Partial Cloning and DNA Sequencing of Terpene Synthase-4 Gene (*TPS-4*) in *Origanumonites* L.

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Abstract

Thyme plant is commercially widely produced plant species that used in many fields as drug, cosmetic and spice. Especially Origanumonites L. plant species belonging to the Lamiaceae family is most cultivated thyme species in the world. Many thyme species contain plenty of volatile oil within. Terpenes are compounds that providing aromatic flavor to plants and the smell of their own. Terpenes constituted basis structure of volatile oil in aromatic plants were cultivated for years. Terpene synthases synthesize various terpenes in volatile oil produced as secondary metabolite. The aim of this study is to determine existence of terpene synthase 4 (TPS-4) gene in O. onites L. and to clone. The degenerative primers were designed for TPS-4 gene. RNA isolation from O. onites was performed with TRIzol reagent. cDNAs were obtained by revers transcription from mRNAs. The partial DNA fragments of TPS-4 gene were amplified by RT-PCR method using designed primers. Amplified TPS-4 gene fragments were added to pGEM-T Easy vector with ligation reaction. Recombinant vectors including TPS-4 gene fragments were transformed to XLI-Blue strain of E. coli. The amplified vectors in host bacteria were isolated for DNA sequencing. DNA sequence analysis was performed and DNA

sequence was analyzed by using NCBI database. DNA sequence data were confirmed for TPS-4 gene. The sequence data presented in this study is the first report for a TPS-4 gene in O. onites. The DNA sequence and primers obtained from this study can be used as an important initial data in TPS-4 gene expression and biotechnology studies in the future.

Keywords: *Origanumonites*, Terpene sythase-4, Gene cloning, DNA sequencing

1. Introduction

Origanumonites(Labiate, Lamiaceae) is commercially cultivated aromatic plant species in the world. It is known to have antiseptic, antibacterial and antispasmodic characteristics due to its volatile oils and terpenic materials (Başer et al., 1993; Aligiannis et al., 2001; Lambert et al., 2001; Manohar et al., 2001). In several studies, it was reported that environmental factors affect the yield and the content of the essential oils in *Thymus piperella* L. (BoiraandBlanquer, 1998) and *O. onites*(Baydar, 2005). Terpene synthases are the primary enzymes in the formation of low-molecular-weight terpene metabolites. Rapid progress in the biochemical and molecular analysis of terpene synthases has allowed significant investigations of their evolution, structural and mechanistic properties, and regulation (Tholl, 2006;et al., 2016). Terpenes are not only the largest group of plant natural products and comprising at least 30.000 compounds, but also contain the widest assortment of structural types (Degenhardt et al., 2009; Yang et al., 2013). Terpene synthases are thus a principal reason for the diversity of terpene products in the plant kingdom, and these catalysts have been the subjects of detailed studies (Köllner et al., 2004).Terpenes/terpenoids have great industrial uses as flavors, fragrances, high grade lubricants, biofuels, agricultural chemicals, and medicines (Jiang et al., 2016). The global industry recovers about 3 million tons of terpenes annually (Mewalal et al., 2016). Therefore, the researches about characterization andgenetically identification of various terpene synthases from different aromatic plantsprovide advantages in increasing of terpene production in industry.

2. Material and Methods

2.1. Plant material

O. onites seeds were provided by Professor Hasan BAYDAR, SuleymanDemirel University, Isparta, Turkey. The plants were grown on potting mixture containing peat, perlite and vermiculite (3:1:1) at 25°C under 16h photoperiod of cool white fluorescent illumination in a growth chamber (Sanyo, MLR, Japan).

2.2. RNA isolation and reverse transcription

Total RNA was isolated using a modified guanidine isothiocyanate method (Poyraz et al., 2010). *O. onites* leaves were ground to fine powder and transferred to an eppendorf tube. Total RNA was extracted with a TRIzol reagent at room temperature and recovered by isopropanol precipitation. The quantity and purity of the RNA samples were determined following spectrophotometric measurements and integrity of total RNA was also confirmed by denaturing agarose gel electrophoresis. Reverse transcription was performed according to the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) and the harvested complementary DNA (cDNA) was stored at -20°C until use.

