex, Frankfurt, Germany; 148 kDa = phosphorylase B; 60 kDa = glutamate dehydrogenase; 42 kDa = carbonic anhydrase; 30 kDa = myoglobin). Equal amounts of each fraction were loaded on to the gel corresponding to 30 μg protein of the cleared cell lysate.

Table 2. Biochemical properties of purified MyrS. Where applicable, values are mean ± SD ($n = 3$).

Temperature optimum[a]	40 °C; activities measurable up to 55 °C
Activation energy E_a (Arrhenius plot) [a]	113 kJ·mol^{-1}
Mean Michaelis constant K_m for myrcene formation from GDP[a, b]	84 ± 14 μM
Native molecular mass according to SDS/PAGE and deduced amino-acid sequence of the gene	64.3 kDa
Product formation of the expressed MyrS protein from GDP in μkat·(kg protein)$^{-1}$ and (% of total)[a]	
α-Pinene	0.5 ± 0.3 (0.5%)
Sabinene	0.9 ± 0.1 (0.8%)
β-Pinene	1.0 ± 0.1 (0.9%)
Myrcene	100.5 ± 8.9 (96.6%)
Limonene	1.2 ± 0.1 (1.2%)
Products from DMADP[a]	No isoprene

[a] Enzyme assays were performed under the conditions described for monoterpene synthases [12] and isoprene synthase [16] from *Q. ilex* and *Q. robur*, respectively, except the parameter varied in the certain tests. Under the experimental conditions, 2–8% of the substrate GDP was converted into myrcene. [b] The mean Michaelis constant for myrcene was calculated according to Hanes, Lineweaver–Burk, and Eadie–Hofstee.

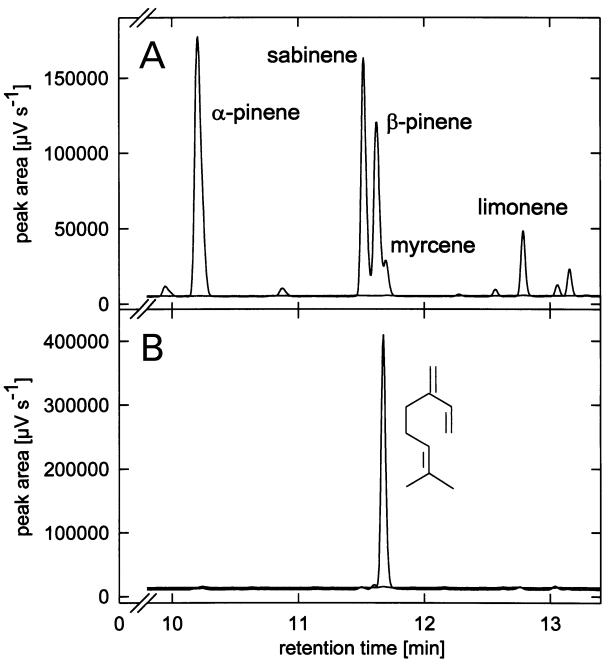

Fig. 4. Gas chromatographic analysis of monoterpenes generated from GDP (a) by leaf extracts of *Q. ilex* at the time of RNA isolation and (b) by the recombinant enzyme MyrS. For comparison, chromatograms of enzyme assays with active and heat-denaturated protein are shown. Tests were performed as described previously [12]. Traces of α-thujene (9.8 min), *E*-ocimene (13.1 min), β-phellandrene (13.2 min), and *Z*-ocimene (13.4 min) were detected.

to 250 mM, no isoprene production was detected. The major product from GDP was myrcene (97% of total hydrocarbon product) followed by minor amounts of the cyclic monoterpenes limonene (1.2%), α-pinene, sabinene, and β-pinene (each less than 1%). Because of the dominance of the myrcene product, the enzyme encoded by *myrS* is designated as a myrcene synthase.

Extracts of induced *E. coli* TG1(pQE30-*myrS*) contained myrcene synthase activity of ≈1.2 ± 0.1 μkat·(kg protein)$^{-1}$ (mean ± SD; three independent repetitions). After purification, the enzyme activity increased to 100.5 ± 8.9 μkat·(kg protein^{-1}), corresponding to a 90-fold purification. Biochemical characterization of this partially purified recombinant 'pseudo-mature' myrcene synthase MyrS revealed a temperature optimum of 40 °C with activation energy, $E_a = 113$ kJ·mol^{-1} and an apparent Michaelis constant of 84 μM for GDP (Table 2). Under the experimental conditions chosen, between 8 (at 1 μM GDP) and 2% (at 250 μM GDP) of the substrate GDP was converted into myrcene, indicating that the calculated velocity of this enzyme is close to the initial velocity. In addition, the measured concentrations of the expressed MyrS protein are in good agreement with previous data for monoterpene synthase activity of crude *Q. ilex* leaf extracts [12].

The *Q. ilex* myrcene synthase is the first functionally characterized single-product monoterpene synthase from the Fagaceae (Fig. 2). Another single-product myrcene synthase cloned previously from the conifer *Abies grandis* (Dougl. ex D. Don) Lindl. [21] belonged to the *TPSd* group [7], which comprises terpene synthases from gymnosperms. Recently a mixed mycrene/ocimene synthase was cloned from *Arabidopsis thaliana* L. [8], distinguishing this enzyme functionally from the *Q. ilex* myrcene synthase. Nevertheless, the two genes belong to the same subfamily *TPSb* (Fig. 2), indicating that sequence relatedness allows assignment of genes to *TPS* gene subfamilies, but identification of the metabolic function requires functional characterization.

ACKNOWLEDGEMENTS

The authors thank Professor H. Rennenberg, University of Freiburg, for critical reading of the manuscript. The work was supported by the European Community in the frame of the ECOVOC project (Parameterization of Environmental and Physiological Controls of Volatile Organic Compound Emissions from European Forests) as part of the 4th Framework Programme on Environment and Climate 1994–1998.

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