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SHORT COMMUNICATION

Molecular cloning and expression analysis of a putative sesquiterpene synthase gene from tea plant (*Camellia sinensis*)

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Abstract A putative gene encoding germacrene D synthase (GenBank Accession No. JQ247185) from tea plant (*Camellia sinensis*) was isolated by rapid amplification of cDNA ends for the first time, which was designated as tea plant gene terpene synthase 1 (*Cstps1*). This full-length cDNA was 2,090 nucleotides, and the open reading frame was 1,704 bp encoding polypeptides of 568 amino acids. The deduced amino acid sequence contained two characteristic conserved motifs of RRx_8W and DDxxD, which showed high homology with sesquiterpene synthases of angiosperms, and the highest level of similarity was 73 % to germacrene D synthase from *Actinidia deliciosa* (Accession NO. AAX16121.1). Phylogenetic analysis indicated that *Cstps1* belonged to the terpene synthase-a subfamily of plant, which was the largest clade of terpene synthases and mainly composed of sesquiterpene synthases. The *Cstps1* expression was detected in different organs, with high expression levels in leaf and flower, weak expression in stem and root. Real-time qRT-PCR indicated that *Cstps1* could be induced by insect damage, this result suggested *Cstps1* might have potential ecological function during tea plant defense response.

Keywords Tea plant · *Camellia sinensis* · *Cstps1* · Terpene synthase · Gene

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Introduction

Terpenes, or terpenoids comprise a very large class of plant secondary metabolites (approximately 25,000 terpene structures reported), that serve a variety of different functions in basic and specialized metabolism (Chen et al. 2004; Gershenzon and Dudareva 2007). Terpenes are one of the major volatile components of flower and fruit volatiles, they also have important roles in ecology such as direct and indirect defense, allelopathy, and tritrophic interactions (Gershenzon and Dudareva 2007; Nieuwenhuizen et al. 2009). When attacked by herbivores, plants may increase the emission of volatile blends; the volatile organic compounds (VOCs) influence herbivore behavior directly or indirectly through attracting the natural enemies (Kessler and Baldwin 2001). Terpenes are the key members of induced VOCs (Howell et al. 2000; Van Poecke et al. 2001; Schnee et al. 2006; Lin et al. 2008), which have versatile protective functions in plants.

In higher plants, terpenes are biosynthesized via two separate pathways: the cytosol-localized mevalonic acid (MVA) pathway and the methyl-D-erythritol 4-phosphate pathway in plastids (Rohmer et al. 1993; Chappell et al. 1995; Chen et al. 2011). In both pathways, terpene synthases (TPSs) are the key regulatory enzymes during terpenoids biosynthesis (Chappell et al. 1995), TPSs catalyze the divergent reactions that produce kinds of terpenoids in plants. Analysis of the several sequenced and annotated plant genomes indicated that the TPS gene family is a mid-size family, with gene numbers ranging from approximately 20 to 150 (Tholl and Lee 2011; Chen et al. 2011). Under biotic stress, terpene synthase genes in plants could be activated, then released terpene volatiles which repel insects or attract natural enemies against herbivore damage (Van Poecke et al. 2001; Lin et al. 2008).

Tea, *C. sinensis* (L.) O. Kuntze, originated in China, now is grown in almost 60 countries (Hazarika et al. 2009). Various cultivars of tea plant are grown in southern China and a few provinces in northern China, which constitute one of the major supply sources of tea products worldwide. Tea plant is one perennial woody evergreen plant with a characteristic secondary metabolite, and there are approximately 100 kinds of terpenes in tea secondary metabolites and tea products (Zhang et al. 2000). To our knowledge, most of the tea terpenes are only looked as tea aromas and indexes of tea biochemical quality, few other functions were involved (Cai et al. 2009; Huang et al. 2009), even none terpene synthase gene was isolated from tea plant.

In this paper, we cloned a putative terpene synthase gene for sesquiterpene from *C. sinensis* (designated as *Cstps1*) for the first time. The expression pattern of *Cstps1* in different organs of tea plant and under insect treatment also investigated. All the results were helpful to understand terpenes biosynthesis pathway in tea plant, the mechanism of induced defense response, and tritrophic interaction among insect tea enemy.

