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Cloning of a Sesquiterpene Cyclase and Its Functional Expression by Domain Swapping Strategy

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Sesquiterpene cyclase, the first committed step enzyme from the general isoprenoid building block farnesyl pyrophosphate (FPP) for the synthesis of phytoalexin capsidiol, was isolated from the UV-C treated leaves of *Capsicum annuum*. This sesquiterpene cyclase, termed as CASC2 showing 77% amino acid identity with the previously cloned sesquiterpene cyclase CASC1, was composed of 560 amino acids with a calculated molecular mass of 64,907. The mRNA expression pattern of CASC2 was very similar to that of CASC1 during the time course of UV-C irradiated leaves of pepper on RNA blot analysis by using each specific probe. The heterologous expression in *Escherichia coli* using the CASC2 full length failed; however the chimeric construct of CASC2 in which the amino terminal 164 amino acid substituted by the equivalent portion of either CASC1 or tobacco sesquiterpene cyclase was capable of expressing the functional sesquiterpene cyclase activities. The radio-labeled enzymatic products catalyzed by the partially purified chimeric CASC2 were comigrated with authentic radio-labeled sesquiterpene on thin layer chromatography.

Keywords: *Capsicum annuum*; Capsidiol; Domain Swapping; Sesquiterpene Cyclase.

Introduction

Sesquiterpene synthases refer to a group of enzymes that are capable of synthesizing a myriad of sesquiterpenes from single precursor farnesyl pyrophosphate (FPP). More than 7,000 different sesquiterpene compounds have been reported to date (Bohlmann *et al*., 1998; Connolly and Hill, 1991). All of them were derived from FPP. Many of sesquiterpenes reported were demonstrated to play significant ecological roles as pollination attraction, antifeedant action, and defense-related action by the production of hormonal, pheromonal, and antimicrobial compounds (Harborne, 1991; Marby and Gill, 1979). Unlike the myriad of sesquiterpenoids identified, few sesquiterpene synthases, the first committed step enzymes responsible for the synthesis of 7,000 sesquiterpenoids from FPP, have been cloned and characterized to date at the molecular level (Bohlmann *et al*., 1998). Most of these sesquiterpene synthases, such as tobacco 5-epi-aristolochene synthase (Back *et al*., 1998; Facchini and Chappell, 1992), *Hyoscyamus muticus* vetisiradiene synthase (Back and Chappell, 1995), and cotton δ–cadinene synthase (Chen *et al*., 1995), are involved in the biosynthetic pathway leading to the production of antimicrobial phytoalexins. The enzymatic mechanisms of the above sesquiterpene synthases have a lot in common, such as ionization-oriented initial cyclization followed by hydride shifts, methyl migrations, and final reconstitution resulting in all different types of sesquiterpene structures (Cane, 1990; Whitehead *et al*., 1990).

Based on similar intrinsic enzymatic mechanism among different sesquiterpene synthases, the domain reshuffling hypothesis among sesquiterpene synthases was proposed, in which the mutual substitution of possible exon domain positioned in corresponding sequence between sesquiterpene synthases can give rise either to functional chimeric terpene synthases without perturbing the integrity

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Abbreviations: CASC, *capsicum annuum* sesquiterpene cyclase; EDTA, ethylenediaminetetraacetate; FPP, farnesyl pyrophosphate; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEAS, tobacco 5-epi aristolochene synthase; TLC, thin layer chromatography; Tris, tri(hydroxymethyl)aminomethane.

of enzyme activity, or to new terpene synthases (Back and Chappell, 1995).

In our continuation of cloning sesquiterpene cyclases from pepper, we isolated another sesquiterpene cyclase (CASC2) from the UV-C irradiated leaves of pepper, in which CASC2 showed high amino acid homology (77%) with CASC1. Also, we showed the induction of CASC2 mRNA in response to UV and the functional expression of CASC2 in *E. coli* by using the domain swapping strategy.