2.3. Cloning of the middle region of cDNA

We analyzed their conservative regions by CLUSTAL Omega software in EBI (European Bioinformatics Institute)database using other plant *TPS-4* sequence in NCBI (National Center for Biotechnology Information) database, and then designed a pair of degenerative primers Forward: 5'-CTTCGWTTYCGWTTGCTTAGACAR-3' and Reverse: 5'-CAGTTCATCAAG RGTWGCATABTC-3'. The primers were used for polymerase chain reaction (PCR). The 25 µl PCR reaction mixture contained 10× PCR buffer 2.5 µl , 25 mM MgCl2 3 µl, 2.5 mM deoxyribonucleotide triphosphates 2 µl, 2.5 µM P1 and P2 2.5 µl, respectively, cDNA 5 µl, ddH2O 7.3 µl, and 1 U Taq polymerase (Promega, WI, USA). PCR reaction conditions were as follows: pre-denaturalization at 94°C for 2 min, denaturalization at 94°C for 40 s, annealing at 53°C for 50 s, extension at 72°C for 50 s, 35 cycles and final extension at 72°C for 7 min. PCR product was purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The harvested fragment was cloned in pGEM-T Easy vector system (Promega, WI, USA) and then introduced into Escherichia coli XL1B strain. Positive clones were selected after growing on LB medium supplemented with ampicillin (50 mg/ml), X-Gal (0.008%). Positive plasmids were obtained from E. coli cultures using the Qiagen Plasmid Miniprep Kit (Qiagen, Hilden, Germany), and subsequently used to sequence the middle region of the cDNA. DNA sequencing was carried out by commercial sequencing service (Macrogene, Korea).

2.4. Bioinformatics analyses

DNA sequencing analysis was performed and obtained sequence data was verified for *TPS-4* genes in the NCBI database. The NCBI database was used as a sequence source of different plant species *TPS-4* gene (www.ncbi.nlm.nih.gov/). The multiple protein sequence alignments were carried out with the Clustal W program (www.ebi.ac.uk/clustalw/) and GeneTool^{TM}(Biotools Inc., USA). The

phylogenetic dendrogram was drawn with MEGA 6.06 software for comparison with the other plant *TPS-4* genes using Maximum Likelihood Analysis Method.

3. Results and Discussion

Plant biologists and natural products chemists have long marveled over the huge variety of secondary metabolites produced by plants (Köllner et al., 2004; Jiang et al., 2016). Engineering the activities of terpene synthases provides opportunities for detailed functional evaluations of terpene metabolites in planta (Tholl, 2006). Terpenes comprise a very large class of plant secondary metabolites that serve a variety of different functions in basic and specialized metabolism (Chen et al., 2004). The diversity and variability of terpenes is due to the terpene synthases, a family of enzymes which, unlike the majority of the enzymes involved in the biosynthesis of secondary metabolites, are renowned for being able to convert a single substrate into many different products (Keszei et al., 2010). *O.onites* is an aromatic plant and the most commercially cultivated species in Turkey (Poyraz, 2007). In this study, terpene synthase-4 (*TPS-4*) is first *O.*onites L. cloning of the gene sequence and determination of the mRNA sequence information was performed. We performed PCR and obtained the expected size (~616 bp) PCR yield (Figure 1).

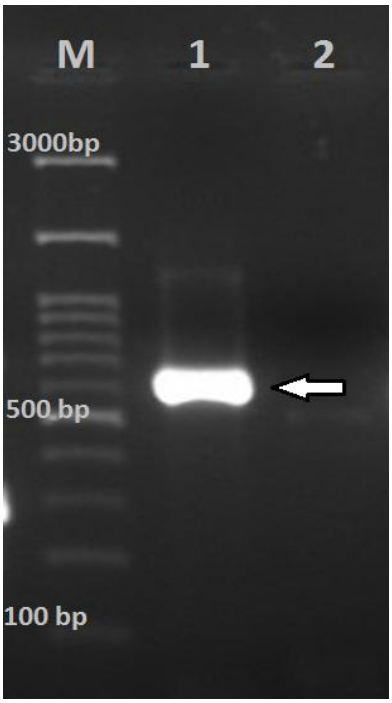

Figure 1. PCR result for partial terpene synthase 4 (*TPS-4*) from *O. onites*.4: ~616 bp PCR bands, M: 100 bp plus DNA ladder.