Materials and methods

Plant materials and reagents

Tea plants (*Camellia sinensis.* cv. Longjing 43) were cultured from seeds in commercially available vermiculite kept in light incubator under controlled conditions (25 °C and 10/14 h light/dark photoperiods) and 85 % relative air humidity. All the harvested organs (roots, stems and leaves were collected from seedlings, flowers were picked from flowering field plants) were immediately frozen in liquid nitrogen and stored at −80 °C until nucleic acid were extracted. Super SMART^{TM} PCR cDNA Synthesis Kit and Advantage® Polymerase Mix were purchased from Clontech Laboratories Inc., USA. QIAquick PCR Purification Kit was purchased from QIAGEN Co., Germany, pGEM-T Easy vector was purchased from Promega Co., USA. Other reagents were obtained from Sigma or Aldrich Chemical Co., USA. Primers were synthesized by Invitrogen Corporation Shanghai Representative Office, China. DNA sequencing was performed by Hangzhou Genomics Institute, China.

Plant treatment and RNA extraction

Tea plant materials were first lysed with TRIzol, then RNA were collected with RNeasy spin column (polysaccharide and polyphenol total RNA isolation kit, BioTeke Corporation, China). Three-week-old seedlings were used for the treatments, intact leaves were used as control. Three-third instar larvae of tea geometrids (*Ectropis obliqua* hypulina) were starved for 24 h, two or three larvae were placed on the foliage for feeding damage. All the infested leaves were harvested at 6, 12, 24, 48, 72 and 96 h after geometrid removal. The quality and concentration of the all RNA products were checked via NanoDrop spectrophotometer and formaldehyde agarose gel electrophoresis.

Cloning of *Cstps1* full-length cDNA

Total RNA from intact leaves was used to reverse transcribe the first-strand cDNA with an oligo (dT) primer designed with an adaptor sequence, which was performed according to the protocol of the SMART^{TM} RACE cDNA Amplification Kit. The 5′ and 3′ RACE PCR nest primers was designed and synthesized based on the sequence obtained from a suppression subtractive hybridization (SSH) cDNA library of geometrid-injured tea plants. Primers of *Cstps1*-5′RACE (5′-GGCAATCACTTTGGTTAGCATCCTTCT-3′) and universal primer A mix (UPM, long: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′; short: 5′-CTAATACGACTCACTATAGGGC-3′) provided in RACE kit were used to carry out the 5′-RACE-PCR under the recommended condition by the kit. The 3′RACE-PCR were performed with the 3′-RACE CDS Primer A (5′-AAGCAGTGGTATCAACGCAGAGTAC(T)30 N_{-1}N-3′) in kit and specific primer *Cstps1*-3′RACE (5′-AGTGATTGCCTTGACTTCCA-3′) under the same condition as above-mentioned. The PCR products was purified by kit and subcloned into the pGEM-T Easy Vector, transferred into *E. coli* DH5α and selected by colony PCR. The plasmids were extracted and sequenced bidirectionally by ABI 3730 automated sequencer (Applied Biosystems, USA) with the universal pair primers of M13.

The 5′-RACE and 3′-RACE products were automatically aligned and assembled, and the full-length cDNA sequence of *Cstps1* gene was obtained by splicing. A pair of primers (*Cstps1*F, 5′-AGGTCTGCCACAGGCAAGAAGTGAA-3′; *Cstps1*R, 5′- CTCAACAAAAAAATTCCTTTATTAA) designed according to 5′ and 3′ end sequences were used to amplify the full-length sequence with 3′RACE-Ready cDNA as template under the following conditions: denaturing for 3 min at 94 °C, followed by 30 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min) and extension at 72 °C for 10 min. The PCR product was purified, then introduced into the pGEM-T Easy vector and sequenced. To avoid PCR-induced errors, three positive clones were sequenced by primer walking method and repeated for two times separately. The result was aligned again with spliced sequence above to ensure the two sequences were totally identical.

Sequence analysis

The 5′ and 3′ sequence was analyzed and spliced by DNAMAN program, primers were designed by Primer Premier software package. The *Cstps1* cDNA sequence, deduced amino acid sequence and open reading frame (ORF) were analyzed online or by corresponding bioinformatics software. The sequence comparison was performed based on web database via BLAST program (NCBI, National Center for Biotechnology Services, http://www.ncbi.nlm.nih.gov). The *Cstps1* and other *tps* genes retrieved from GenBank were aligned with CLUSTAL W online (http://www.ebi.ac.uk/Tools/msa/clustalw2). The phylogenetic tree was constructed by MEGA 4 program based on the converted format of CLUSTAL W alignments.

