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Cloning and Characterization of Terpene synthase 3 (*SoTPS3*) Gene from Leaves of Garden Sage (*Salvia officinalis*)

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Abstract

Salvia officinalis plant produce various terpene compounds have many roles and useful biological properties in plants. In different salvia species structure of sesquiterpenes was remarked to be the main group of compounds at various tissues and development stages. However, some genes responsible for sesquiterpene synthese in *S. officinalis* remain unclear. In this study, we clone the full-length of sesquiterpene synthase (*SoTPS3*) gene from *S. officinalis*. The full-length cDNA of *SoTPS3* contains a 1668-bp open reading frame that putatively encoded a protein of 555 amino acids which resembled a sesquiterpene synthase in sequence. The deduced *SoTPS3* shared an overall homology with other known sesquiterpene genes from other plants. Analysis of *SoTPS3* amino acid sequence revealed that it contained two domains and four types of motifs such as, DDxxD, NSE/DTE, RRx8W and RXR. Bioinformatics and phylogenetic analyses revealed that *SoTPS3* clustered in clade subfamily TPS-a, and the genes that belong to this subfamily can encode mono- and sesquiterpene. Functional complementation of *SoTPS3* in *Arabidopsis thaliana* demonstrates that *SoTPS3* is responsible for the production of Germacrene D-4α-ol. Overexpression of *SoTPS3* in *A. thaliana* enhanced flower formation in transgenic plants compared with wild type. Our study will provide a basis for understanding the role of *SoTPS3* in the biosynthesis of sesquiterpene in *S. officinalis.*

Key words: Essential oils - *S. officinalis* - Terpene synthase - *Arabidopsis* - Functional characterization

1. Introduction

Terpenoids, the largest group of natural products, are secondary metabolites that are discovered in the Kingdom plantae with ~40,000 structures (Nema and Omimah, 2013; Abdul-Rahim and Taha, 2011; Ibrahim, 2011; Bohlmann *et al*. 1998). Isopentenyl diphosphate (IPP), the backbone molecule for terpenoids, consists of five carbon atoms (C5) (Xi *et al*. 2016; Abbas *et al*. 2019). Some terpenes play a solemn role in the plant metabolism (e.g. plant hormone gibberellin, pigments, Steroid, tryptophan, Brassinosteroid, Carotenoid, unsaturated fatty acids; hence, they affect plant growth, flowering and development (Trapp and Croteau, 2001; Gershenzon, 1999). However, most non-volatile and volatile terpenes are categorized as secondary metabolites, and they are crucial for the processes of protection against biotic and abiotic stresses (Sujatha and Bollipo, 2013; Dorothea *et al.* 2006)

The Salvia genus belongs to the Lamiaceae family and encompasses about 1,000 species of small shrubs. The members of this family are cultivated around the world for their medicinal and volatile oil properties. Salvia plant species have a sub-cosmopolitan distribution, and are largely present in three areas of the world such as Central and South America (about 500 species), Mexico (about 250 species), West Asia (about 200 species), East Asia (about 100 species) and Southern Africa (about 30 species) and includes several culinary, medicinal and ornamental plant species. In the low-lying tropical areas *e.g.*, the Amazon, other Salvia species are widespread (Ali *et al.* 2018; Ali *et al*. 2017; Sarrou *et al*. 2017; Kamatou et al., 2008; Alziar, 1988–1993). Moreover, Chinese folk medicine utilizes numerous salvia species as sedative, antibacterial, anti-inflammatory, antioxidant, antiseptic, cardiovascular, anti-cholinesterase activities, antitumor activities spasmolytic, analgesic and antidiabetic. (Zhenqing *et al*. 2018; Li *et al*. 2015; Wang *et al.* 2015a; Takano and Okada, 2011; Kamatoua *et al*., 2008).

The main composites of salvia's fragrant oil are monoterpenes and sesquiterpenes. The monoterpenes and sesquiterpenes composition in salvia species vary depending on the type of tissues on salvia species (Makris et al., 2007; Aziz *et al*. 2008; Loizzo *et al*. 2010; Atsuko and Hiroshi 2011; Hua *et al*. 2011; Nadaf *et al*. 2012; Fateme *et al*. 2013; Ali *et al*. 2017; Ali *et al*. 2018). In *S. officinalis*, the main sesquiterpenes are alpha-caryophyllene, isocaryophyllene, (−)-germacrene D and caryophyllene oxide, though, their biological or physiological function is unclear (Ali *et al*. 2017). To our knowledge, many genes responsible for synthesizing

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** **Abbreviations**: OE: Overexpression; EOs: Essential oils; FPP: Farnesyl diphosphate (FPP); TPS: Terpene synthase; *SoTPS3*: *S. officinalis* Germacrene D-4α-ol synthase; Semi-RT-PCR: Semiquantitative RT-PCR.

sesquiterpenes in salvia are still obscure. In our study, we cloned a full-length cDNA of *SoTPS3* from *S. officinalis*. A following step was established using the *Arabidopsis* floral-dip transformation method to clarify its role in sesquiterpene biosynthesis. The aforementioned strategies revealed that *SoTPS3* synthase gene actively catalyzed the Farnesyl pyrophosphate (FPP) substrate to Germacrene D-4α-ol. Taken together, these results will enhance our understanding of *SoTPS3* and its role in the biosynthesis of sesquiterpene in *S. officinalis* at the biologically level.