Materials and Methods

Materials Cold farnesyl pyrophosphate (FPP) was purchased from Sigma (St. Louis, MO). [1-^{3}H] FPP (17.5 Ci/mmol) was purchased from Amersham Pharmacia Biotech. All other chemicals were of analytical grade and obtained from standard sources. The oligonucleotides were synthesized from Bioneer (Korea).

Plant materials and UV treatment Pepper (*Capsicum annuum* cv. Subicho) seeds were sown on plastic pots and grown in a growth chamber at 27°C with daily 14 h light/8 h dark periods. The methods of UV irradiation and leaf harvest were identical as that previously described (Back *et al*., 1998).

Isolation and analysis of nucleic acids Total RNA was isolated by using TRI REAGENT (Sigma, St. Louis, MO, USA). RNA blot analyses were performed as before (Back *et al*., 1998), except that blots were hybridized with 3′ untranslated region (UTR) of CASC1 and CASC2, respectively. Specific probes were prepared as follows. For CASC1, the 3′ UTR was PCR amplified with a forward primer 5′-d(GAAGATGGATACACTCATCC)-3′, a reverse primer 5′-d(GGTCGACTTGAGGTGTAC)-3′ and pBSK-CASC1 as a template (Back *et al*., 1998) by standard polymerase chain reaction (Sambrook *et al*., 1989). CASC2 specific probe was also prepared as above except using a different reverse primer 5′-d(GGGGTCACTTATCGGACC)-3′ with CASC2 cDNA as a template.

Screening of cDNA library The entire coding region of a tobacco 5-epi aristolochene synthase (TEAS) amplified by PCR (Back *et al*., 1994) was used as a heterologous probe for screening pepper sesquiterpene cyclases from the cDNA library, which was constructed with poly(A)$^+$ RNA isolated from 24 h UV-irradiated leaves of pepper. The screening procedures and sequencing of CASC2 cDNA clone were identical as described previously (Back *et al*., 1998).

Chimeric gene construction Pepper sesquiterpene cyclases (CASC1 and CASC2) were originally *in vivo* excised as Bluescript phagemids (Strategene, La Jolla, CA, USA) containing the full length genes at *Eco*RI/*Xho*I sites (thereafter denoted by pBSK-CASC1 and pBSK-CASC2). Initially, the full length CASC2, which was PCR amplified with the forward primer 5′-d(GCGAATTCC**ATG**GCCTCAGCTATAGTTGACAAT)-3′ (*Nco*I restriction site underlined and the translation start codon in bold), and the reverse primer 5′-d(GAGTGCGGCCGC-AATTTTGATGGAGTCCAC)-3′(*Not*I site underlined), were subcloned into pET28b. The mutated CASC2 (CASC2-ATA5ATC), in which isoleucine codon (ATA) in the fifth amino acid position was point-mutated into (ATC) without changes in amino acid, was constructed using the forward primer 5′-d(GCGAATTCC**ATG**GCCTCAGCTATCGTTGACAAT)-3′ (*Nco*I restriction site underlined, the translation start codon in bold and the mutated nucleotide in italic) and the same reverse primer above. The amino terminal portion of chimeric gene (CASC2-CH1) was obtained from CASC1 as followings. The full length of CASC1 was first amplified using the forward primer 5′-d(GGCGAATTCC**ATG**GCCTCAGTTGCAGTTGAA)-3′ harboring the *Eco*RI and *Nco*I restriction sites (underlined), the translation initiation site (bold) and subsequent 18 bases corresponding to the coding portion of the CASC1 gene, the T_7 reverse primer, and pBSK-CASC1 as a template. The around 1.8 kbp PCR product was digested with *Nco*I and *Hin*dIII, and the 492 bp size DNA fragment corresponding to one third of N-terminal portion of CASC1 was gel purified. As for CASC-CH2, the amino terminal *Nco*I/*Hin*dIII fragment (456 bp) of TEAS was prepared as above, but using pBSK-TEAS (Back *et al*., 1994). The carboxyl terminal portion of CASC2 corresponding to bases 459-1683 was prepared as follows. The PCR amplified products with the forward T_3 primer, and the reverse primer 5′-d(GAGTGCGGCCGCAATTTTGATGGAGTCCAC)-3′ (*Not*I site underlined), and a pBSK-CASC2 as template was digested with *Hin*dIII and *Not*I, followed by the gel purification of 1,191 bp *Hin*dIII and *Not*I fragment. Both gel-purified fragments were double ligated into pET28b bacterial expression vector (Novagen, WI, USA) predigested with *Nco*I and *Not*I. The sequence integrity of chimeric genes was confirmed by DNA sequencing using the dideoxynucleotide chain termination method with Sequenase (Amersham Pharmacia Biotech).

