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Domain swapping of *Citrus limon* monoterpene synthases: impact on enzymatic activity and product specificity

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Abstract

Monoterpene cyclases are the key enzymes in the monoterpene biosynthetic pathway, as they catalyze the cyclization of the ubiquitous geranyl diphosphate (GDP) to the specific monoterpene skeletons. From *Citrus limon*, four monoterpene synthase-encoding cDNAs for a β-pinene synthase named Cl(−)βPINS, a γ-terpinene synthase named ClγTS, and two limonene synthases named Cl(+)LIMS1 and Cl(+)LIMS2 were recently isolated [J. Lücker et al., Eur. J. Biochem. 269 (2002) 3160]. The aim of our work in this study was to identify domains within these monoterpene synthase enzymes determining the product specificity. Domain swapping experiments between Cl(−)βPINS and ClγTS and between Cl(+)LIMS2 and ClγTS were conducted. We found that within the C-terminal domain of these monoterpene synthases, a region comprising 200 amino acids, of which 41 are different between Cl(−)βPINS and ClγTS, determines the specificity for the formation of β-pinene or γ-terpinene, respectively, while another region localized further downstream is required for a chimeric enzyme to yield products in the same ratio as in the wild-type ClγTS. For Cl(+)LIMS2, the two domains together appear to be sufficient for its enzyme specificity, but many chimeras were inactive probably due to the low homology with ClγTS. Molecular modeling was used to further pinpoint the amino acids responsible for the differences in product specificity of ClγTS and Cl(−)βPINS.

Keywords: Monoterpene synthase; γ-Terpinene; β-Pinene; Limonene; Domain swapping; Product specificity; Molecular modeling

Terpenoids form one of the most important classes of plant secondary metabolites consisting of more than 36,000 different compounds [1]. Throughout the plant kingdom, terpenoids are known to have a wide range of functions. They can act as defense compounds in plants as they deter herbivores or attract natural enemies of herbivores [2]. Plant hormones, such as cytokinins, gibberellins, and abscisic acid, are often derivatives of terpenoids [3,4]. In nature, terpenes can function in communication between plants and animals, including humans [5,6]. The terpenoid structure consists of an integral number of five-carbon (isoprene) units. Two such units can form C_{10} atom monoterpene structures (e.g., limonene), three units form the C_{15} atom sesquiterpenes (e.g., 5-epi-aristolochene), four units form the C_{20} atom diterpenes (e.g., taxol), six units form the C_{30} atom triterpenes (e.g., β-amyrin), and eight units form the C_{40} atom tetraterpenes (e.g., β-carotene). Finally, polyterpenoids, of many more than C_{40} atoms, also occur in nature (e.g., rubber) [7].

The fundamental chain elongation of terpene biosynthesis is achieved by the condensation of isopentenyl diphosphate (IPP)[1] and its isomer dimethylallyldiphosphate (DMAPP). Geranyl diphosphate (GDP) formed

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[1] *Abbreviations used*: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; TEAS, tobacco 5-epi-aristolochene synthase; HVS, *Hyoscyamus muticus* vetispiradiene synthase

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from the condensation of one molecule of IPP with one molecule of DMAPP is a precursor for monoterpene biosynthesis. In the presence of a metal cofactor (such as Mg^{2+} or Mn^{2+}), monoterpene synthase enzymes use GDP as a substrate for the first step in monoterpene biosynthesis. These synthases act as templates to fix the conformation and stereochemistry during the cyclization process, and, on binding to the hydrophobic substrate, the result is a closed solvent-inaccessible active site pocket that protects and stabilizes reactive carbocation intermediates from attack by water. The degree of stabilization of carbocationic intermediates determines to what extent rearrangements can occur and thus which terpene structures are finally formed [8].

So far only one plant terpene synthase, the tobacco 5-epi-aristolochene synthase (TEAS), has been crystallized and its 3D structure determined. This structure provides a basis for the understanding of the stereochemical selectivity displayed by this and other terpene synthases, together with more insight into the involvement of acidic and aromatic amino acids in carbocation stabilization [9]. TEAS was shown to consist entirely of α helices, short connecting loops and turns, and to be organized in two structural domains. The crystal structure of TEAS with the docked farnesyl diphosphate substrate suggests that the specificity of the synthases depends on the presence of certain amino acid residues in the active site but also in the surrounding layers [9–11]. The study of chimeras derived from homologous proteins having different specificities constitutes a powerful tool to identify functions of structural domains [12]. Back and Chapell [13] swapped the two structural domains of the TEAS and those of a *Hyoscyamus muticus* vetispiradiene synthase (HVS), both sesquiterpene synthases, and characterized the resulting chimeric enzymes expressed in *Escherichia coli*. The domain swapping between subdomains within the N-terminal and C-terminal structural domains of TEAS and HVS resulted in novel enzymes capable of synthesizing the products of either one of the two, or both, parent enzymes. The authors proposed that the evolution of terpenoid synthases is based on recombinations of functional domains encoding parts of cyclase genes. If the active site topology of a protein is in some way altered (e.g., by recombination between genes), the ability to select a single product could be lost, resulting in multiproduct chimeric enzymes [14]. For monoterpene synthases, site-directed mutagenesis or domain swapping experiments and, so far, a crystal structure have not been published.