2. Materials and Methods

2.1. Plant materials.

S. officinalis seeds were donated by the Egyptian Desert Gene Bank (EDGB), Desert Research Center (DRC), Egypt. Seeds were grown in the greenhouse at National Research Centre, Cairo, Egypt. Three leaves from 2-years- old plants were used for RNA isolation and gene cloning.

2.2. Sequence characterization of SoTPS3.

The sequence of *SoTPS3* gene was selected on the basis of the highest sequence similarity found with the known plant sesquiterpene syntheses genes (see Appendix S1). The physical and chemical property of the *SoTPS3* was assessed using PROTPARAM Server (http://web.expasy.org/protparam/). Putative transit peptide for open-reading-frames (ORF) of *SoTPS3* was analyzed by iPSORT prediction tool (http://ipsor t.hgc.jp/). NCBI BLASTX tool was used for comparative protein sequence analysis of *SoTPS3* (http://blast.ncbi.nlm.nih.gov/). Clustal Omega Online software with default tool parameters was used for analyzing the sequence alignments (https://www.ebi.ac.uk/Tools/msa/clustalo/). To predict the 3D protein structure of SoTPS3, we used the SWISS-MODEL Server for build the 3D structure based on other homology-modules that have been stored on the site (https://swissmodel.expasy.org). To determine the quality of our predicted 3D structure, we used PROCHECK tool (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). The evolutionary relationships of SoTPS3 protein and other plant TPS proteins were inferred using the PhyML Server without changing the tool parameters (http://www.phylogeny.fr/) (Dereeper *et al*. 2008; Mehmood *et al*., 2021).

2.3. Putative tissue expression pattern of SoTPS3 gene and its subcellular localization.

Tissue-specific expression data from thirty six tissues were analyzed based on *Arabidopsis thaliana* transcript expression database. We used Arabidopsis eFP browsers (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) to generate expression profiles, while, the putative sub-cellular localizations of *SoTPS3* gene from *S. officinalis* was analyzed using the Cell-eFP browsers (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) as described by (Makhadmeh *et al*., 2022a and 2022b). The arrow points the expression scale (e.g. red color= high expression & yellow color = low expression).

2.4. RNA extraction and cDNA synthesis

Six tissues with three replicates from two-year-old of *S. officinalis* were used for RNA extraction using TransZol Reagent for gene cloning and qRT-PCR. Also, leaves from wild and transgenic *A. thaliana* were used for RNA extraction for semi-quantitative RT-PCR. For cDNA Synthesis 1 µg from each RNAs was used to synthesize the first-strand cDNA using reverse transcriptase TransScript® First-Strand cDNA Synthesis Super Mix kit as described by (Ali *et al*. 2017; Hussain *et al*., 2017)

2.5. Isolation of full-length S. officinalis sesquiterpene synthase gene (SoTPS3).

The entire *SoTPS3* cDNA was used as a template to magnify the full-length by short-gene-specific forward (5′-ATGGCTCAAATATATGCATCGG-3′) and reverse (5′-AGTTCACGGGCTCTACGAGC-3′) primers and KOD-polymerase enzyme and amplification was done at 3 min at 96°C; 33 cycles for 10 s at 98°C; 30 s at 60 °C; 90 s at 68°C, and then 10 min at 68°C. The first PCR product was used as a model for the second PCR using long-gene-specific forward (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATG GCTCAAATATATGCA-3′) and reverse (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTC ACGGGCTCTACGA-3′) primers with the same polymerase enzyme and PCR program conditions to clone into Gateway vectors. The successful amplicon was purified and cloned into a pDONR221vector (Invitrogen, Carlsbad, CA, USA), then pB2GW7 vector (Invitrogen, Carlsbad, CA, USA) and sent for sequencing as depicted by (Ali *et al*. 2017 and Ali *et al*., 2018).

2.6. Growth conditions of Arabidopsis plants and transformation using Agrobacterium.

Arabidopsis thaliana seeds from ecotype Columbia-0 (Col-0) were grown in our Lab growth chamber as reported by (Ali *et al*., 2018). After 2 months from the growth, healthy plants at pre-flowering stage were selected for floral-dip transformation experiment. Moreover, to characterize the function of *SoTPS3* gene, the vector pB2GW7-*SoTPS3* and pB2GW7 (empty vector) were transformed into *Agrobacterium tumefaciens* strain GV101. *A. tumefaciens* GV101 containing pB2GW7-*SoTPS3* was grown in selective solid LB media supplier with rifampin (Rif) and spectinomycin (Spc) as antibiotics. One positive colony was selected and inoculated into 0.8 ml of liquid-LB-media with Rif and Spc, after one day from incubation period at 28°C the bacterial culture was sub-cultured to conical flask containing about 60 ml LB media and incubation at incubator with shaker until the optical density of *Agrobacterium* cells reached to 0.75 (OD 600) according to (Ali *et al*., 2017; Ali *et al*., 2018 and Darwish *et al*., 2022). On the next day, cell suspension was collected, and the bacteria was re-suspended in fresh-inoculation-medium (5.2% sucrose and 0.055% Silwet). The plasmids pB2GW7-*SoTPS3* and pB2GW7 (control) were introduced separately into *A. thaliana* plant by directly immersing the axis of bud flowers in the fresh floral-dip medium with pressing gently to ensure intake of *A. tumefaciens* GV101 harbouring the pB2GW7-*SoTPS3* and pB2GW7 (control) plasmids into the flower gynaecium (Aharoni *et al*. 2003; Su-Fang *et al*. 201,). A total of 12 *A. thaliana* transgenic lines were obtained and

