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Isolation and characterization of β-cadinene synthase cDNA from *Chamaecyparis formosensis* Matsum

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Abstract

Chamaecyparis formosensis is a precious conifer endemic in Taiwan. To understand the sesquiterpene synthesis mechanism in this tree, full-length cDNA of a putative sesquiterpene synthase (sesqui-TPS), designated *Cf-Cad*, was obtained by rapid amplification of complementary DNA ends-polymerase chain reaction. *Cf-Cad* is 1812 bp in length. To identify its function, recombinant protein from *Escherichia coli* was incubated with farnesyl diphosphate, which produced one major product, the structure of which was elucidated by gas chromatography/mass spectrometry (GC/MS) analysis. GC/MS analysis, GC retention time and MS matching with authentic standards revealed that the major product was β-cadinene. This is the first report of the cloning, functional expression in *E. coli* and identification of a sesqui-TPS from a Cupressaceae conifer.

Keywords: β-cadinene; *Chamaecyparis formosensis*; terpene synthase.

Introduction

Chamaecyparis formosensis (Cupressaceae), commonly called Taiwan red cypress or Formosan cypress, is known as one of the "five precious woods of Taiwan" (along with *Taiwania cryptomerioides*, *Calocedrus macrolepis* var. *formosana*, *Cunninghamia lanceolata* and *Chamaecyparis obtusa* var. *formosana*). *Chamaecyparis formosensis* is endemic to Taiwan and grows at elevations of 1500–2150 m in Taiwan's central mountains (Liu et al. 1988). *Chamaecyparis formosensis* is well known for the outstanding durability of its wood and its attractive fragrance (Wang et al. 2006). The main fragrance of the wood is generated from its essential oils, which are primarily composed of terpenes and their oxygenated derivatives. Since 1931, when Kafuka and Ichikawa (1931) first studied the chemical ingredients of *C. formosensis*, many of its terpeonids have been identified. Forty-one terpenes have been determined in the leaf essential oils of the cypress, including α-pinene, β-pinene, 3-carene, α-terpene, γ-muurolene and kaurene (Fang et al. 1986a). Lin et al. (1999) isolated 18 sesquiterpenes and 30 diterpenes from the leaves. The composition of root, bark, wood and cones of *C. formosensis* has also been studied (Nozoe et al. 1996; Fang et al. 1986b; Hsu et al. 1995; Chen et al. 2008). Several terpenoids from *C. formosensis* have been identified and characterized (Lin et al. 1999; Wang et al. 2005, 2006), but almost nothing is known about the biosynthesis of terpenoids in *C. formosensis* or how essential oil production is regulated. Plant terpenoids may be involved in the direct defense of plants against herbivores, and microbial pathogens. Terpenes and their oxygenated derivatives are not only responsible for the fragrance of *C. formosensis*, but also have antibacterial and antifungal activities. For example, the growth of the wood decay fungi *Laetiporus sulphureus* and *Trametes versicolor* were inhibited in the presence of essential oil of *C. formosensis* (Wang et al. 2005).

There are few genetic studies concerning metabolite biosynthesis of terpenes and terpenoids *C. formosensis*. To date, only one monoterpene synthase (mono-TPS) from *C. formosensis* has been cloned and characterized (Chu et al. 2009). Terpenes in plants are synthesized by TPSs from one of three common prenyl diphosphate precursors (Chappell 1995). These precursors, geranyl diphosphatte (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are generated from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). TPSs are the key enzymes leading to the diversity of terpenes. Mono-TPS, sesquiterpene synthases (sesqui-TPS) and diterpene synthases (di-TPS) catalyze the cyclization reactions (cyclases) converting GPP, FPP or GGPP into any of the 20 000 known hydrocarbon skeletons (Davis and Croteau 2000). These enzymes are typically either monomeric or homodimeric with molecular masses ranging from 40 kDa to 65 kDa and require Mg^{2+} for catalysis.

The production of single or multiple compounds from a single TPS enzyme may provide an evolutionary advantage through increased resistance against potential herbivores or pathogens (Huber et al. 2004; Keeling and Bohlmann 2006; Gershenzon and Dudareva 2007). In the present study, the terpene synthase(s) of *C. formosensis*,

a sesqui-TPS, cDNA has been cloned and investigated. The full-length clone was characterized and identified as β-cadinene synthase. The results of cloning and identification will be reported.