The isolation of four different monoterpene synthase cDNAs from *Citrus limon* L. Burm. peel, all of which belong to the *tpsb* monoterpene gene family, one encoding a γ-terpinene synthase named ClγTS (GenBank Accession No. AF14286), another encoding a β-pinene synthase named Cl(−)βPINS (AF14288), and two encoding (+)-limonene synthases named Cl(+)LIMS1 and Cl(+)LIMS2 (AF14287 and AF14289, respectively), was recently reported [15]. The main products of the enzymes in assays with GDP as substrate were (−)-β-pinene, γ-terpinene, and (+)-limonene (two cDNAs), respectively, but also a number of minor products were found. Cl(+)LIMS2 and ClγTS are 50% identical at the amino acid level. Cl(−)βPINS and ClγTS share a higher sequence homology (85% amino acid identity) (Fig. 1). The availability of these monoterpene synthases with varying homology and different product specificities presents an excellent opportunity to study product specificity of monoterpene synthases. Three out of these four monoterpene synthases catalyzing the formation of three different major products were used in a study of the effects of domain swapping on product specificity.

Materials and methods

cDNA clones. Monoterpene synthase cDNAs, encoding Cl(−)βPINS, ClγTS, and Cl(+)LIMS2 truncated and cloned in the multiple cloning site of the pRSETB expression vector (that included an ATG translation initiation codon and a series of six histidine residues (His tag) (Invitrogen, Groningen), were described earlier [15]. Further cDNA analysis was conducted using the Megalign software (DNA Star Inc, Madison, WI, USA), and the ClγTS and Cl(−)βPINS proteins were modeled on the Swissmodel online service [16], using TEAS (Swissprot Accession No. Q40577) as the template for modeling.

Site-directed mutagenesis. To create restriction enzyme recognition sites at the same relative position within the three genes, a site-directed mutagenesis approach was adopted using the Quickchange Site Directed Mutagenesis PCR kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's recommendations. The *Eco*RI restriction site of the expression vector pRSETB was substituted with an *Eco*RV restriction site using the sense primer 1

(5′-GAGATCTGCAGCTGGTACCATGGATATCGAAGCTTGATCCGGCTGCTAA-3′) and an antisense primer 2 (5′-TTAGCAGCCGGATCAAGCTTCGATATCCATGGTACCAGCTGCAGATCTC-3′). The altered nucleotides are underlined. Cl(+)LIMS2 was mutated twice without changing the integrity of the encoded amino acid sequence. Using the sense primer 3

(5′-AGAGGACAAGAACCACCTTTTACTCGAGCTCGCTAAGATGGAGTTTAAC-3′) and the antisense primer 4 (5′-GTTAAACTCCATCTTAGCGAGCTCGAGTAAAAGGTGGTTCTTGTCCTCT-3′), an *Xho*I restriction site was introduced at position 799. Primers 5