survived the successive subculture process under BASTA resistance. The leaf morphology, flowering time and terpene metabolic of the previous lines were subjected to assessment.

2.7. Semi-Quantitative RT-PCR (sqRT-PCR) analysis

To ensure the success of our gene transfer process qRT-PCR was implemented using a PCR system from Biometra. The *At-B-actin* gene forward primer 5′-GGCTGAGGCTGATGATATTC-3′ and reverse primer 5′-CCTTCTGGTTCATCCCAAC -3′ were used as housekeeping with 155 bp, and *SoTPS3* forward primer 5′-ACGTCTAGGAGTTGCCTATC-3′and reverse primer 5′-CGGTAACCTTGCTGTCTAAG-3′ with 150 bp length. We used IDTdna online website (http://www.idtdna.com/scitools/Applications/RealTimePCR/) to design our target primers. The qRT-PCR conditions program as follows: 94°C for 5 min, 33 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, 72°C for 10 min. PCR products were screening on 1.4 % agarose gel to determine our genes expression levels.

2.8. Terpenoids extraction and GC-MS analysis.

For a rapid survey of terpenoids in transgenic and non-transgenic *A. thaliana* plants, intact leaves of various lines were frozen in liquid nitrogen (LN_2), powdered with a ceramic mortar and pestle, and directly inundation in n-hexane as a solvent for 72 h to ensure complete removal of terpenoids leave contents as previously described (Ali *et al*. 2017; Ali *et al*. 2018; Ali et al., 2022a, 2022b, 2022c). And an approximately 1µl aliquot of each extract was analyzed by Shimadzu-GC-MS system with three replicates. Terpenoid component identification was made by reference to Wiley GC/MS Library, the Volatile Organic Compounds (VOC) Analysis S/W software, and the NIST Library as previously described (Ali *et al*. 2017; Ali *et al*. 2018).

2.9. Quantitative real-time PCR (qRT-PCR) analyses.

To analyze the expression of *SoTPS3* in various *S. officinalis* tissues (e.g., flowers, bud flowers, stems, young leaves, old leaves and roots), tissues were collected with three biological replicates. QRT-PCR was performed to represent the scale of *SoTPS3* transcript involved in the production of sesquiterpene. A 157 and 150 bp fragments in the 3' region of *SoACTIN* and *SoTPS3* were amplified using the following couple of primers: for *SoACTIN* forward 5′-GGCAGTTCTCTCCCTCTAT-3′ and reverse 5′ GAGGTGGTCGGTGAGAT-3′ was used as a housekeeping gene, and *SoTPS3* forward 5′-ACGTCTAGGAGTTGCCTATC-3′and reverse 5′-CGGTAACCTTGCTGTCTAAG-3. The qRT-PCR experiments were performed using IQTM5 System, SYBR Green and the cycler program as follows (95°C for 10s, 60°C for 30s, and 72°C for 20s), then 65°C for 5s and 95°C for 5s). The expression levels were enumerated by comparing our target gene cycle thresholds (CTs) with the housekeeping gene *SoACTIN* using the $^{2\text{-}\Delta\Delta}$Ct method (Wise *et al*., 1998; Anders & Huber, 2010; Hussain *et al*., 2017; Rehman *et al*., 2017;). Values are offered as means ± SE of three different RNA pools replicates.