The DNA fragment was ligated to PGEM-Teasy vector and transformed to XL1-B *E. coli* strain. The positive colonies were selected on transformation plate and transferred to master plate for confirmation. The ligated DNA fragment was confirmed with clony PCR methods and observed same DNA fragment size (Figure 2).

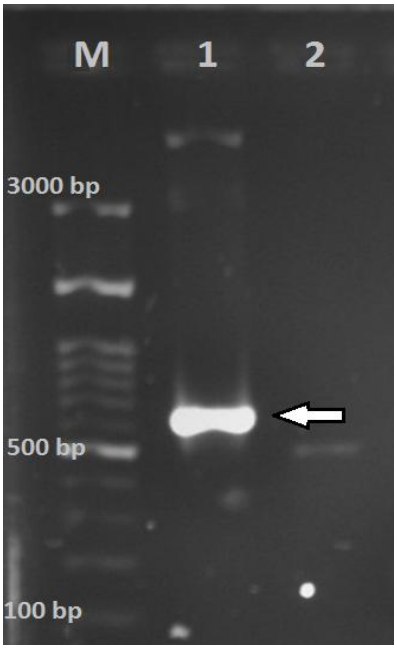

Figure 2. The confirmation of the cloned *TPS-4* DNA fragment in recombinant plasmid vector.