Bacterial expression, affinity purification and sesquiterpene cyclase assay pET28b harboring the chimeric genes were transformed into *E. coli* strain BL21(DE3) (Novagen, WI, USA). Cell culture and purification procedures were previously described (Mathis *et al*., 1997). In brief, 100 ml of Terrific Broth medium supplemented with 50 μg/ml Kanamycin (Sambrook *et al*., 1989) was employed for time course assay and affinity purification. After addition with 0.3 mM IPTG, cells were further grown at 28°C for indicated time. Ten μl of cells was used for the Western blot analysis. For measuring sesquiterpene cyclase activity, one mL of cells at each time course was collected by centrifugation (5000 × *g* for 5 min at 4°C), and further enzyme assay was followed as described previously (Back *et al*., 1994). Affinity purification steps by His-Bind column was performed according to the manufacturer's recommendations (His-Bind Kit, Novagen, WI, USA). The eluted protein with 1 M imidazole was reconstituted with 20 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$ using a Ultrafree-4 Centrifugal Filter (Millipore, Bedford, MA, USA). The final sample was adjusted to storage buffer [10 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, and 50% glycerol], and stored at −20°C. The radio-labeled enzymatic products were generated by using purified proteins with 10 mM $MgCl_2$, 100 mM Tris·HCl (pH 7.0), and 10 μM [^{3}H] FPP (4 × 10^5 dpm).

Gel electrophoresis and immunoblotting Total proteins of *E. coli* cells (10 μl) were separated by SDS-PAGE (Back *et al*., 1994). After electrophoresis in an 11% (w/v) acrylamide/bis gel, the proteins were transferred to nitrocellulose membrane and

immunodetected with sesquiterpene cyclase monoclonal antibodies (gifts from Dr. Chappell at University of Kentucky, USA), as described previously (Vogeli *et al*., 1990).

Thin layer chromatography TLC was performed according to Meigs and Simoni (1997) using silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany). Radioactive enzymatic products (10,000 to 15,000 dpm) generated in the *in vitro* assays were separated using hexane:ethylacetate (4:1) as the developing solvent. After drying at room temperature, the chromatogram was treated with En3Hance surface fluorography spray (Dupont, Boston, MA) and exposed to Kodak XAR-5 film for 3 d at –80°C. Control 5-epi aristolochene and vetaspiradiene were produced by the purified enzymes (Mathis *et al*., 1997). Radioactive farnesol was generated by calf intestinal alkaline phosphatase (Gibco BRL, Gaithersburg, MD, USA) treatment of [^{3}H]-FPP.

Results and Discussion

Cloning of sesquiterpene cyclase and its molecular comparison to terpene synthases The prior paper had shown that the similarity based screening, using a tobacco 5-epi aristolochene synthase (TEAS) as a heterologous probe, had identified six positive sesquiterpene cyclases, in which the clone possessing the largest insert was characterized first, and its *E. coli* overexpression revealed that the clone functioned a 5-epi aristolochene synthase activity, a sesquiterpene cyclase found in UV-irradiated leaves of *Capsicum annuum* (CASC1). Another positive clone showing a 1.9 kb in size was now selected for its functional characterization. The overall sequence information revealed that the cDNA clone with a 1,941 bp in length consisted of 41 bp of a 5′ untranslated region, 1,683 bp of an open reading frame, and 217 bp of a 3′ untranslated region (GenBank accession number AF212433). The predicted protein of this open reading frame was made up of 560 amino acids with a calculated molecular mass of 64,907. The deduced amino acid sequence was found to have a 77% identity with CASC1 (Fig. 1), 74% with TEAS and 71% with a vetispiradiene synthase, respectively. As for comparison with other related terpene synthases, the homology ranged from 46% with delta cadinene synthase (Chen *et al*., 1995), to 43% with germacrene C synthase (Colby *et al*., 1998) and 37% with farnesene synthase (Crock *et al*., 1997). Due to its high sequence homology to terpene cyclases, we named this new clone as CASC2 (*Capsicum annuum* sesquiterpene cyclase 2).