Materials and methods

Plant material and RNA isolation

The sample of a 25-year-old *C. formosensis* was obtained from the Chi-Tou Tract of the Experimental Forest of National Taiwan University and was identified by Sheng-Yang Wang (Department of Forestry, National Chung-Hsing University). Total RNA was isolated following the method reported in Chu et al. (2009). Briefly, the leaf tissue was frozen in liquid nitrogen, ground into powder and then suspended in an extraction buffer with 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidinone K30, 100 mM Tris-HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid (EDTA), 2.0 M NaCl, and 0.5 g spermidine l^{-1} and 2% (v/v) β-mercaptoethanol. The homogenate was extracted twice with an equal volume of chloroform/isoamyl alcohol (24:1). Total RNA was precipitated overnight with 10 M LiCl at 4°C and then dissolved in RNase-free water and stored at -80°C.

Isolation of partial- and full-length TPS cDNA

To isolate sesqui-TPS cDNAs, partial cDNA fragments were obtained using degenerate primers, TPSs-F [5′-CA(T/C)-ACGACATGTGGAG(A/C)TTCTACT–3′] and TPSs-R [5′-CATG(A/T/C)ATCTAAAGCTTGC(A)TTCCT-3′] designed from nucleotide alignments of conifer TPS sequences. The full-length cDNA of putative terpene synthase was isolated by means of the method "rapid amplification of complementary DNA ends (RACE)", which was performed by the SMART RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ, USA) with the primers, TPS-5′RACE: 5′-GGTTCTTCGTTGTTGATATTCTGCAGAGC-3′, and TPS-3′RACE: 5′-ACTCCCTTCATGCACATTGAAGCATCAG-3′ for polymerase chain reaction (PCR) amplification. The PCR products were cloned by means of pGEM-T Easy Vector System (Promega, Madison, WI, USA) and sequenced with ABI 377 automatic sequencer (Perkin Elmer, Waltham, MA, USA).

Detection of *Cf-Cad* transcript in different tissues

Cf-Cad transcripts in different tissues were detected by reverse transcription (RT)-PCR. To perform RT-PCR, 1 μg of total RNA was isolated from different tissues to make cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT) primer (Invitrogen) following the manufacturer's protocol. To amplify *Cf-Cad*, forward and reverse primers were applied: 5′-AAAGAAGATTCTAGTTGAACCTAT-3′ and 5′-GTCACATTCCTTTACAATCCTATA-3′. The forward primer was 372 bp upstream from the stop codon and the reverse primer included the stop codon and a 9-bp three prime untranslated region (3′UTR). Gene-specific primers were designed for RT-PCR to analyze the expression pattern of the TPS genes in different tissues, including the leaves, stems, xylems, phloems and roots. PCR conditions: 2 min denaturation at 94°C followed by 28 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and then 72°C for 7 min. An actin gene was amplified simultaneously as a control with the forward and reverse primers: 5′-CCGGTATTGTTCTCGATTCTGG-3′ and 5′-GTAGGTTGTCTCATGGATACCTG-3′.

Protein expression and purification

The truncated open reading frame (ORF) of the *Cf-Cad* gene was synthesized with cDNA as a PCR template. The product of the PCR was ligated to the fusion protein expression vector, pTYB12 (New England BioLabs, Ipswich, MA, USA) by different cloning sites (*Spe*I and *Xho*I) using the IMPACT™-CN protein purification system. The resultant construct was expressed in *Escherichia coli* BL-21 (DE3) (Novagen, Madison, WI, USA). The transformed *E. coli* cells were cultured overnight at 37°C in Luria-Bertani medium and induced with 0.4 mM isopropyl-b-D-thiogalactoside (IPTG) at 16°C for 20 h. pTYB12 is a N-terminal fusion vector in which the N-terminus of the *Cf-Cad* is fused to the intein tag. The intein-*Cf-Cad* fusion protein was purified on an affinity chitin bead resin column (New England BioLabs). The intein underwent specific self-cleavage when 50 mM cysteine and 1 mM dithiothreritol (DTT) were added for 20 h at 16°C. The *Cf-Cad* was released from the chitin-bound intein tag column (Chong et al. 1997). The eluted protein was collected and dialyzed against phosphate buffered saline (PBS buffer: 13.7 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4; pH 7.0). The molecular mass of the purified protein was determined under denaturing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined with a Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA).