The DNA sequence information of partial *TPS-4* gene was obtained using a commercial DNA sequencing service. Nucleotide sequence was translated to amino acid (AA) sequence using GeneTool™ software and confirmed in NCBI database (Figure 3).

```
     L   R   F   R   L   L   R   Q   Q   G   Y   R   V
  1 ctt cga ttt cgt ttg ctt aga cag caa ggt tac cgt gtc

     P   C   D   V   F   R   K   F   T   D   G   E   G
 40 cca tgc gat gtt ttc cgc aaa ttc acc gac ggt gaa ggc

     N   F   A   T   A   L   T   N   D   V   E   G   L
 79 aat ttc gcg acg gcg ctg acg aac gac gtc gag ggt ttg

     L   E   L   Y   E   A   S   H   L   A   T   R   G
118 ctg gaa tta tac gag gcg tcg cat ctc gcg aca cgt ggc

     E   E   I   L   D   R   A   M   E   F   S   S   S
157 gag gag ata ctg gat aga gcg atg gag ttc tct tct tcc

     H   L   Q   A   F   L   N   Q   Q   L   V   G   S
196 cat ctc caa gca ttt ctc aat cag cag ttg gtg gga agt

     V   S   L   S   K   R   V   D   E   A   L   T   M
235 gtt tct ctc tct aaa cgt gtt gac gaa gct ctg acg atg

     P   I   H   K   T   L   T   R   L   G   A   R   K
274 cca ata cac aag act ctc acg aga tta gga gcg aga aag

     F   I   S   L   Y   Q   E   D   E   S   R   N   E
313 ttc atc tct cta tac caa gaa gat gaa tcg cgt aac gaa

     I   L   L   N   F   A   K   L   D   F   K   M   V
352 ata ctc ttg aat ttt gct aaa ttg gat ttc aaa atg gtg

     Q   K   M   H   Q   R   E   L   S   D   A   T   R
391 cag aag atg cac cag aga gag ctc agt gat gcc acg agg

     W   W   K   K   L   E   V   A   N   R   M   P   Y
430 tgg tgg aag aaa ttg gag gtg gcg aat aga atg cct tat

     A   R   D   R   I   V   E   L   F   F   W   M   V
469 gca aga gac aga ata gta gag ctc ttc ttt tgg atg gtg

     G   V   Y   F   E   P   C   Y   A   T   A   R   R
508 ggt gtc tac ttc gag cca tgc tat gct act gca aga aga

     I   L   I   K   A   I   S   M   A   S   I   I   D
547 ata cta att aaa gcc ata agt atg gct tca att att gac

     D   T   Y   E   Y   A   T   L   D   E   L
586 gac acg tac gaa tat gca acc ctt gat gaa ctg
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Figure 3. DNA Sequence information and the translated amino acid sequence of partial *TPS-4* gene.

For comparison with the other plant *TPS-4* AA sequences, the multiple protein sequence alignment was performed using Clustal W program (Figure 4).

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Zea_mays_TPS4                QRFYLLRKNGYDVPSDVFLSFKTEEG-GF-ACAAADTRSLLSLYNAAYLRKHGEE-VLDE
Thymus_vulgaris_TPS4         LGFRLFREAGFSVSQEVFDRFKNEESSGFEECLGEDTKGMLQLYEASFLLREGED-TLEL
Matricaria_chamomilla_TPS4   LKFRILRQSGYRVSQDFQRKFKESRGG-----LTGGLKELLSIYEASYLSLEGEP-DLHE
Populus_trichocarpa_TPS4     LHFRLLRQHAFSVSTDVFGKFRSNRDGKFKDSIRTDVAGLLSLYEASYLGVPGEDHVLEE
Copaifera_officinalis_TPS4   LLFRLLRQQGYHVSPDVFNKFKD-EQGKISETIANDVEGMLSLYEAAHLRIHGED-ILDE
Origanum_onites_TPS4         LRFRLLRQQGYRVPCDVFRKFTD-GEGNFATALTNDVEGLLELYEASHLATRGEE-ILDR
Origanum_vulgare_TPS4        LRFRLLRQQGYRVPCDVFRKFTD-GEGNFATALTNDVEGLLELYEASHLATRGEE-ILDR
                               * ::*: .: *  :.   *                  :*.:*:*:.*   **   *.

Zea_mays_TPS4                AISSTRLRLQD-LLGRLL-PESPFAKEVSSSLRTPLFRRVGILEARNYIPIYETEATRNE