Unlike other sesquiterpene synthases identified to date, CASC2 revealed some intriguing sequence features: it has the glutamic acid rich region with 5 consecutive glutamic acids at amino terminal very end (amino acid residues 15–19) and additional putative substrate binding motif (amino acid residues 111–115), besides the known central substrate binding site DDXXD at amino acid residues 313–317 (Starks *et al*., 1997).

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CASC1   MASVAVENNV VNHIAEEI-- IRPVADFSPS LWGDRFLSFS IDNQVETKYA 048
CASC2   ***AI*D**F ****E**EEI *****N*A** ***NS***** *N*H*****V 050
             ↑
CASC1   QEIEPLKEQT RSMLLASGRK LSETLNLIDV IERLGIAYHF EKEIDEILDR 098
CASC2   ****V***** *****DT*** *V******** *********Y *****D**KK 100

CASC1   IYNENSNFEG DVYNEDLCTC RLQFRLLRQH GYNISLKIFS KFLDGNGRLK 148
CASC2   NFLD****DE *DF*-***** A********* **K******* *LQ*E**K** 149
                      ▽
CASC1   ESLASDVLGL LSLYEASHVR SHGEDILEDA LAFSTTHLES ATPHLEYPLK 198
CASC2   ********** ********** T**D****** ********** *A***NS*** 199

CASC1   EQVRHALEQS LHKGIPRIEI QFFISSVYDK QAIKNDVLLR FAKLDYNMLQ 248
CASC2   ***T****L* *******A*T R*****I*E* *ET******* *****F*L** 249

CASC1   MLHKQELAEV SRWWKDLNFV NTLPYARDRV VECYFWALGV YYEPQYSQAR 298
CASC2   ********** *********M A********A ******T*** ********** 299

CASC1   VMLVKTIAMI SIVDDTYDAY GTVDELAIYT DVIQRWDIKE IDSLPDYMKI 348
CASC2   I**A****** ******F*S* ***K**DT** NA******S* **R******* 349

CASC1   SYKALLDLYK DYEKEMSRDG RSHVVYYAKE RLKELVKSYN IEAKWFIEGH 398
CASC2   ******Q**E *****L*SN* ******H**Q *M**I*R*** V**R***K*Y 399

CASC1   MPPASEYLRN AFVTTTYYYL ATTSYLGMKY AKEQQFEWLS KNPKILEGCV 448
CASC2   **LV****S* *LI*S***L* T********S ST**D***** ******KANA 449

CASC1   TICRVIDDIA TYEVEKNRGQ LSTGIECYMR DYSVSTKEAM AKFQEMGESG 498
CASC2   ********** ******S**E IA******R* E*G***E*G* I**S*IR*AA 499

CASC1   WKDINEGMLR PTPIPMEFLS RILNLARLVD VTYKHNEDGY THPEKVIKPH 548
CASC2   ********** *SSVT****T P****V**I* *V****Q*** ******L*** 549

CASC1   IIAMVVDSFK I   559
CASC2   *V*LL***I* *   560
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Fig. 1. Comparison of the predicted amino acid sequence between CASC1 and CASC2. Asterisks indicate identical amino acids. A single nucleotide mutation site is denoted by the arrow. The reverse triangle denotes a conserved *Hin*dIII restriction site found in sesquiterpene cyclases which was used in constructing various chimeric genes (see Fig. 3). The possible candidates of substrate binding sites are underlined. The known substrate binding site (DDXXD) is shown in bold. The cDNA sequence reported in this paper has been submitted to the GenBank under accession number AF212433.