3. Results

3.1. Full-length isolation of Terpene synthase 3 (SoTPS3) gene and sequence characterization

The full-length-ORF of *SoTPS3* gene with 1668 bp encoded a 555 amino acid protein with a predicted theoretical isoelectric point (pI) of 5.52 and molecular mass of 64.24 kDa. The amino-acid of SoTPS3 have stumpy signal peptide compare with monoterpene synthases (600–650 aa) and matched with a lot of sesquiterpene synthases of 550–580 aa in the thirty amino acid existence at N-terminal sequence. Using 'iPSORT' program revealed that *SoTPS3* is localized in the chloroplast (plastid) where FPP originates, and biosynthesis takes place. NCBI-BLASTX analysis in (Table 1) indicated that *SoTPS3* shared 79.35% identity and ≤76.12% identity with its homologue sesquiterpene synthase protein from *Salvia splendens* and other plants respectively. The sequence alignment of *SoTPS3* gene with putative and recognized TPS genes from Lamiaceae and other plants aided the prediction of its putative function. Based on this prediction, *SoTPS3* protein have various motifs such as: spartate-rich-DDxxD (residues 313–317), NSE/DTE (residues 457–465) motifs, RXR motif (residues 276–278), RR (X8) W (residues 22–32) and GTLxEL (residues 320–325) region that are predominant in similar sesquiterpene synthases involved in (-)-germacrene D synthesis (Abbas *et al*. 2019; Su-Fang *et al*. 2014; Degenhardt *et al*. 2009; Ali *et al*. 2017; Ali *et al*. 2018; Ali *et al*., 2022a, 2022b) (Figure 1). Comparable to other sesquiterpene synthases, *SoTPS3* has two domains, which were specified by InterPro database. So, *SoTPS3* protein has three terpene synthase family domains, N-terminal-domain (IPR001906: from 32-207 aa), C-terminal-domain (IPR005630: from 238-499 aa) and metal-binding-domain (IPR034741: from 233-554aa) (Figure 2). The SoTPS3 was tabulated to the TPS-a subfamily of angiosperm sesquiterpene synthases based on the phylogenetic analysis results (Figure 3).

Table 1. BLASTX analysis of *SoTPS3* was compared with the NCBI protein database for gene identification purposes.

NCBI Accession	[a]Descriptiona	Organism	*E* value	Identity (%)
TEY90111.1	(-)-germacrene D synthase	*Salvia splendens*	0.0	79.35%
ADK73619.1	Terpene synthase 3	*Origanum vulgare*	0.0	76.12%
Q5SBP6.1	Germacrene-D synthase;	*Ocimum basilicum*	0.0	69.01%
RVW14969.1	(-)-germacrene D synthase	*Vitis vinifera*	0.0	56.85%
TEY69656	(-)-germacrene D synthase	*Salvia splendens*	0.0	53.36%

[a] Description—homology search using BLASTX.

3.2. 3D modeling and analysis of active site

The 3D protein model for *SoTPS3* was constructed using (+)-δ-cadinene synthase from *Gossypium arboreum* [PDB accession: 3g4f] as a template (Gennadios *et al*. 2009) (Figure 4). We used Ramachandran plot analysis to check the quality of *SoTPS3*-3D protein model (Laskowski *et al*. 1993) (Figure 4). The model showed a valid fit

versus the reference geometry (Resolution: 2.65Å, R-factor: 0.206 and R-free: 0.256). Accordingly, *SoTPS3* entirely consisted of *α*-helices with loops, turns, long and short connecting. Both of the C- and N-terminal conserved domains were manifested in the 3D model that was foretell using SWISS-MODEL server (https://swissmodel.expasy.org). This model illustrates the active site that catalyzes the FPP substrate. (K¨ollner *et al.* 2008) (Figure 4).

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                                                                  RR   (X8)   W
NsGDS           ----------------------MDFSKGLPV-----------GVHEVSRPSANYHRSIWGD     28
VvGDS           -----------------------------MSVQSSVVLLA--------PSKNLSPEGAHV     23
ADR74196.1      ----------------------------MSGQVLASPLGQFPELENRPVVQYHPSIWGD     31
ADR74197.1      MELAKLFLSYLPIHHSRYSAVLSLSQGINMSTQVSACSLAQIPKPKNRPVTNFHPNIWGD     60
TEY69656.1      ---MEMCA-------------------------PHVSAMKNGKSLDEIRKSATFHPSIWGD     33
sp|Q5SBP6.1     --MTNMFA-------------------------SAAPISTNNTTVEDMRRSVTYHPSVWKD     34
ADK73619.1      --MAEICA-------------------------SAAPISTKNTSVEELRRSVTYHPSVWRD     34
SoTPS3          --MAQIYA-------------------------SAVPISTKNTNVDNIRRSVTYHPSVWRD     34
TEY90111.1      ------------------------------------------------------------      0

NsGDS           YFLDCVS-DSTIINPLERKQVQDLREEVRKMLMAVHDTSSEKVELIDKIQRLGVSYHFEE     87
VvGDS           YFINCLNLTVQNTDDHLKQHVQQLKEEVRKMLMAADDDSAQKLLLIDAIQRLGVAYHFES     83
ADR74196.1      QFLSYT-PEDEVTRACKEKQLEDLKEEIRRKLMNTAGNTSQQLKFIDAVQRLGVAYHFER     90
ADR74197.1      QFITYT-PEDKVTRACKVEQIEDLKKEVKRKLTAATANHSLLLNFIDAVQRLGVAYHFEQ    119
TEY69656.1      FFLKYDS-NNTKITDAEQEELAKHKEMVRKMLSQTPNDSTCKLELIDEIQRLGVEYHFEK     92
sp|Q5SBP6.1     HFLDYAS-GITE---VEMEQLQKQKERIKTLLAQTLDDFVLKIELIDAIQRLGVGYHFEK     90
ADK73619.1      HFLSYTN-DVTEITAAEKEELEKQKEKVKNLLDQTPNDSTLKIELIDAIQRLGFGYHFEE     93
SoTPS3          HFLKYTD-DVTKITTAEKQELEKQKEEVKKLLAQTPDDSKVKMELIDAIQRLGVAYHFSK     93
TEY90111.1      -------------------MLEKQKVEVKKLLAQTPDDSTLKMDLINAIQRLGVAYHFSK     41