Functional identification

The activity of TPS was measured in vitro by gas chromatography/mass spectrometry (GC/MS) analysis as described by Lewinsohn et al. (1991) and Martin et al. (2004) with slight modifications. Briefly, the purified protein (30 μg) was added to sesqui-TPS buffer (25 mM HEPES, pH 7.2, 100 mM $MgCl_2$, 10% glycerol, 10 mM DTT) with 30 μM FPP, 1 mM ascorbic acid, and protease inhibitor. The recombinant plasmid pTYB12/sesqui-TPS was transformed into *E. coli* BL21 (DE3) for protein expression. The soluble intein-Cf-Cad fusion protein was purified on an affinity chitin column. After a specific on-column cleavage of intein, nsp-Cf-Cad was cleaved, eluted from the column and molecular weight was confirmed by SDS-PAGE. Most of the intein-Cf-Cad fusion protein was cleaved by a cysteine and DTT mixture at 16°C. The reaction mixture was then covered with 1 ml of pentane to trap volatile products and incubated at 30°C for 1 h. After extracting with pentane, the extractions were combined and passed through a silica gel column (500 mg, 1 cm×1 mm i.d.). Sesquiterpene products were analyzed by ITQ Series GC/MS system equipped with a DB-5 capillary column (30 m×0.25 mm i.d., film thickness 0.25 mm). The oven temperature was held at 60°C then programmed to increase from 60°C to 120°C at a rate of 5°C min^{-1}, and then programmed to increase to 300°C at a rate of 15°C min^{-1} and held for 10 min. Other parameters were: injector temperature 250°C; ion source temperature 230°C; EI 70 eV; carrier gas He at a flow rate of 1 ml min^{-1}; split ratio 1:50; mass range 50–400 *m/z*. Identification of the major compound synthesized by sesqui-TPS of *C. formosensis* was confirmed by comparison with authentic standards (from Y. H. Kuo, China Medical University), and comparison of its Kovats index (Adams 2001), and on the basis of its MS fragmentation [Wiley (V. 7.0) and National Institute of Standards and Technology (NIST) V.2.0 GC–MS library].

Bioinformatic analysis

Presequence region predictions were carried out with the TargetP 1.1 Server (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al. 2000). Multiple sequence alignment was performed with the