(5′-CAATCATTAAGAAGGAACTGGAATTCCTAGAAAGTAATCCAGATATAGTT-3′) and 6

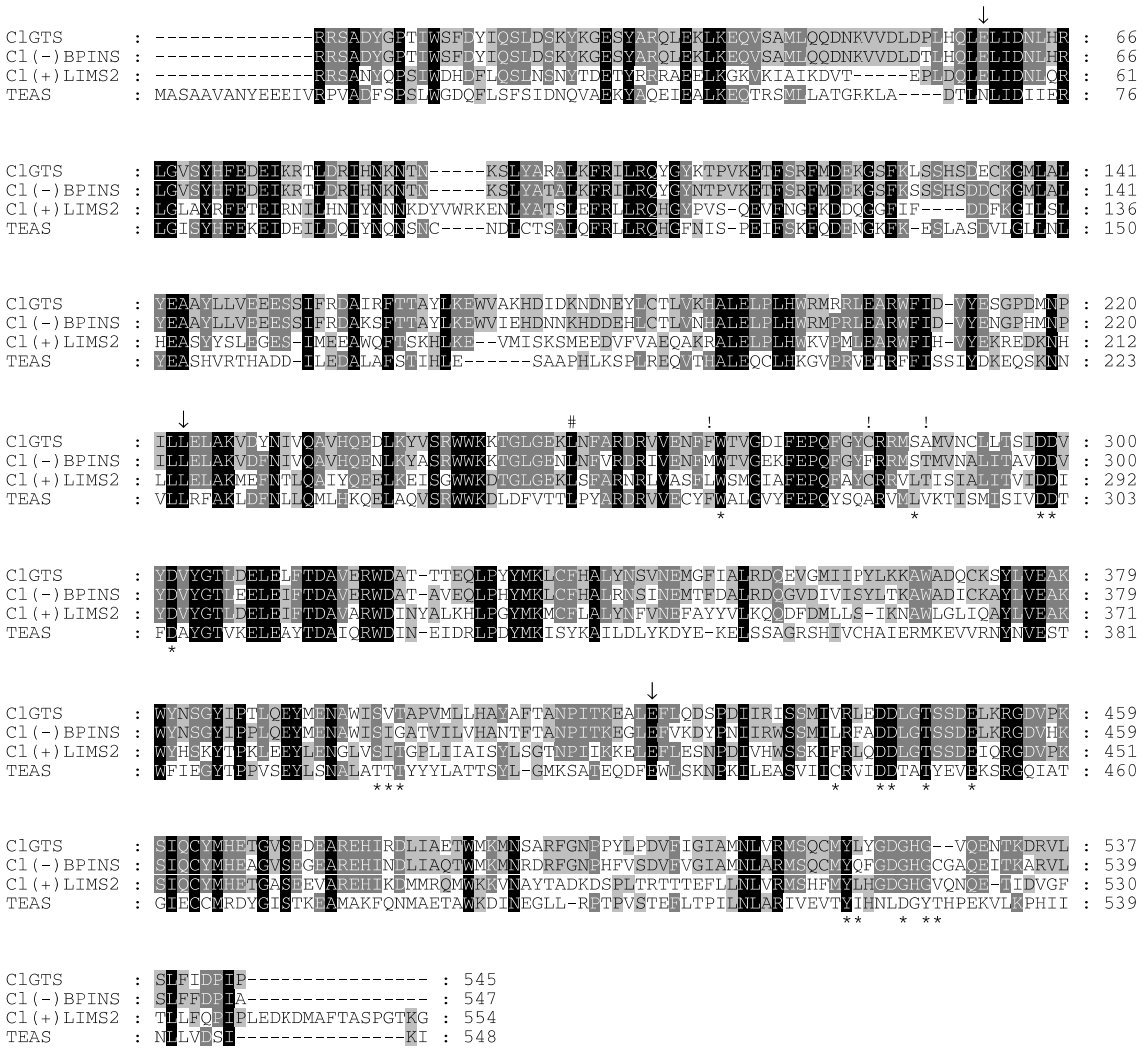

Fig. 1. Protein alignment of TEAS, Cl(−)βPINS, ClγTS, and Cl(+)LIMS2. Domain borders of the monoterpene synthases used in this study are indicated by ↓. The amino acid residues marked with an asterisk correspond to the ones in TEAS that were hypothesized by Starks et al. [9] to be important for the formation of 5-epi-aristolochene. The borders between the two structural domains of TEAS are indicated by #, while amino acids marked by ! are discussed in the text.

(5′-AACTATATCTGGATTACTTTCTAGGAATTC CAGTTCCTTCTTAATGATTG-3′) were used to create an *Eco*RI restriction site at position 1399.

Creation of chimeric enzymes by restriction digestion and domain swap approach. The parent Cl(−)βPINS and ClγTS cDNAs cloned in pRSETB were digested with the enzyme combinations *Nhe*I/*Sac*I, *Sac*I/*Xho*I, *Xho*I/*Eco*RI, *Eco*RI/*Eco*RV, and *Xho*I/*Eco*RV. *Nhe*I is a unique restriction site located exclusively in the pRSETB vector and upstream of the cloned monoterpene synthases. The correct size DNA bands were isolated from agarose gel using GFX columns (Amersham Pharmacia Biotech, Freiburg, Germany) and ligated to subsequently create the chimeric cDNAs 1 to 8 (Table 1). The parent Cl(+)LIMS2 and ClγTS cDNAs were digested either with *Xho*I/*Eco*RI, or *Eco*RI/*Eco*RV, or *Xho*I/*Eco*RV. In the same way as described above, the chimeric cDNAs called 9, 10, 11, 12, 13, and 14 were created (Table 1). In addition to restriction digestion analysis, all chimeric genes were sequenced completely.

Gene expression and protein purification. All plasmids containing the chimeric cDNAs, as well as the parental cDNAs including the mutated Cl(+)LIMS2, were used to transform *E. coli* BL21-CodonPlus-RIL strain (Stratagene), using the original pRSETB vector as a negative control. Clones were inoculated into 5 mL LB supplemented with 100 mg L^{-1} ampicillin and grown overnight. Aliquots of 0.5 mL were taken to inoculate 250-mL Erlenmeyer flasks containing 50 mL LB with ampicillin (50 μg mL^{-1}) and cultures were grown at 37 °C to an OD_{600} of 0.6. For induction of expression, IPTG was added to a final concentration of 1 mM and the cultures were grown at 16 °C overnight with shaking at 250 rpm. Cells collected by centrifugation and resus-

Table 1
Product formation of parent and chimeric enzymes generated by swapping of domains I to IV between lemon monoterpene synthases

Name	D I	D II	D III	D IV	α-Thujene	α-Pinene	Sabinene	β-Pinene	Myrcene	α-Terpinene	ρ-Cymene	Limonene	γ-Terpinene	Terpinolene	α-Terpineol	Total
Cl(-)βPINS					-	2.5	5	30	-	-	-	**0.5**/ 4	-	-	1	43
1					-	2	4.5	28.5	-	-	-	4.25	-	-	-	39.25
2					-	2.5	5.5	29.5	-	-	-	4.25	-	-	-	41.75
3					-	1	1.5	8.5	-	-	-	3.5	-	-	<0.25	14.75
4					0.5	1	0.25	1.25	0.5	<0.25	<0.25	3.25	15	-	<0.25	22.25
5					1.5	1	0.5	1	-	<0.25	-	3.5	2	<0.25	<0.25	10
6					-	1	2.5	6	-	-	-	3.25	-	-	-	12.75
7					-	0.25	0.5	1	-	-	-	2.25	-	-	-	4
8					0.25	0.5	0.25	0.75	-	-	-	3	1.5	-	-	6.25
ClγTS					0.5	1.5	0.25	1.25	0.25	0.25	<0.25	**0.5**/2	16.25	1	0.5	24.25
9					-	-	-	-	-	-	-	-	-	-	-	
10					-	<0.25	-	-	-	-	-	**3.25**/ 0.75	-	-	-	4.25
11					-	<0.25	-	-	-	-	-	**1.5**/ 1	-	-	-	2.75
12					-	-	-	-	-	-	-	-	-	-	-	
13					-	-	-	-	-	-	-	-	-	-	-	
14					-	-	-	-	-	-	-	-	-	-	-	
Cl(+)LIMS2					-	<0.25	-	-	0.5	-	-	**24**	-	-	-	24.75