Expression of sesquiterpene cyclase transcripts in response to UV-irradiation Full-length cDNA clones for CASC1 and CASC2 were separated by agarose gel electrophoresis, and two identical blots were probed independently with radiolabeled specific CASC1 and CASC2 probes as shown in Fig. 2A. The conditions of hybridization stringency used in this study were all identical. The specific probes generated from sequence of each 3′ untranslated region share a > 49% nucleotide sequence identity. Thus, the CASC1 and CASC2 probes were hybridized exclusively to their counterpart full length cDNAs (Fig. 2A). The expression patterns of CASC mRNA transcripts in UV-treated leaves of pepper were shown in Fig. 2B. Both mRNA transcripts showed significant induction within 3 h of treatment, reached maximum by approximately 9–12 h, then declined to lower levels by 24 h. At 36 h, the levels of both transcripts rose again. The transient induction and expression patterns of CASC1 transcript had synchronized with those of CASC2 in response to UV, suggesting both CASC genes may function similarly *in vivo*. No complementary transcripts were detected in RNA samples from control, 0.5 h, and 1 h of treatments.

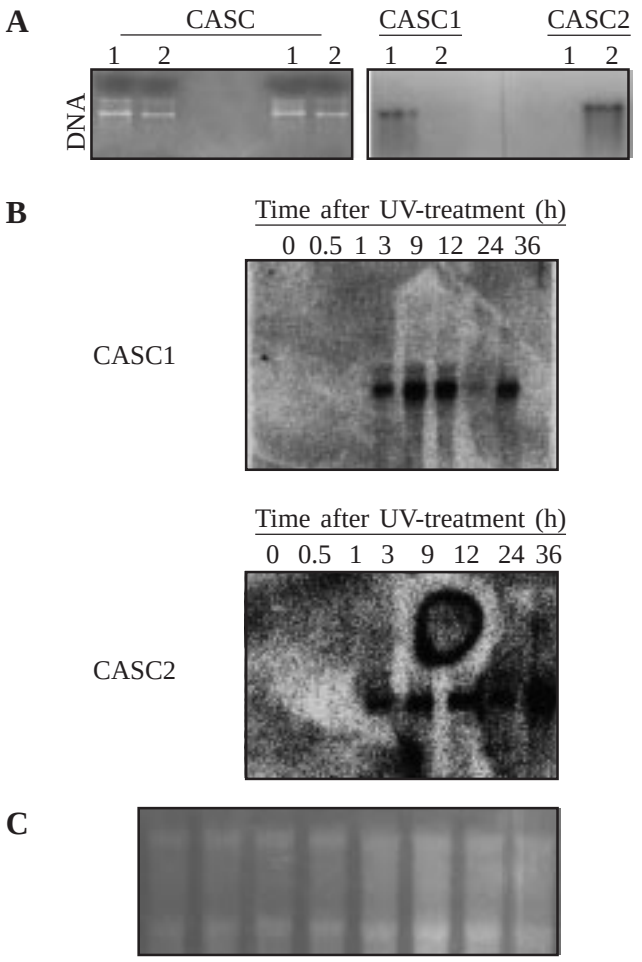

Fig. 2. Specificity of CASC probes and induction pattern of CASC genes. **A.** Each 20 ng of DNA representing CASC1 and CASC2 was stained with ethidium bromide before blotting. Blots were hybridized with specific probes described at materials and methods. **B.** RNA gel blot analysis of CASC mRNA levels. Pepper leaves were UV-C irradiated for 15 min, and leaves were collected at the indicated time points for total RNA isolation. RNA samples (10 μg) were subjected to electrophoresis, and then hybridized with CASC1 and CASC2 specific probes. **C.** The equal loading of total RNA was shown by ethidium bromide staining.