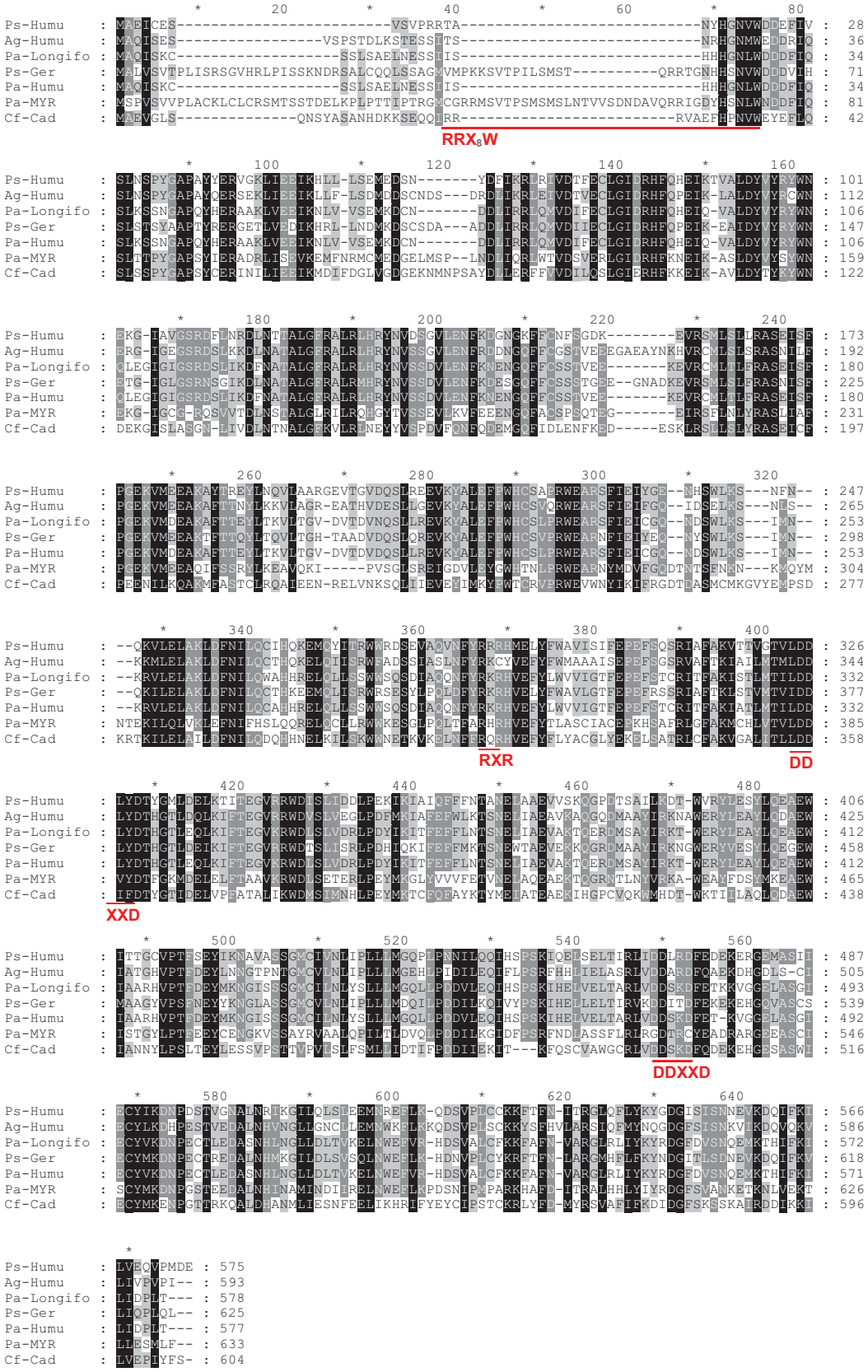

Figure 1 Amino acid alignments of *Cf-Cad* from *Chamaecyparis formosensis* with other reported coniferous terpene synthases by ClusterlX and GeneDoc. The RRX_8W, DDXXD and RXR motifs are shown. Conserved similarity shading is based on 100% (black), 70% (dark gray) and 60% (light gray).

ClustalW program at EMBL (http://www.ebi.ac.uk/) and GeneDoc. Homologs of sesqui-TPS were identified by BLAST algorithms at National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic and molecular evolutionary analyses of the terpene synthases data was performed using MEGA v.4.1 (Tamura et al. 2007). Phylogenetic analysis was performed by ClustalW and a phylogenetic tree was generated based on the neighbor-joining algorithm from Phylip3.6 software. The TreeView (v.1.6.6) program was used to visualize the format of the phylogenetic tree. Distance analyses were completed on 1000 bootstrap replicated data sets (SEQBOOT) using PRODIST. The protein structure predictions of the *C. formosensis Cf-Cad* was processed by the SWISS-MODEL Workspace (http://swissmodel.expasy.org/) (Arnold et al. 2006). Validation of the predicted structure models was performed using the PROCHECK program (Laskowski et al. 1996) and the Verify 3D web server (Luthy et al. 1992). Illustrations of the tertiary structure and interaction prediction were viewed and edited by the Swiss-Pdb Viewer (Guex and Peitsch 1997).