Expression analysis

The *Cstps1* transcript in different organs (roots, stems, leaves and flowers) of tea plant were investigated by semiquantitative one-step reverse transcriptase (RT)-PCR, expression profiles under biotic treatments were detected by real-time qRT-PCR. Aliquots of 1 μg total RNA were isolated from 100 mg of roots, stems, leaves and petals, first-strand cDNAs were synthesized and used as templates in RT-PCR (F, 5′-GATGACAAAGCCAACGATGA-3′; R, 5′-CCCTAAGATGTGCAGCCTC-3′) reactions. RT-PCR amplifications were performed in 25 μl reaction volumes under the following conditions: denaturing at 94 °C for 3 min, followed by 30 cycles of amplification (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s), then extension at 72 °C for 10 min. Aliquots of 1 μg total RNA from damaged leaves were used as templates to perform real-time qRT-PCR following the manual of PrimeScript® RT reagent Kit. The 18SrRNA gene (18SrRNA-F, 5′-CGGCT ACCACATCCAAGGAA-3′; 18SrRNA-R, 5′-GCTGGAA TTACCGCGGCT-3′) was used as the internal control gene in both the experiments (Sun et al. 2010).

Results and discussion

Two fragments, a 1,074 bp long 3′-flanking region with poly (A) and a 1,026 bp long 5′-region (10 bp overlap) were obtained by RACE method, respectively. Blast results showed both sequences were high homologous with sesquiterpene synthase genes of angiosperms. Primers *Cstps1*F and *Cstps1*R based on 5′ and 3′ end sequences were used to amplify the full-length sequence, followed by sequence analysis as a further verification. The complete nucleotide sequence was termed tea plant gene terpene synthase 1 (*Cstps1*) and registered in GenBank (Accession NO. JQ247185). The full-length cDNA of *Cstps1* was 2,090 bp long including a 5′-untranslated region (UTR) of 99 bp and a 3′-UTR of 287 bp, and the ORF was 1,704 bp with 568 deduced amino acids. The deduced amino acid sequence of *Cstps1* (CsTPS1) contained motifs of RRx_8W and DDxxD, which were commonly conserved in sesquiterpene synthase genes. The theoretical isoelectric point (pI) and molecular weight (MW) of CsTPS1 were 5.35 and 65.6 kDa calculated by the online Computer pI/Mw Tool (http://cn.expasy.org/tools).

The deduced amino acid sequence CsTPS1 was compared by Blast P online (http://www.ncbi.nlm.nih.gov/), which showed higher homology with conserved elements of mono- and sesquiterpene synthases including the most highly conserved metal ion-binding motif of DDxxD (Liat et al. 2003; Chen et al. 2004, 2011; Nieuwenhuizen et al. 2009). The CsTPS1 was most similar to sesquiterpene synthases of angiosperms, with the highest level of similarity (73 %) to germacrene D synthase from *Actinidia deliciosa* (Accession NO. AAX16121.1). A phylogenetic tree was constructed based on the CsTPS1 and other plant TPSs via CLUSTAL W2 online (http://www.ebi.ac.uk/) and MEGA 4 software. The TPSs superfamily was subdivided into seven subfamilies including TPS-a, b, c, d, e/f, g and h based on their amino acid sequence relatedness (Chen et al. 2011; Tholl and Lee 2011). The phylogenetic result indicated that CsTPS1 was a TPS-a member (Fig. 1).

The one-step RT-PCR result showed that *Cstps1* was expressed in all tested organs, the highest transcript level was in leaves and flowers with no obvious difference, followed by stems, and the weakest expression was in roots (Fig. 2). Most of the TPS-a members were sesquiterpene synthases with roles in ecological plant interactions, rather than roles in primary plant metabolism (Chen et al. 2011). In several plants, the germacrene D synthase gene were found to be induced by insects hurt, such as in hybrid poplar (Populus trichocarpa × deltoides), the germacrene D synthase gene *PtdTPS1* was significantly induced after 24 h of tent caterpillars feeding (Arimura et al. 2004). To assay the function of *Cstps1* in tea plant defense, seedlings were treated with starved tea geometrids, RNA were isolated and detected, then investigated by real-time qRT-PCR. The result showed that *Cstps1* was significantly induced after 6 h of herbivore feeding. *Cstps1* transcript level was from 1.61 to 2.64-fold as that of intact control after infested 6, 12, 24, 48 and 72 h, and decreased obviously as 0.53-fold of control after 96 h of being damaged (Fig. 2). Our observation suggested that *Cstps1* had potential defense function in tea plant against insect attack.