Note. The hatched columns depict domain composition for wild-type β-pinene synthase (Cl(−)βPINS), γ-terpinene synthase (ClγTS), (+)-limonene synthase (Cl(+)LIMS2), and chimeric (1–14) monoterpene synthase cDNAs. Values for products formed are given in ng product μg^{-1} eluted protein; <0.25 = product formation was detectable but below 0.25 ng/μg eluted protein; — = product not detectable. Values for limonene are in normal typeface for (−)-limonene and in boldface for (+)-limonene.

pended in buffer were sonicated and proteins were isolated using His tag purification by passing the lysate over Ni-NTA spin columns (Qiagen, Chatsworth, CA, USA). After washing, the bound protein was eluted using 50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH 8. For protein concentration measurements, the proteins were first precipitated in 10% trichloroacetic acid on ice for 15 min, followed by centrifugation for 10 min. The resulting pellet was washed twice with acetone and, after drying, dissolved in 5 mM Tris, pH 6.8, 0.2% (w/v) sodium dodecyl sulfate, and 1% glycerol. Protein concentration was determined using the BCA Protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as reference, according to the manufacturer's recommendations. One hundred fifty nanograms of each of the eluted proteins was loaded on a 12% polyacrylamide gel and the remainder was supplemented with glycerol to 30% and stored at −70 °C until used.

Enzymatic assays and GC–MS analysis. Each (8.3 μg) of the eluted enzyme proteins and 6 μg of the eluted negative control proteins were used in a total reaction volume of 1 mL of assay buffer containing 15 mM MOPSO buffer (pH 7), 10% glycerol, 1 mM ascorbic acid, 0.6 mM $MnCl_2$, 2 mM dithiothreitol, and 10 μM GDP [15]. After the addition of a 1-mL redistilled pentane overlay, the tubes were carefully mixed and incubated for 1 h at 30 °C. Following the assay, the tubes were vortexed, and the organic layer was removed and passed over a short column of aluminum oxide (Al_2O_3) overlaid with anhydrous Na_2SO_4. The assay mixture was reextracted with 1 mL of pentane:diethyl ether (80:20), which was also passed over the aluminum oxide column, the column was washed with 1.5 mL of diethyl ether, and the organic fractions were pooled. For quantification, 10 ng of Δ-3-carene was added to the eluant as internal standard. Two microliters of each pentane/diethyl ether fraction was injected into a HP 5890 series II gas chromatograph (Hewlett–Packard, Agilent Technologies, Alpharetta, GA, USA) and an HP 5972A Mass Selective Detector essentially as described before [17]. The gas chromatograph was equipped with a HP-5MS column (30 m × 0.25 mm i.d., film thickness = 0.25 μm) and programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C min^{-1} to 280 °C, and final time of 10 min. Products were identified by comparison of retention times and mass spectra with authentic reference compounds.

MDGC–MS. The enantiomeric composition of the limonene produced by the parent and chimeric enzymes was analyzed using MDGC–MS. MDGC–MS analyses were performed with a Fisons 8160 gas chromatograph (GC1) connected to a Fisons 8130 gas chromatograph (GC2) and a Fisons MD 800 quadrupole mass spectrometer and analyzed with Fisons MassLab software (Version 1.3; Fisons, Manchester, UK). The system setup was as described previously [18] but with the following settings. The fused silica capillary column in GC1 (J&W, Folsom, CA, USA) DB-Wax 20 M (25 m × 0.25 mm i.d.; film thickness = 0.25 μm) was maintained at 40 °C then programmed to 240 °C at 1 °C min^{-1} with He gas flow at 3 mL min^{-1}. The compounds of interest were transferred from GC1 to GC2 from 2 to 15 min. The fused silica capillary column in GC2 (30% 2,3-diethyl-6-*tert*-butyl-dimethyl-β-cyclodextrin/PS086; 25 m × 0.25 mm i.d.; film thickness = 0.15 μm) was maintained at 40 °C (9 min), then programmed to 200 °C at 2 °C min^{-1} with He gas flow at 3 mL min^{-1}. The MS operating parameters were ionization voltage 70 eV (electron impact ionization) and ion source and interface temperatures 230 and 240 °C, respectively.