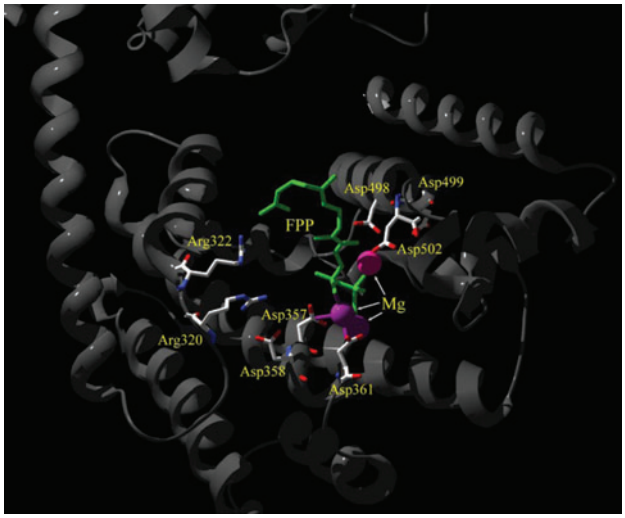

Figure 2 Predicted three-dimensional structure of *Cf-Cad*. The side chains illustrated are the motifs $D^{357}D^{358}XXD^{361}$ and $D^{498}D^{499}XXD^{502}$. Mg^{2+} ions are shown as small pink spheres and the FPP complex is shown in green.

Results and discussion

cDNA cloning of sesquiterpene synthases from *C. formosensis*

The full-length cDNA of a putative sesqui-TPS, designated as *Cf-cad* (GeneBank accession no. JN715077) was obtained by PCR and RACE extension. *Cf-Cad* has an 1812-bp ORF which encodes a 603-a.a. protein (Figure 1). The predicted molecular weight of the polypeptide was 71.0 kDa and the theoretical isoelectric point was 5.12. Highly conserved regions of

Table 1 Similarity and identity analysis of *Cf-Cad* with other known terpene synthases.

Enzyme	Species	Accession number	Similarity (%)
Monoterpene synthase			
Phellandrene	*Abies grandis* (Ag)	AAF61453	62 (38)
pinene	*Abies grandis*	AAB71085	59 (37)
Limonene	*Abies grandis*	AAB70907	60 (35)
Limonene/pinene	*Abies grandis*	AAF61455	59 (35)
Myrcene	*Abies grandis*	AAB71084	61 (38)
Pinene	*Chamaevyparis formosensis* (Cf)	ABW80964	15 (6)
Myrcene	*Picea abies* (Pa)	AAS47696	60 (37)
limonene	*Picea abies*	AAS47694	59 (37)
phellandrene	*Picea abies*	AAK39127	58 (36)
pinene	*Picea sitchensis* (Ps)	AAP72020	59 (37)
Linalool	*Picea sitchensis*	ABA86247	58 (37)
pinene	*Pinus tabuliformis* (Pt)	ABY65904	58 (37)
pinene	*Pinus taeda* (Pta)	AAO61225	59 (36)
Sesquiterpene synthase			
γ-humulene	*Abies grandis*	AAC05728	63 (40)
bisabolene	*Abies grandis*	AAK83562	59 (41)
Longifolene	*Picea abies*	AAS47695	62 (40)
γ-humulene	*Picea abies*	AAK39129	61 (39)
bisabolene	*Picea abies*	AAS47689	60 (40)
selinene-like	*Picea sitchensis*	ABA86249	61 (39)
1(10),5-germacradien-4-ol	*Pinus sylvestris* (Psy)	ABV44453	61 (39)
farnesene	*Pinus sylvestris*	ADH29869	58 (38)
Bisabolene	*Pseudotsuga menziesii* (Pm)	AAX07266	59 (39)
Farnesene	*Pseudotsuga menziesii*	AAX07265	57 (38)
Diterpene synthase			
Levopimaradiene	*Ginkgo biloba* (Gb)	AAS89668	57 (36)
taxa-4(5),11(12)-diene	*Taxus canadensis* (Ta)	AAR13860	57 (36)
taxadiene	*Taxus wallichiana* var. *chinensis* (Twc)	AAG02257	56 (36)
taxadiene	*Taxus wallichiana* var. *mairei* (Twm)	AAY16197	56 (36)

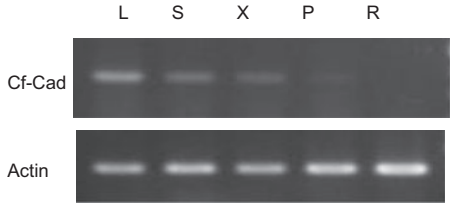

Figure 3 Expression of *Cf-Cad* in different tissues. RT-PCR analysis of *Cf-Cad* expression using the constitutive gene actin as an internal control. Total RNA was extracted from leaves (L), stems (S), xylem (X), phloem (P) and roots (R).