NsGDS           EIEASLQRMYEAYRE--CNMYGDDLYLVAIGFRLLRQQGHFVSCDVFKKFKDNEGNFDKA    145
VvGDS           EIDEVLKHMFDGSV----VSAEEDVYTASLRFRLLRQQGYHVSCDLFNNFKDNEGNFKES    139
ADR74196.1      EIEEVLQHIYDSYPN--GDDMEGDIYNVALQFRLLRQAGFNISCGLFNEFKDEKGNFKKA    148
ADR74197.1      EIEEALQHIYESFHD--LNDIDGDLYNVALGFRLLRQQGYSISCGILKKFTDERGRFKEV    177
TEY69656.1      EIEESLKHIHDSYMQRNCKD-NDDLHIVALRFRLLRQQGYNVPCGVFCKFTDSEGNYEAS    151
sp|Q5SBP6.1     EINHSLRQIYDTFQISSK---DNDIRVVALRFRLLRQHGYPVPSDVFKKFIDNQGRLDES    147
ADK73619.1      VIDESLGEVYDRYEMPSGKDDEDEIRVRSLRFRLLRQQGYRVPCDVFEKLLDDKGNFKDS    153
SoTPS3          EIDESLRKIHDTYQIQSRTD-KDDARVLALRFRLLRQQGYRVSSDVFNGLVDEEGNLKEW    152
TEY90111.1      EIDDSLRKIHHNYESQSSKD-KNNVGVLALRFRLLRQHGYRVSCDVFNGLVDKEGNLKES    100

NsGDS           LTSNVPAMLSLYEAAHMRVHGEDILEEALVFISNHLKSM-IPILSDSFRVQVLHALNQPI    204
VvGDS           LSSDVRGMLSLYEATHFRVHGEDILDEALAFTTTHLQSA-TKYSSNPLAEQVVHALKQPI    198
ADR74196.1      LVSDVRGMLGLYEAAHLRVHGEDILAKALAFTTTHLKAM-VESLGYHLAEQVAHALNRPI    207
ADR74197.1      LITNVRGLLGLYEAAHLRVHGEDILAEALTFTTTHLKAM-VESLGYPLAEQVVHALNRPI    236
TEY69656.1      LQNDVEGLLNLYEAAHLLTHDEGILENAIEFCSSHLHASLHKLDDVSLSKRVGEALEMPN    211
sp|Q5SBP6.1     VMNNVEGMLSLYEASNYGMEGEDILDKALEISTSHLEPL------ASRSRRINEALEMPI    201
ADK73619.1      LITDVEGLLSLYEASNYGINGEEIMDKALKFSSSHLEGSIH-KMPTSLSRRVKEALDMPI    212
SoTPS3          LISDVEGMLSLYEASNYGINGDEILEKVLQFTSSHLES-LLPQMSTSLSNRVKEALEMPI    211
TEY90111.1      LIDDVEGMLSLYEASNCGINGEDILDKALEFSSSHLRNSLHKTMSTSLSNRVKEALEMPI    160