Results

Construction of the chimeric enzymes

The amino acid sequences of the truncated forms of monoterpene synthases Cl(−)βPINS, ClγTS, and Cl(+)LIMS2 were aligned with TEAS, from which a three-dimensional structure is available, using the ClustalV algorithm (Fig. 1). We picked three restriction sites that are either conserved among the genes or could easily be introduced by mutagenesis without changing encoded amino acids, to define four domains for swapping. These domains and the relative homology between the proteins for each domain are shown in Fig. 2. Domains I and II together overlap, though not completely, with the N-terminal structural domain of TEAS [9]. The first 33 amino acids of domain III align with the N-terminal structural domain of TEAS, while the remaining amino acids correspond, along with domain IV, to the second, C-terminal, structural domain of TEAS. DNA fragments corresponding to these domains were swapped by restriction digestion and ligation in the corresponding position of the counterpart cDNAs and transformed to *E. coli*. Further DNA restriction digestion and sequencing revealed that all chimeric cDNAs with the intended swapped domain retained the correct full amino acid sequence and consequently the proteins of expected size (around 65 kDa) were produced on induction (Fig. 3). For all proteins, using GDP as a substrate, all detectable enzymatic products were identified and quantified using GC-MS analysis (Table 1).

Hybrid enzyme-catalyzed product formation

As expected, the β-pinene synthase, Cl(−)βPINS, produced β-pinene as the major product and the γ-terpinene synthase, ClγTS, γ-terpinene (Fig. 4). Both enzymes produced also a number of minor products partially unique for one of the two enzymes (Fig. 4, Table 1). When domain I or domain II of Cl(−)βPINS were separately substituted by their counterparts from ClγTS (chimeras 1 and 2, respectively), the product specificity and enzyme activity remained the same as in Cl(−)βPINS. The exchange of domain I and domain II together had a similar effect (chimera 3), although with a 2.5-fold decrease in the overall major product amount. The reverse substitution of domains I and II together in ClγTS with their counterparts from Cl(−)βPINS did not change product specificity of ClγTS (chimera 4), γ-terpinene being the major product formed (Table 1). These results suggest that product specificity is determined predominantly by either the third or fourth domain, or both. The separate replacement of domains III or IV of Cl(−)βPINS by their respective counterparts from ClγTS (chimeras 5 and 6) resulted in an overall decrease in enzyme activity (9- and 4-fold respectively). But only the replacement of domain III changed the product specificity to γ-terpinene (Fig. 4, Table 1). Also, the pattern of minor products of chimera 5 was indicative of the specificity of the parent ClγTS (the presence of, for example, α-thujene, α-terpinene, and terpinolene). This

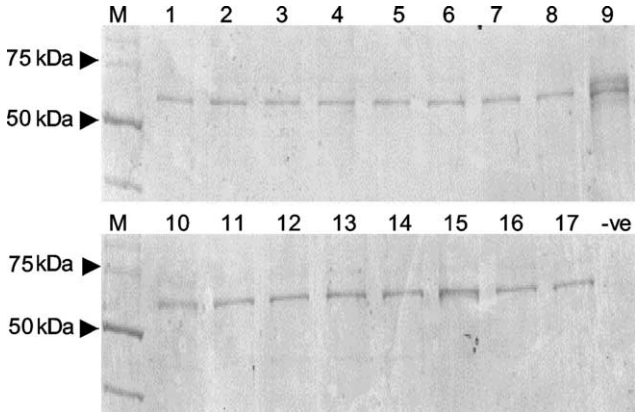

Fig. 3. SDS–PAGE analysis of proteins produced by parental and chimeric genes expressed in *E. coli* and purified using Ni-NTA spin columns. M, Marker; lane 1, Cl(+)LIMS2; lane 2, Cl(−)βPINS; lane 3, ClγTS; lanes 4–17, chimeras 1–14. -ve; pRSETB negative control.

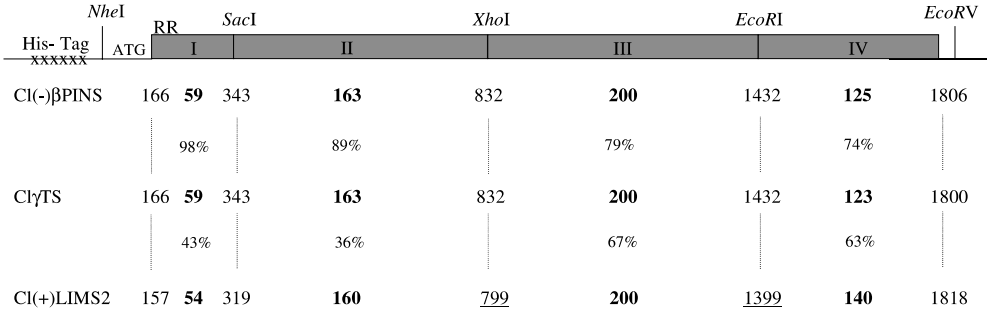

Fig. 2. Primary structure of the three monoterpene synthases, Cl(−)βPINS, ClγTS, and Cl(+)LIMS2, cloned in the pRSETB vector and divided into domains I to IV. The nucleotide positions bordering the domains are indicated. Whenever restriction sites were introduced by site-directed mutagenesis, these positions are underlined. The number of amino acids constituting each domain is given in boldface. The percentage of amino acid identity for each domain between two sets of enzymes (Cl(−)βPINS and ClγTS on the one hand, and ClγTS and Cl(+)LIMS2 on the other hand) is indicated.