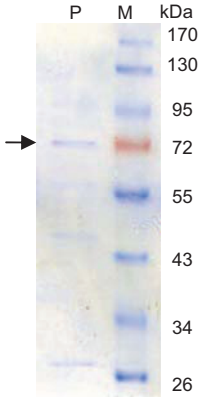

Figure 4 SDS-PAGE analysis of the proteins expressed in *E. coli* pTYB12 system by IPTG induction. Lane M: protein molecular marker. Lane P: purified protein. The gel is stained with Coomassie blue.

terpene synthase genes included a conserved RXR ($R^{320}XR^{322}$) motif and two aspartate-rich DDXXD ($D^{357}D^{358}XXD^{361}$ and $D^{498}D^{499}XXD^{502}$) motifs. Typically, terpene synthases include two motifs, DDXXD and (N/D)DXX(S/T)XXXE (NSE/DTE) (Christianson 2006; Noel 2007); however, several conifer sesquiterpene synthases have two aspartate-rich motifs (DDXXD) without a NSE/DTE motif (Davis and Croteau 2000). The following sesqui-TPS proteins, including Humulene synthase from *Pinus sylvestris* (ABV44452), Humulene synthase from *Abies grandis* (AAC05728), Longifolene synthase from *Picea abies* (AAS47695), 1(10),5-germacradien-4-ol synthase from *P. sylvestris* (ABV44453), γ-humulene synthase-like protein from *P. abies* (AAK39129) and Myrcene synthase from *P. abies* (AAS47696) were selected for analysis of this sesquiterpene synthase (Figure 1). The sequences analyzed are listed in Table 1. Homology analysis (similarity and identity) was performed by means of the BL2SEQ program at NCBI and is presented in Table 1. The amino-acid sequence of *Cf-Cad* (Figure 1) shows a high degree of similarity with several sequences, in particular with *A. grandis* γ-humulene synthase (GenBank accession AAC05728) and a longifolene synthase (GenBank accession AAS47695) from *P. abies* (63% and 62%, respectively) (Table 1).

Protein structure predictions

The protein structure of *Cf-Cad* was predicted by SWISS MODEL with a (+)-δ-cadinene synthase from *Gossypium arboreum* (GeneBank accession no. Q39761; Gennadios et al. 2009). *Cf-Cad* contains two DDXXD motifs ($D^{357}D^{358}XXD^{361}$