NsGDS           HMSLTRVEARRFLSTYQSYDTKNELLLEFAKLDFNLLQKVHRKELSSITRWWKDLDIVTK    264
VvGDS           RKGLPRLEARHYFSVYQADDSHNKALLKLAKLDFNLLQKLHQKELSDISAWWKDLDFAHK    258
ADR74196.1      RKGLERLEARWYISVYQDEAFHDKTLLELAKLDFNLVQSLHKEELSNLARWWKELDFATK    267
ADR74197.1      RKGLERIEARWYISVYQDEAFHDKTLLELAKLDFNLVQSLHREELSNLARWWKELDFATK    296
TEY69656.1      RWSLARLGARKFISAYQEDEAHNEILLNFAKLDFNLLQKMHQRELSDATRWWKKLDVANK    271
sp|Q5SBP6.1     SKTLVRLGARKFISIYEEDESRDEDLLKFAKLDFNILQKIHQEELTHIARWWKELDLGNK    261
ADK73619.1      SKTLTRLGARKFISLYQEDESHNELLLKFAKLDFNIVQKMHQRELHHITRWWEGLEFGKK    272
SoTPS3          SKTLMRLGARKFISIYQEIESHNELLLNFAKLDFNVMQKIHQRELHHITRWWVDLEFGKK    271
TEY90111.1      SKSLIRLGAKKFISMYQLDESHNQTLLNFAKLDFNIVQKIHQRELHHITRWWEDLEFGKK    220
                    RxR                                       DDxxD  GTLxEL
NsGDS           CPFARDRLVESYFWALGVYFEPKFVIARRMLAKVIALATIIDDIYDAYGSYDEHMCFTEA    324
VvGDS           LPFARDRVVECYFWILGVYFEPQFFFARRILTKVIAMTSIIDDIYDVYGTLEELFTEA    318
ADR74196.1      LPFARDRLVECYFWMLGVYFEPQYLRARRILTKVIAMISILDDIHDAYGTPEELKLFIEA    327
ADR74197.1      LPFARDRLVEGYFWILGVYFEPQYLRARRILTKVIAMTSILDDIYDAYGNPEELKLFTEA    356
TEY69656.1      IPHARDRIAELYLWVLGVFFEPCYAKARRILLKCISMASIADDTYE-YATLEELRILTDA    330
sp|Q5SBP6.1     LPFARDRVVECYFWILGVYFEPQYNIARRFMTKVIAMTSIIDDIYDVHGTLEELQRFTDA    321
ADK73619.1      LPFARDRVVECFFWILGVYFEPKYEIARRFLTKVISMTSILDDIYDVYGSLDELRRLTHA    332
SoTPS3          LSFARDRMVECYFWILGVYFEPQYATARIFLTKVIALTSTLDDIYDVYGTLDELRCFTDA    331
TEY90111.1      LPFARDRVAECYFWIVGVYFEPQYDTARVFMTKVIALTSIIDDIYDVYGTLDELRLFTHA    280
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NsGDS                LERWDVSA-IDGLPPYMKSCYLAILGVYAEMEEELAKRGESYRVDYAKNEMKKLTRAYFE    383
VvGDS                VERWDISA-IDQLPEYMRVCYQALLYVYSEIEEEMAKEGRSYRLYYAKEAMKNQVRAYYE    377
ADR74196.1           IERWDINS-IDQLPEYMKLCYAALLDVYKEIEEEMEKEGNQYRVHYAKEVMKNQVRAYFA    386
ADR74197.1           IERWDINS-IDQLPEYMKLCYAALLDVYKEIEEEMEKEGNQYRVHYAKEVMKNQVRAYFA    415
TEY69656.1           IERWDVNETLEDSPPYIQMLYRSLIKTYTEIEDEMAKTGESYRVQYAKQEMKKLLVSYFD    390
sp|Q5SBP6.1          IRSWDIRA-IDELPPYMRLCYEALLGMYAEMENEMVKQNQSYRIEYARQEMIKLVTTYME    380
ADK73619.1           IQRWDISV-GDELPPYMRICYEALLGVYSEMEDEMAKKGQSYRLLYARQEMIKLVMAYMV    391
SoTPS3               IRRWDISA-ADELPAYMKICYEALLGIYAEMEDEMAKRGESFRLQYAKKEMIKLVMAYMK    390
TEY90111.1           IRGWDISA-AEELPPYMRICYEALLGVYSEMEDEMAKRGQSYRLQYARKEMIKLAGVYME    339

NsGDS                EAKWSHSASYIPTFEEYMKVALDSSGYMMAATTSLVGIGDNLINKNVMDWVTHEPLIVQA    443
VvGDS                EAKWLQVQ-QIPTMEEYMPVALVTSAYSMLATTSLVGMGDA-VTKETFDWIFSKPKIVRA    435
ADR74196.1           EAKWLHEE-HVPTFEEYMRVALVSSGYCILATTSFVGMGEI-ATKEAFDWVTSNPKIMSS    444
ADR74197.1           EAKWLHEE-HVPTIEEYLRVALVSSGYCMLATTSLVGMGEI-ATKEAFDWVTSDPKIMSS    473
TEY69656.1           EVKWLYNDNYIPTPGEYLKVSVVSGGYMMLSTTSLVGMGDDQVTKNDFDWIVNEPLIDRA    450
sp|Q5SBP6.1          EAKWCYS-KYIPNMDEYMKLALVSGAYMMLATTSLVGILGDPITKQDFDWITNEPPILRA    439
ADK73619.1           EAEWCFS-KYFPTMEEYMKQALVSGAYMMLSTTSLVGMEDLNITKHDFDWITSEPPMLRA    450
SoTPS3               EAEWCYG-KYVPTMEEYMKLALVSGAYMMLSTTSLVGMED-PITKNDFEWITNEPPILQA    448
TEY90111.1           EAEWCYS-KHVPTMDEYMKLALVSGAYMMLSTTSLVGMGD-PITQDDFDWITTEPPIVRA    397
                              NSE/DTE
NsGDS                STVIARLMDDMAGHEFEQERGHEPSAVECYMKQHGTSKEEVLLELQKLVSNAWKEVNRQC    503
VvGDS                SAIVCRLMDDMVSHKFEQKRGHVASAVECYMKQHGASEQETHNEFNKQVRDAWKDINEEC    495
ADR74196.1           SNFIARLMDDIKSHKFEQKRGHVASAVECYMKQYGVSEEQVYKEFQKQIENAWLDINQEC    504
ADR74197.1           SNFIARLMDDISSHKFEQKREHVASAIECYMKQYGVSEEQAYSEFRKQIENAWMDINQEC    533
TEY69656.1           SALLARLLDDLVGDEYEEK----PSSIHCYMRQYGMSEDDARAKIKQQMKNAWKDMNQEC    506
sp|Q5SBP6.1          ASVICRLMDDVVGHGIEQK----ISSVDCYMKENGCSKMEAVGEFSKRVKKAWKNLNEEW    495
ADK73619.1           ASVICRLMDDMVGHGIEQK----IISVDCYMRENGCSKEEACREFWNRVKKAWKCMNEEC    506
SoTPS3               ASVVCRLMDDMVGHGIEQK----ITSVDCYMREHCCSKTEASSEFWKRVKKAWKDMHEGC    504
TEY90111.1           ASVICRLMDDMVGHGIEAK----TTSVDCYMKENGCSKMEAFDEFWKRVKKAWKDINEEC    453