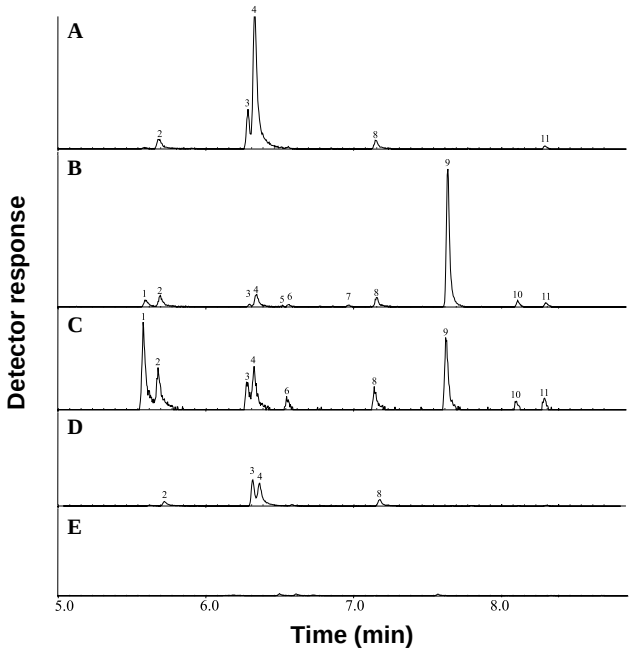

Fig. 4. GC–MS chromatograms of *m/z* 93 showing the product profiles of Cl(−)βPINS (A), ClγTS (B), chimera 3 (C), and chimera 4 (D) and empty pRSETB vector negative control (E). Peak identities were confirmed using authentic standards: (1) α-thujene; (2) α-pinene; (3) sabinene; (4) β-pinene; (5) myrcene; (6) α-terpinene; (7) ρ-cymene; (8) limonene; (9) γ-terpinene; (10) terpinolene; (11) α-terpineol.

suggests that while both domains III and IV of ClγTS were required for optimal enzyme function, specificity for the production of γ-terpinene and other related minor products is determined predominantly by domain III. In the reaction catalyzed by chimera 7, where domain III of ClγTS was substituted by its counterpart from Cl(−)βPINS, no formation of γ-terpinene was observed. In all parent and chimeric enzymes, limonene was always found as a side product.

MDGC-MS analysis confirmed that the limonene cyclase enzyme Cl(+)LIMS2 produced exclusively (+)-limonene as the major product of the enzymatic catalysis, in contrast to Cl(−)βPINS and ClγTS, which produced mainly (−)-limonene as a side product and only a small amount of (+)-limonene [15] (Table 1). In the chimeric enzyme 10, substitution of ClγTS domain III and domain IV by their counterparts from Cl(+)LIMS2 changed product specificity to that of Cl(+)LIMS2, (+)-limonene being the major product formed, although with an approximately sevenfold decrease in product formation. This decrease in enzyme efficiency was accompanied by some decrease in product specificity indicated by the formation of (−)-limonene. Substitution of only domain IV in ClγTS by domain IV from Cl(+)LIMS2 (chimera 11) also led to the formation of some (+)-limonene. Hence, also in these chimeras it seems that domains III and IV are determining predominantly product specificity, here the formation of (+)-limonene by Cl(+)LIMS2. All the remaining chimeric enzymes were inactive and no product was detected using GC-MS analysis.

Discussion

In the present study, we have demonstrated that in monoterpene synthases the C-terminal domains III and IV are essential for proper enzyme activity but that the γ-terpinene specificity of ClγTS is determined predominantly by domain III (see chimera 5 in Table 1). The data suggest that monoterpene synthase product specificity lies within the amino acids of domain III but that the ratio with which the different enzyme-specific monoterpenes, including minor products, are produced by the enzyme is codetermined by amino acids in the fourth domain. This supports the hypothesis that the active site of catalysis previously suggested for sesquiterpene synthases [9] is located in a similar region of the monoterpene synthases. Starks et al. obtained the 3D structure of TEAS and have designated the amino acids that are likely to play a key role in the catalysis process leading to the formation of the sesquiterpene 5-*epi*-aristolochene from farnesyl diphosphate. These amino acids are Trp 273, Leu 290, Asp 301, Asp 302, Asp 305, Glu 379, and Thr 403 (aligning with amino acids in domain III of the lemon monoterpene synthases) and Cys 440, Asp 444, Thr 448, Glu 452, Thr 519, Tyr 520, Asp 525, and Tyr 527 (aligning with amino acids in domain IV of the lemon monoterpene synthases) (Fig. 1).

In contrast to ClγTS and Cl(−)βPINS, ClγTS and Cl(+)LIMS2 have a relatively low homology and belong to separate phylogenetic clusters [15]. Domain substitutions between these two enzymes resulted in either a strong reduction in the enzyme efficiency or even complete loss of activity. Also in many of the other chimeras, enzymatic efficiency was reduced. This became more pronounced whenever either domain III or IV was substituted separately (Fig. 4, Table 1). This suggests that, even though some or all of the amino acids in the first and second domains need not be directly involved in product specificity, proper interactions between amino acids from different domains are essential for enzyme function. A decrease in enzymatic activity was also described in many other studies, such as on site directed mutagenesis [12,13,19].

The outcome of the GDP cyclization process catalyzed by the monoterpene synthases is determined by the migration and quenching of the carbocation in the enzyme-bound intermediate. For the formation of γ-terpinene, a mechanism was proposed by Croteau and co-workers [20] that involves the cyclization of the acyclic GDP, a 1,2-hydride shift (from C-4 to C-8), and the subsequent loss of a proton from C-5 to form the Δ^4

double bond (Fig. 5). A similar mechanism, but with formation of a cyclopropane ring and loss of the proton from C-6, would yield one of the side products, α-thujene [20]. The elimination of a proton from the α-terpinyl cation allows the formation of limonene [19]. Both wild-type Cl(−)βPINS and ClγTS enzymes and all their chimeric enzymes produced (−)-limonene as a side product at almost similar rates. If the cyclohexenyl double bond of the α-terpinyl cation undergoes an intramolecular Markovnikov addition, the pinyl cation is formed (Fig. 5). Subsequent deprotonation of this pinyl cation produces β-pinene [21].