***In vitro* mutagenesis and bacterial expression studies** The privious report had shown that the intact CASC1 was able to express the functional sesquiterpene cyclase enzyme in *E. coli* system (Back *et al.*, 1998). As for CASC2, several trials to express CASC2 in *E. coli* were subjected to failure. These included different expression vectors, host strains, and low temperature induction (Das, 1990; Kim *et al.*, 1998; data not shown). The initial modification of the CASC2 gene was performed by point mutation at the fifth amino acid isoleucine**,** at which a native isoleucine codon ATA was replaced with an isoleucine codon ATC, the most favorite codon in *E. coli*, because the ATA codon is known as a very rare codon (Gribskov *et al.*, 1984). The point mutated CASC2 (CASC2-ATA5ATC) gene was inserted in several expression vectors, and examined for its functional expression. However, it was unlikely that active sesquiterpene cyclase was detected when judged by the cyclase activity and the Western blot analyses.

Chimeric CASC2 genes by domain swapping strategy and their functional expression in *E. coli* Domain swapping strategy between structurally related terpene synthases offers a promising alternative tool in order to express functional terpene synthases without interrupting enzyme integrity in *E. coli* or to generate new chimeric enzymes which are capable of catalyzing a series of new terpene products. Two chimeric constructs were generated and assessed as to whether these chimeric genes give rise to functional cyclase enzyme in bacteria (Fig. 3). The chimeric gene 1 (CASC2-CH1) prepared by ligating the 5′-terminal portion of the CASC1 cDNA (corresponding to the *Nco*I-*Hin*dIII fragment) with the 3′-terminal portion of CASC2 cDNA (corresponding to the *Hin*dIII-*Not*I) into the bacterial expression vector pET28b (Novagen, Madison, WI, USA). The CH1 gene was transformed into BL21(DE3) host strain. The immunodetectable cyclase polypeptide and cyclase activity were detected in extracts of *E. coli* expressing the CH1 gene, whereas neither cyclase polypeptide nor enzyme activity were found in *E. coli* harboring the pET28b vector only (Fig. 4A). Maximum levels of the cyclase protein and enzyme activity were observed 3 h after IPTG addition to cells. The chimeric gene2 (CASC2-CH2) generated by substituting the 5′-terminal portion of the CASC2 cDNA (corresponding to the *Nco*I-*Hin*dIII fragment) with the TEAS gene was assayed in bacteria. Significantly greater amounts of cyclase polypeptide and enzyme activity were detected in extracts of *E. coli* expressing the CH2 gene (Fig. 4B). The cyclase protein and enzyme activity reached

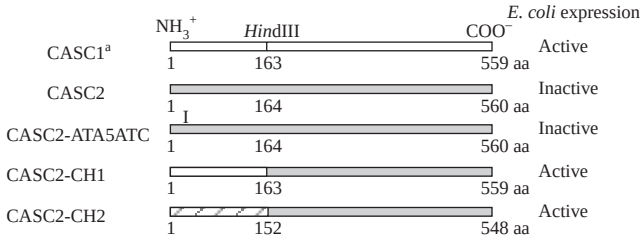

Fig. 3. Schematic diagram of chimeric gene construction. CASC1 was expressed functionally in *E. coli* (Back *et al.*, 1998). A single nucleotide change was made at the fifth amino acid, in which isoleucine codon (ATA) was mutated into isoleucine (ATC) for the favorite codon. The conserved *Hin*dIII restriction sites were employed for construction of CH1 comprising CASC1 amino terminal 164 amino acids fused with *Hin*dIII downstream portion of CASC2. Instead, 152 amino acids of TEAS (hatched box) was substituted in amino terminal region of CASC2 as for CH2.

a peak at 5 h after adding IPTG into the culture. It is worth noting that the stability of recombinant cyclase decreased at a much slower rate, showing a relatively high activity even at 10 h after IPTG addition. It was also shown that expression of both CH1 and CH2 were initiated after IPTG addition, whereas barely detectable cyclase enzymes were found before IPTG addition into the culture, suggesting the inducibility of recombinant cyclase in response to IPTG. The mechanism by which the CASC2 cDNA was not expressed in bacteria is not understood at this time. However, this phenomena may be related to protein structure formed by N-terminal CASC2 than the N-end rule (Looman *et al*., 1987; Tobias *et al*., 1991)