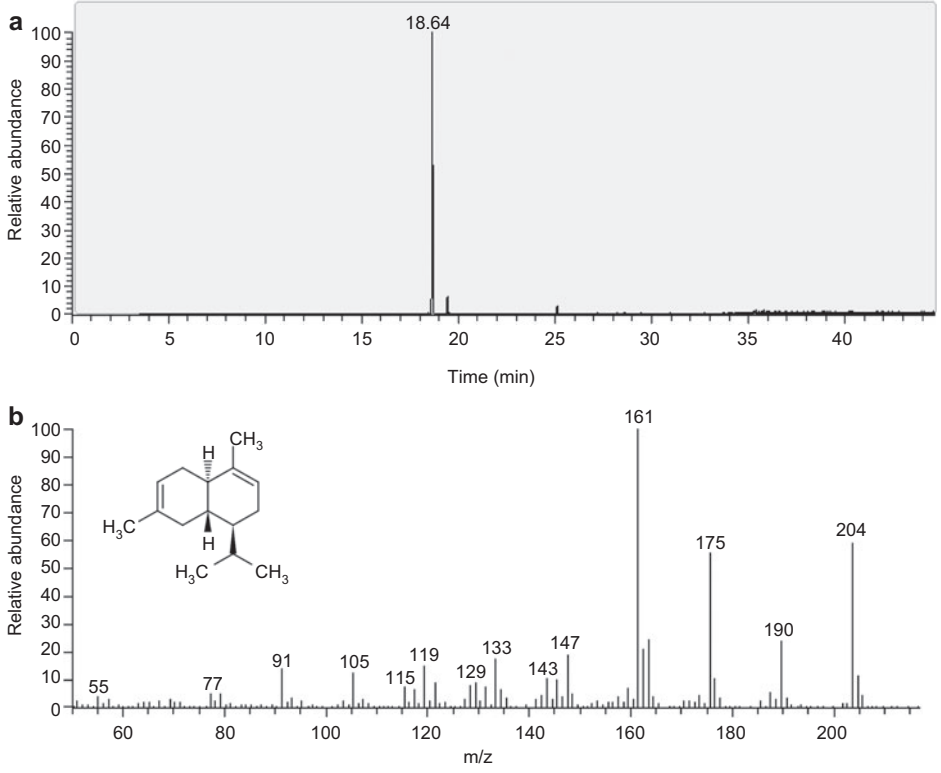

Figure 5 (**a**) GC/MS analysis of sesquiterpene products of recombinant protein product of *Cf-Cad* with farensyl diphosphate as the substrate. (**b**) MS of the product of *Cf-Cad* is β-cadinene.

and $D^{498}D^{499}XXD^{502}$), which are shown in Figure 2. The $D^{357}D^{358}XXD^{361}$ motif, which has been previously implicated in binding the substrate via divalent metal ion bridges (Cane et al. 1996), and the highly conserved RXR ($R^{320}XR^{322}$) motif, which was implicated in the complexation of the diphosphate function of the ionized substrate to prevent nucleophilic attack on any of the carbocationic intermediates (Starks et al. 1997), were found. The deduced amino acid sequences of *Cf-Cad*, when compared with other conifer terpene synthases, showed the two aspartate-rich DDXXD motifs which are involved in coordinating the bivalent metal ion for substrate binding (Köpke et al. 2008).

Expression of *Cf-Cad* in different tissues

A semi-quantitative RT-PCR method was employed to measure the steady-state transcript levels of the *Cf-Cad* gene in different tissues, including leaves, stems, xylem, phloem and roots. The leaves of *C. formosensis* showed high levels of expression of *Cf-Cad*. The stems and xylem had only a low level of expression (Figure 3). The phloem and roots showed no detectable expression of *Cf-Cad*.

Functional identification

A NCBI BLAST query identified *Cf-Cad* to be 1812 bp in length and it was predicted to function as a mono-TPS, sesqui-TPS or di-TPS. To determine the function of this gene, the activity of the full-length and truncated recombinant protein was tested with the substrates GPP, FPP and GGPP. The protein only showed activity with FPP. The recombinant plasmid pTYB12/sesqui-TPS was transformed into *E. coli* BL21 (DE3) for protein expression. The soluble intein-*Cf-Cad* fusion protein was purified using an affinity chitin column. After a specific on-column cleavage of intein, nsp-*Cf-Cad* was cleaved, eluted from the column and a molecular weight of 71.0 KDa was confirmed using SDS-PAGE (Figure 4). The KI value of *Cf-Cad* is 1476. Based on comparison of