ClβPINS and ClγTS have much lower product and enantiomer specificity than Cl(+)LIMS2 (Table 1) [15]. This low enzyme selectivity might be explained by the high K_m values of both ClβPINS and ClγTS, compared with Cl(+)LIMS2 [15], suggesting a lower binding affinity. Also, ClβPINS and ClγTS produce α-terpineol as a side product, suggesting that the α-terpinyl cation is not protected well enough against quenching by a water molecule. Rynkiewisz and co-workers [22] have postulated that the tighter the active site is around the substrate, the more specialized the enzyme is. Therefore, we assume that stabilization of the enzyme-bound intermediates in the active site of ClβPINS and ClγTS is less controlled than in the case of Cl(+)LIMS2, leading to the formation of more different monoterpene side products and sometimes even different enantiomers as in the case of the side product limonene of ClγTS (Table 1).

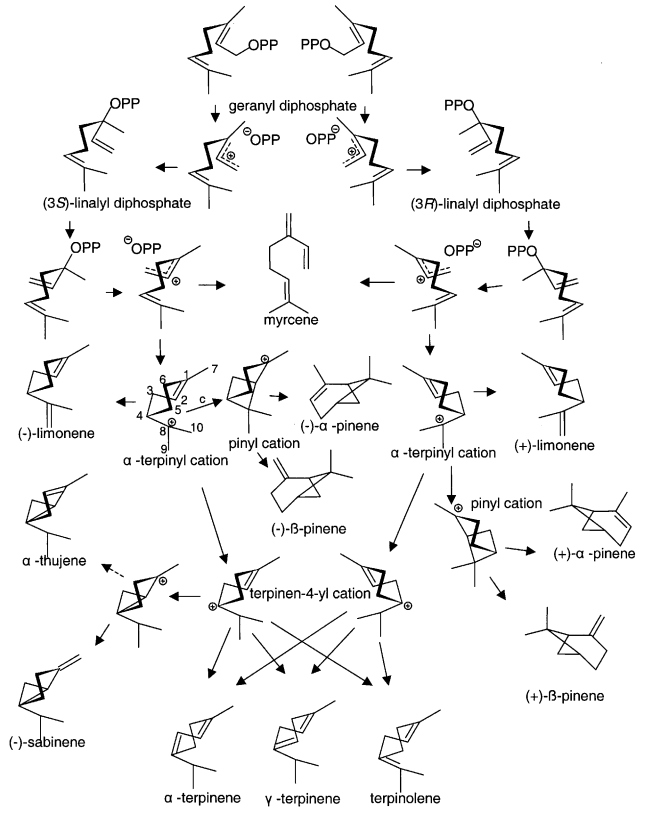

Fig. 5. Cyclisation mechanism leading to (+)-limonene, β-pinene, or γ-terpinene and other minor products catalyzed by Cl(−)PINS, ClγTS, and Cl(+)LIMS2. Adapted with permission from [8]. Copyright (1987) American Chemical Society. Some compounds are drawn in somewhat unfavorable conformations for reasons of clarity.

In terpene synthases, acidic or aromatic amino acids are known to stabilize positive charges and carbocationic intermediates, whenever positioned around the active site [11]. In the case of acidic amino acids, this is due to the presence of a carboxylate anion. In the case of aromatic amino acids, the π electrons of the aromatic ring and the phenolic oxygen can readily accept a proton. Thus, they stabilize a cation and participate in the rearrangements of terpene structures [9,23–26]. The amino acid residues in the enzyme that stabilize the specific position of the positive charge in the carbocationic intermediates are likely to be highly important for product outcome. The high level of amino acid identity between the β-pinene synthase and the γ-terpinene synthase in domain III (80%) forms a good lead to further explore the role of specific amino acids determining product specificity. Of the 41 amino acids of domain III that are different between these two cDNAs, only three (F269M, C283F, A288T) have different physicochemical properties and are within 3 Å of the modeled active site. Of these three, two are aromatic, have carbocationic stabilizing properties, and can be involved in deprotonation. Interestingly, they are differentially positioned in ClγTS and Cl(−)βPINS at positions 283 and 269, respectively (Fig. 6). F283 in ClγTS is substituted by the non-proton-abstracting amino acid C283 in Cl(−)βPINS, whereas F269 in Cl(−)βPINS is substituted by M269 in ClγTS. These amino acid substitutions have moved the carbocation stabilizing property from one side of the active site to the other (Fig. 6). If the initial spatial positionings of GDP in both enzymes are identical, this change may be responsible for the migration