NsGDS                LYPREVPMLILMRVLNLARVIELLYKDGDAFTHSTTKLKNIITSILVDPVP---------    554
VvGDS                LIPTAVPMPILMRVLNLARVIDVIYKNEDGYTHSGTVLKDFITSMLIDPVPI--------    547
ADR74196.1           LKPTAVSMPLLARILNLTRAADVVYKEQDSYTHVGKVMRDNIASVLINAVI---------    555
ADR74197.1           LKPTAVPMPLLARVLNLTRAADVIYKEQDSYTHVGKVMKNNIAAFFINPII---------    584
TEY69656.1           LEPTPASMPILMRIINLARAAQLIYSNGDCYTDPN-KAKEWVKMLLIEAVPI--------    557
sp|Q5SBP6.1          VEPRAASMVILVRVVNLARVINLLYVGEDSYGNSSVKTKELIKGVLVHPIK---------    546
ADK73619.1           LEPRAASMAILARVLNLARVINLLYVGEDAYGSSSTKTKNFIKSVLVDPIHSIEYHI---    563
SoTPS3               LEPRAASMAILMRVVNLARVINLLYVGEDAYGSSSTKTKELIESVLVEPVN---------    555
TEY90111.1           LEPRAASMAILVRIVHLARVINLLYVGEDSYGNPKTKTKEMVKMVLVEPVQF--------    505
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Figure 1. Multiple sequence alignment. The concluded amino acid sequence of SoTPS3 was aligned with its homologues from the analysis of BLASTX. The conserved motifs RR(X8)W, RXR, DDXXD, GTLxEL and NSE/DTE are marked. SoTPS3 : sesquiterpene synthase [S. officinalis]; SsGDS:(-)-germacrene D synthase [Salvia splendens:TEY90111.1]; OvTPS3: Terpene synthase 3 [Origanum vulgare:ADK73619.1]; ObGDS: Germacrene-D synthase [Ocimum basilicum: sp|Q5SBP6.1]; NsGDS: (-)-germacrene D synthase-like [Nicotiana sylvestris: XP_009804030.1]; SsGCS: Germacrene C synthase [Salvia splendens: TEY69656.1]; VvGDS: (-)-germacrene D synthase [Vitis vinifera: RVW14969.1]; VvTPS: Terpene synthase [Vitis vinifera: ADR74196.1]; VvGDS: Germacrene D synthase [Vitis vinifera: ADR74197.1]

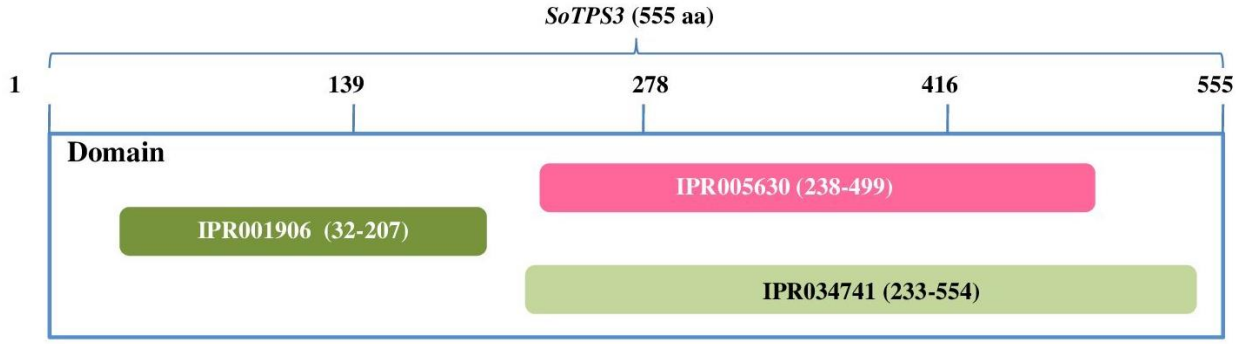

Figure 2. The putative domains' structure and qRT-PCR analysis for *SoTPS3* gene. (A) Three putative domains of SoTPS3 were detected at different amino acids positions, namely Terpene synthase, N-terminal domain (IPR001906), Terpene cyclase-like 1, C-terminal domain (IPR034741) and Terpene synthase, metal-binding domain (IPR005630).