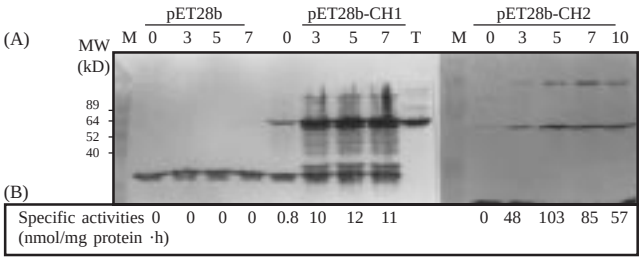

Fig. 4. Immunodetection of chimeric cyclase protein in pET28b-CH1, CH2 (**A**) and determination of the *in vitro* sesquiterpene cyclase enzyme activity (**B**). *E. coli* cultures were harvested at 0, 3, 5, 7, 10 h after IPTG treatment, and cell lysates then used for SDS-PAGE and sesquiterpene cyclase activities. M, molecular weight standard; T, purified recombinant TEAS (1 μg).

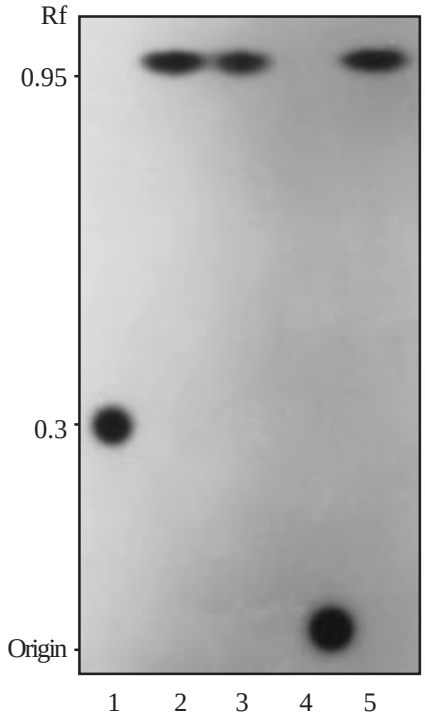

Fig. 5. Thin layer chromatogram of radiolabeled products generated by chimeric sesquiterpene cyclases. Lane 1, radioactive farnesol; lane 2, reaction product(s) of CH1 enzyme; lane 3, reaction product(s) of CH2 enzyme; lane 4, radioactive substrate FPP; lane 5, reaction product (5-epi arstolochene) of TEAS (Back *et al*., 1994).

Affinity purification and *in vitro* enzymatic product determination The two His-tagged chimeric genes were constructed by inserting the CH1 and CH2 genes into *Nco*I and *Not*I restriction sites of the pET28(b) expression vector in frame with codons for six additional histidine residues at the carboxyl termini. Two His-tagged chimeric proteins were purified by the Ni^{2+} affinity column. Coomassie blue staining intensities of the proteins separated by SDS-PAGE showed that the one step affinity chromatographic procedure resulted in purification of the His-tagged proteins to approximately 50% homogeneity (data not shown). The specific cyclase activities ranged from 10- to 20-fold for CH1 and CH2, respectively (data not shown). Thin layer chromatography was used to determine the product(s) catalyzed by the partial purified chimeric protein. In Fig. 5, the reaction product(s) of the chimeric enzymes were compared to those generated by the purified 5-epi aristolochen synthase from tobacco (Mathis *et al*., 1997). The dominant product from the recombinant chimeric enzymes migrated as a single spot with an R_f value of 0.8, similar to the products of the 5-epi aristolochene. This TLC system was unable to distinguish between 5-epi aristolochene and vetispiradiene, only showing that the reaction product(s) generated by the recombinant CH1 and CH2 proteins were like eremophilian-type products. On the analysis of Ag^+-TLC which can easily distinguish 5-epi aristolochene from vetispiradiene, the products of CH1 and CH2 proteins did not migrate on the Ag^+-TLC, suggesting that the enzymatic products seemed to be more hydrophilic than 5-epi aristolochene (R_f 0.4) or vetispiradiene (R_f 0.3) (data not shown). To identify the product(s) catalyzed by the recombinant CH1 and CH2 cyclase proteins, we now are using GC and GC-MS analyses.

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