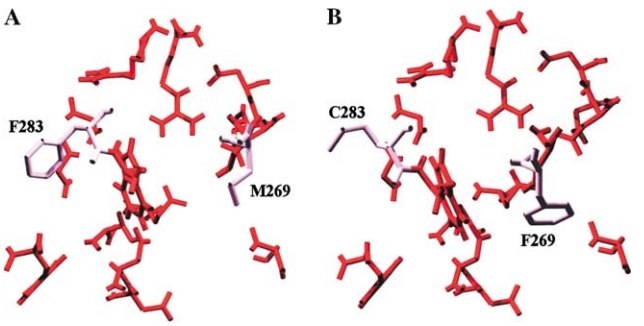

Fig. 6. Protein modeling of the ClγTS (A) and the Cl(−)βPINS (B) proteins. The protein sequences of ClγTS and Cl(−)βPINS were modeled on the tobacco 5-epi-aristolochene synthase (TEAS) crystal structure. The amino acid residues shown here in red were hypothesised by Starks and co-workers [9] to be important for the formation of 5-epi-aristolochene by TEAS and are mostly conserved in ClγTS and Cl(−)βPINS. Amino acids at positions 269 and 281 in ClγTS (A) and Cl(−)βPINS (B), shown here in purple, are discussed in the text.

of the double bond in the α-terpinyl cation, resulting in a carbocation at C1 (the pinyl cation), hence allowing the formation of (−)-β-pinene instead of γ-terpinene (Fig. 5). Site-directed mutagenesis of these two amino acids should confirm this hypothesis. In addition, in the absence of a crystal structure a domain shuffling approach might not only shed light on the biochemical mechanism of the specificity of monoterpene production by monoterpene synthases but may also lead to the formation of new monoterpenes that may not arise in "normal" enzymatic catalysis.

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References

[1] R.A. Hill, Dictionary of natural products on CD-ROM, Ed. version 10:2. Chapman & Hall/CRC, 2002.
[2] S. Trapp, R. Croteau, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 689–724.
[3] D.V.S. Mok, M.C. Mok, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 89–118.
[4] W. Rademacher, Annu. Rev. Plant Physiol. Plant Mol. Biol. 51 (2000) 501–531.
[5] J. Gershenzon, W. Kreis, in: M. Wink (Ed.), Biochemistry of Secondary Metabolism, CRC Press, Boca Raton, FL, 1999, pp. 222–299.
[6] L. Cseke, N. Dudareva, E. Pichersky, Mol. Biol. Evol. 15 (1998) 1491–1498.
[7] J. Ilmberger, Chem. Senses 26 (2001) 239–245.
[8] R. Croteau, Chem. Rev. 87 (1987) 929–954.
[9] C.M. Starks, K. Back, J. Chappell, J.P. Noel, Science 277 (1997) 1815–1820.
[10] K.A. Rising, C.M. Starks, J.P. Noel, J. Chappell, J. Am. Chem. Soc. 122 (2000) 1861–1866.
[11] H.J. Bouwmeester, J. Kodde, F.W.A. Verstappen, I.G. Altug, J.W. de Kraker, T.E. Wallaart, Plant Physiol. 129 (2002) 134–144.
[12] R.A. de Maagd, M.S.G. Kwa, H. van der Klei, T. Yamamoto, B. Schipper, J.M. Vlak, W.J. Stiekema, D. Bosch, Appl. Environ. Microbiol. 62 (1996) 1537–1543.
[13] K. Back, J. Chappell, Proc. Natl. Acad. Sci. USA 93 (1996) 6841–6845.
[14] M.J. Rynkiewicz, D.E. Cane, D.W. Christianson, Biochemistry 12 (2002) 1732–1741.
[15] J. Lücker, M.K. El Tamer, W. Schwab, F.W.A. Verstappen, L. van der Plas, H.J. Bouwmeester, H.A. Verhoeven, Eur. J. Biochem. 269 (2002) 3160–3171.
[16] N. Guex, M.C. Peitsch, Electrophoresis 18 (1997) 2714–2723.
[17] H.J. Bouwmeester, F.W.A. Verstappen, M.A. Posthumus, M. Dicke, Plant Physiol. 121 (1999) 173–180.
[18] J. Lücker, H.J. Bouwmeester, W. Schwab, J. Blaas, L.H.W. van der Plas, H.A. Verhoeven, Plant J. 27 (2001) 315–324.
[19] D.E. Cane, Q. Xue, B.C. Fitzsimons, Biochemistry 35 (1996) 12369–12376.
[20] W.D. Loomis, R. Croteau, in: P.K. Stumpf (Ed.), The Biochemistry of Plants: A Comprehensive Treatise, vol. 4, Academic Press, San Diego, 1980, pp. 363–386.
[21] D.B. Little, R. Croteau, in: Teranishi (Ed.), Flavor Chemistry: 30 Years of Progress, Kluwer Academic/Plenum, New York, 1999, pp. 239–253.
[22] M.J. Rynkiewicz, D.E. Cane, D.W. Christianson, Proc. Natl. Acad. Sci. USA 98 (2001) 13543–13548.
[23] D.A. Dougherty, Science 271 (1996) 163–168.
[24] J. Bohlmann, G. Meyer-Gauen, R. Croteau, Proc. Natl. Acad. Sci. USA 95 (1998) 4126–4133.
[25] M. Seemann, G. Zhai, J.W. de Kraker, C.M. Paschall, D.W. Christianson, D.E. Cane, J. Am. Chem. Soc. 124 (2002) 7681–7689.
[26] T. Maruyama, D. Saeki, M. Ito, G. Honda, Biol. Pharm. Bull. 25 (2002) 661–665.