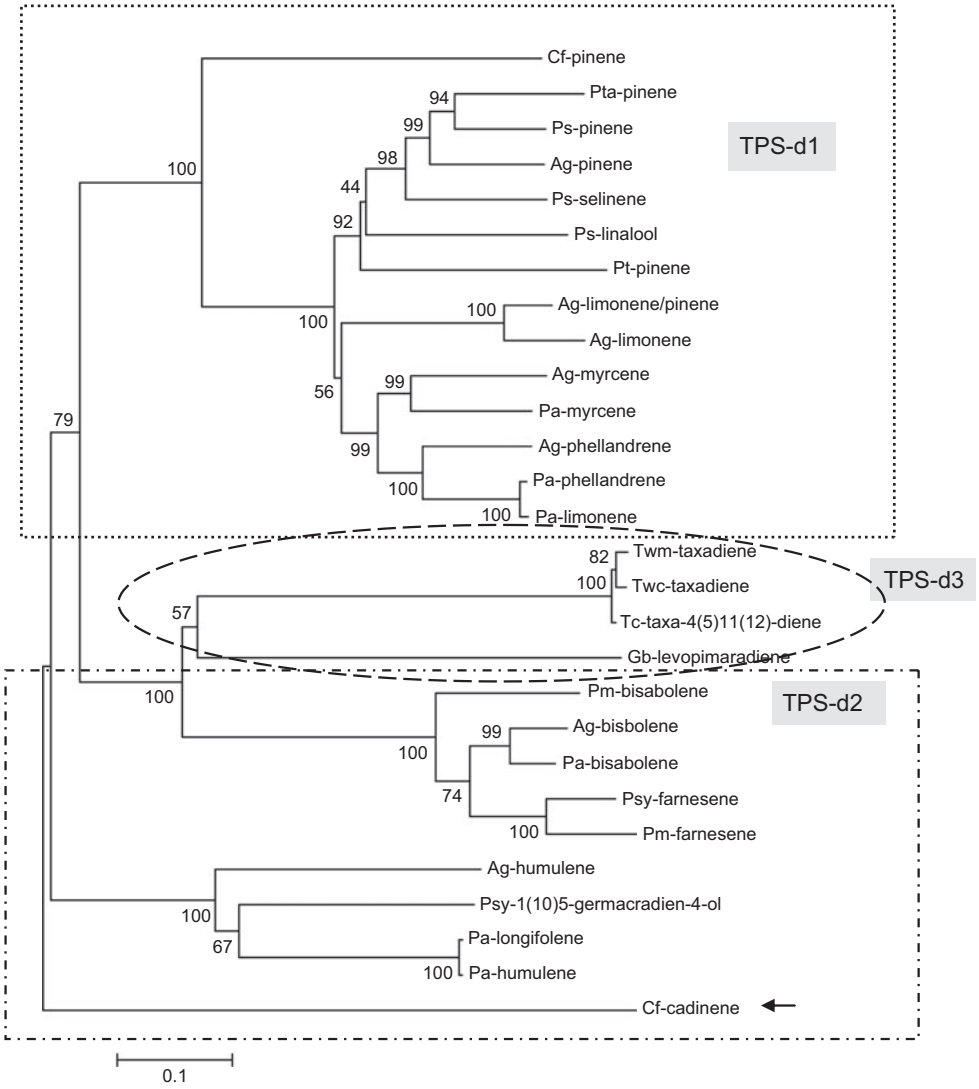

Figure 6 Phylogenetic tree of *Cf-Cad* with other known TPS amino acid sequences. Other terpene synthases compare with Table 1.

the retention time to calculate temperature, KI and MS with a study that investigated the composition of the essential oil extract from *C. formosensis*, the major product was determined to be β-cadinene (Figure 5) (Da Silva et al. 1999; Tellez et al. 1999; Wang et al. 2005; Cheng et al. 2006). According to the search result from NCBI gene bank (http://www.ncbi.nlm.nih.gov), *Cf-Cad* is the first sesqui-TPS to be identified and its function characterized in a Cupressaceae family member. The cloning and identification of this TPS cDNA may provide important information for understanding the molecular genetics and biosynthesis of terpenes in conifers. According to the MetaCyc database (http://metacyc.org/), (*2E,6E*)-farnesyl diphosphate <=> (+)-β-cadinene + diphosphate, but the direction of this reaction is not confirmed.

Homology analysis

The TPS family in plants has been classified into seven subfamilies (TPS-a through to TPS-g), based on sequence relatedness, as well as functional assessment (Martin et al. 2004). To date, all known gymnosperm TPSs are clustered in the TPS-d subfamily, which contains mono-TPS (TPS-d1), sesqui-TPS (TPS-d2) and di-TPS (TPS-d3) (Keeling and Bohlmann 2006). In this study, *Cf-Cad* was obtained from a tree belonging to the Cupressaceae. *Cf-Cad* clustered with the TPS-d2 subfamily, which belongs to a sesquiterpene synthase group (Figure 6), as expected.

Conclusions

A sesquiterpene synthease gene, *Cf-Cad*, was cloned from *C. formosensis*. The full length of *Cf-Cad* was 1812 bp and it was functionally expressed in *E. coli*; the product of the protein was β-cadinene. *Cf-Cad* is the first sesquiterpene synthase gene to be identified from a Cupressace family member that has β-cadinene as a product. Further study of this TPS in *C. formosensi*s, including its physiological function and its interaction with the environment is ongoing in our laboratory.

Acknowledgements

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