Thymus_vulgaris_TPS4         ARQISTKFLQEKLDGTQISDDNNLSSSIRHSLEIPLHWRIQRLEARWFLDAYAARKDMNP
Matricaria_chamomilla_TPS4   AKLFATEKLLK-LTGH---ENEAMKDHVNHALDIPLYRRMLRLEARWYIDAYGKRKDANK
Populus_trichocarpa_TPS4     AKNFSSKHLKS-LMLETIKDE-VLAKQVKQSLEVPLHWKMPRIEARDFIDIYSSSDNTRNL
Copaifera_officinalis_TPS4   ALDFTSTHLKF-LTTQ-LSD--SHAGNVIRSLKRPLRMRLPRLEARHYFFTYQEDPSHIE
Origanum_onites_TPS4         AMEFSSSHLQA-FLNQQLVGSVSLSKRVDEALTMPIHKTLTRLGARKFISLYQEDESRNE
Origanum_vulgare_TPS4        AMEFSSSHLQA-LLNQHLVGSVSLSKRVDEALKMPIRKTLTRLGARKFISLYQEDESRNE
                             *   :   *   :             :  :*  *:   :  : ** ::  *

Zea_mays_TPS4                AVLELAKLNFNLQQLDFCEELKHCSAWWNEMIAKSKLTFVRDRIVEEYFWMNGACYDPPY
Thymus_vulgaris_TPS4         FIFELAKLDFNIIQAAQQQELKDLSRWWKNLSLPEKLPFVRDRLVESYFWAVGLFEPHTF
Matricaria_chamomilla_TPS4   QLLELAILDFNIVQSAHKRDLQEVSKWWEKTGLVRKLDFIRDRLMECFFWSVGMVFEPQY
Populus_trichocarpa_TPS4     DLLELAKLDYNLVQSQHQRELKDLARWWGALGFKKKLSFSRDRLMENYLWAMGMVFEPQF
Copaifera_officinalis_TPS4   TLLAFAKLDFNGLQKLHQREIGNLSKWWKDLDFATKLPFARNRLVEAYFWILGVYFEPCY
Origanum_onites_TPS4         ILLNFAKLDFKMVQKMHQRELSDATRWWKKLEVANRMPYARDRIVELFFWMVGVYFEPCY
Origanum_vulgare_TPS4        LLLNFAKLDFNMVQKMHQRELSDATRWWKKLEVAKRMPYARDRVVECFFWIVGVYFEPCY
                              :: :* *:::  *    .:: . : **       :: : *:*::* ::*  *      :

Zea_mays_TPS4                SLSRIILTKITGLITIIDDMFDTHGTTEDC
Thymus_vulgaris_TPS4         GNQRKIAAKIITLITSLDDVYDIYGTLDEL
Matricaria_chamomilla_TPS4   YTCRVELTKIATLITTIDDIYDVYGSLNEL
Populus_trichocarpa_TPS4     SKCRIGLTKFVCILTAIDDIYDVYGLPEEL
Copaifera_officinalis_TPS4   SLARRIMTKVISLTSIIDDIYDVYGTLEEL
Origanum_onites_TPS4         ATARRILIKAISMASIIDDTYE-YATLDEL
Origanum_vulgare_TPS4        ATARRILSKAINMASIVDDTYE-YATLDEL
                               *    *   : : :** :: :.  ::
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Figure 4. The multiple protein sequence alignment for the partial *O. onites* TPS-4.

The phylogenetic dendrogram was drawn for comparison with the other plant *TPS-4s* (Figure 5). Through cloning and sequencing of the middle region of *TPS-4* gene, this information may be used in quantitative mRNA analysis tests will be held in different stress conditions in future. In this way, it will be pioneering work for the increase of *TPS-4* gene efficiency in *O. onites*.

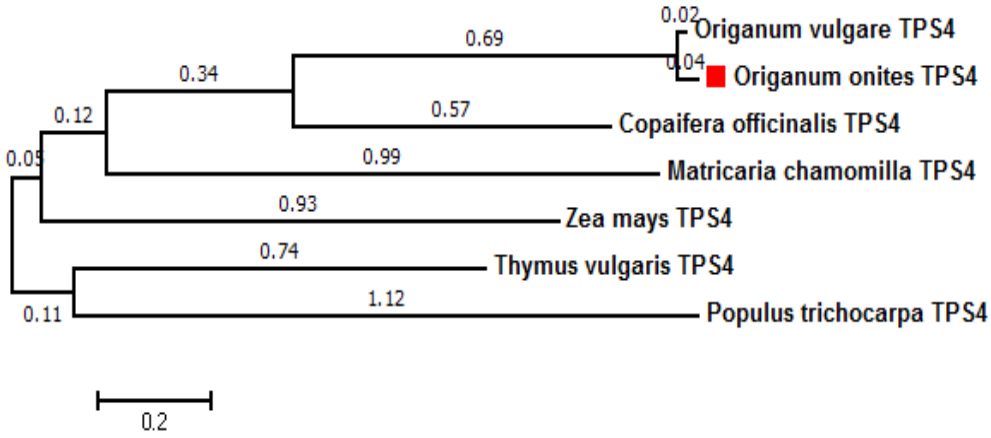

Figure 5. The phylogenetic dendrogram of partial *TPS-4* genes of *O. onites*and the other plants.

Terpene synthase genes in plants having aromatic characteristics encode attendant enzymes in the synthesis of plant essential oil (Bayrak, 2006). The cloning of terpene synthases can be achieved using a method based on the homology within these enzymes (Wallaart et al., 2001; Huber et al., 2005).In 2015, Yahyaa et. al. performed the identification and characterization of terpene synthases in *Daucuscarota* roots. They investigated the role of recombinant *TPS* genes in carrot aroma biosynthesis (Yahyaa et. al., 2015).This and similar studies about genetically identification of different terpene synthases from various aromatic plants will conduceto global science and industry in increasing of terpene production.

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