Germacrene D was a sesquiterpene and produced from farnesyl diphosphate by germacrene D synthases (EC 4.2.3.75) (Picaud et al. 2006). This compound was one of the plant second metabolisms known as an essential oil,

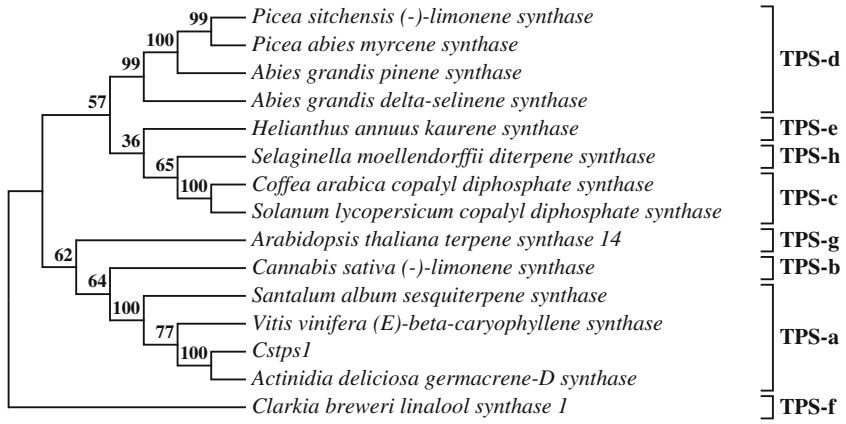

Fig. 1 Neighbor-joining tree based on the degree of sequence similarity between members of TPS clades. *A. deliciosa* germacrene D synthase (AAX16121.1), *V. vinifera* (*E*)-beta-caryophyllene synthase (ADR74192.1), *S. album* sesquiterpene synthase (ACF24768.1), *C. sativa* (−)-limonene synthase (ABI21837.1), *P. sitchensis* (−)-limonene synthase (ABA86248.1), *A. grandis* pinene synthase (AAB71085.1), *A. grandis* delta-selinene synthase (AAK83561.1), *P. abies* myrcene synthase (AAS47696.1), *A. thaliana* terpene synthase 14 (AEE33874.1), *C. breweri* linalool synthase 1 (AAD19838.1), *H. annuus* kaurene synthase (CBL42917.1), *C. arabica* copalyl diphosphate synthase (ACQ99373.1), *S. lycopersicum* copalyl diphosphate synthase (NP_001234008.1), *S. moellendorffii* diterpene synthase (AEK75338.1)

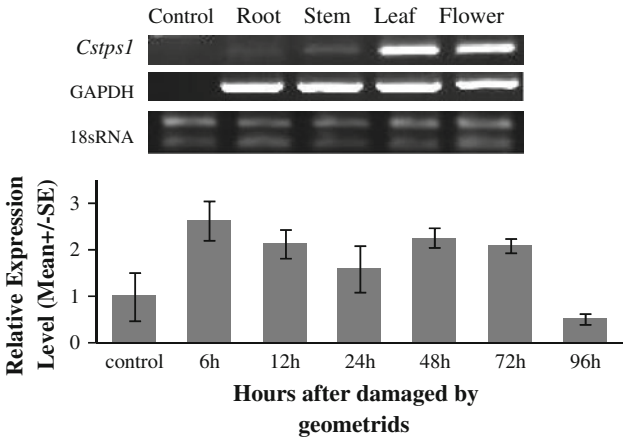

Fig. 2 The Expression profile in organs of tea plant (*above*) and transcription quantification under biotic stress (*below*) of *Cstps1* gene. Gene transcript in roots, stems, leaves and flowers were detected via one-step RT-PCR, control was to check DNA contamination. Transcript levels of *Cstps1* in leaves damaged by geometrids after 6, 12, 24, 48, 72 and 96 h were detected by qRT-PCR. Values are the mean (±SE) of three replicates, 18S rRNA gene was used as the internal control

which was also a volatile with ecological function involved in the interaction among plant insect enemy (Røstelien et al. 2000). Germacrene D synthase belonged to the terpene synthase family, and several germacrene D synthase genes had been identified from plants such as *Vitis vinifera*, *Actinidia deliciosa* and *Zingiber officinale* (Lücker et al. 2004; Picaud et al. 2006).

We first cloned a putative gene *Cstps1* encoding sesquiterpene synthase from *Camellia sinensis*. This gene was most similar to sesquiterpene synthases of angiosperms with both characteristic conserved motifs of DDxxD and RRx_8W. Further, expression profiles suggested that *Cstps1* expression levels were different in tested organs and could be induced under herbivore damage. All the results were helpful to understand the terpenes biosynthesis in the MVA pathway of tea plant and tea-insect interaction at the molecular level.

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