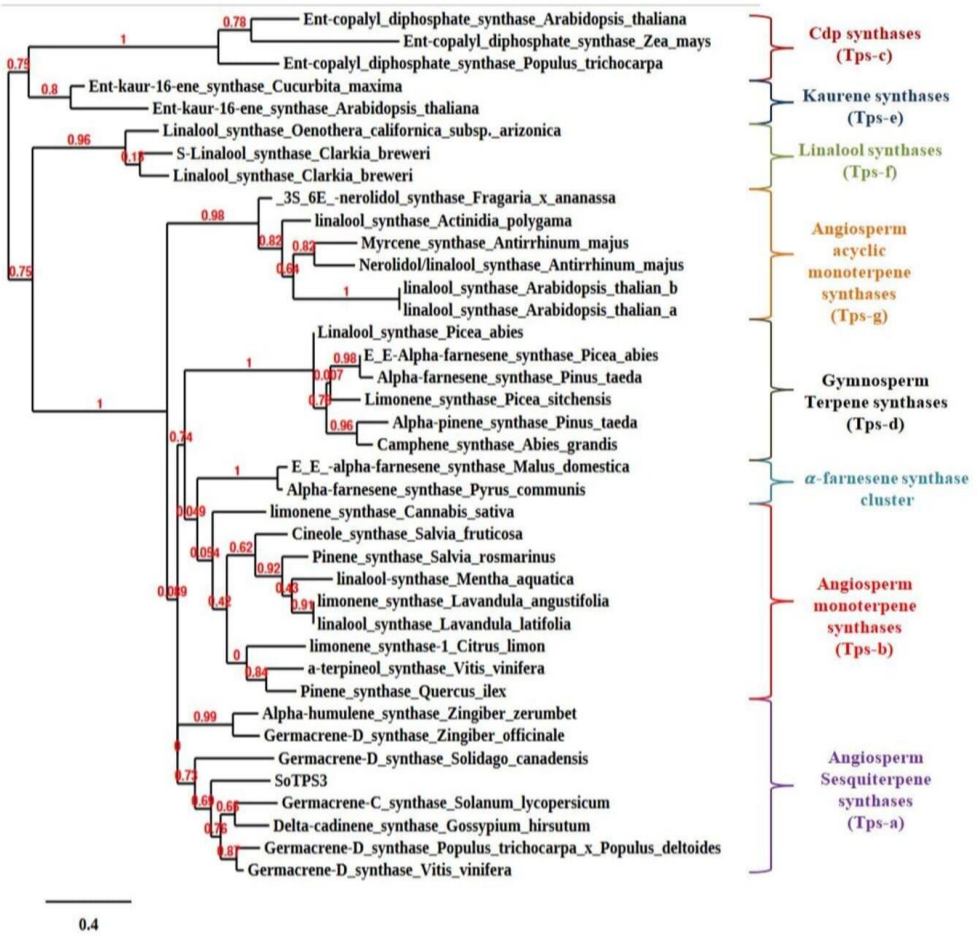

Figure 3. Phylogenetic tree of *SoTPS3* with selected terpene synthases from other plants. Based on the knowledge gained from the work of Bohlmann et al., 1998 and Danner et al., 2011, seven TPS subfamilies (Tps-a to Tps-g) were chosen. However, the Tps-c and Tps-e subfamilies were chosen as out-groups. Those are composed of the copalyl diphosphate (cdp) synthases and kaurene synthases and are involved in primary metabolism. The alignment was performed using the PhyML server. The numbers indicated are the actual bootstrap values of the branches**.**

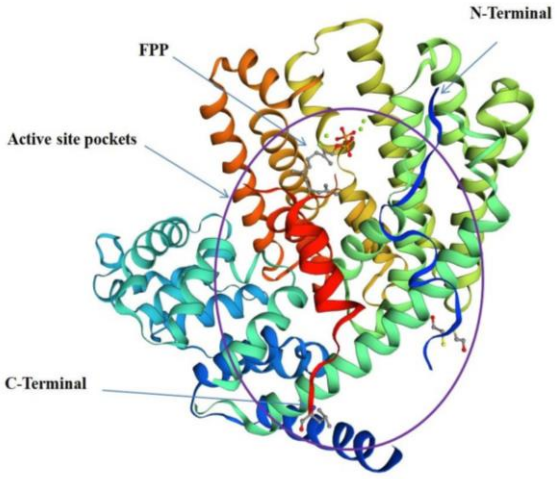

Figure 4. Predicted 3D model of *SoTPS3* generated by the SWISS-MODEL software. The active site pocket with predicted FPP binding residues are indicated through arrows along with the N-terminal domain and the C-terminal domain. Prediction of 3D protein structure and binding residues were generated using online SWISS-MODEL server. An illustration that clarifies the binding between the substrate (FPP) and the active site residues is depicted at the center of the active site in SoTPS3 3D proteins.

3.3. Putative tissue expression and subcellular localization of SoTPS3 gene

To examine the putative tissue expression pattern of *SoTPS3* in the *A. thaliana* genomics, a BlastP search against the *A. thaliana* genomics at Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST&method=Org_Athaliana) was conducted with the protein sequence of *SoTPS3* as a query. This research identified several proteins closely related to the *SoTPS3* sequences specially (AT5G23960) with a high BLAST score and e-Value (351.7 and 3E-113), respectively. The tissue expression of *SoTPS3* gene in Arabidopsis uncovered by our data was analysed across forty seven tissue using BAR database (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and the Arabidopsis Electronic Fluorescent Pictograph Browsers (eFP browsers (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Figure 5a and b).

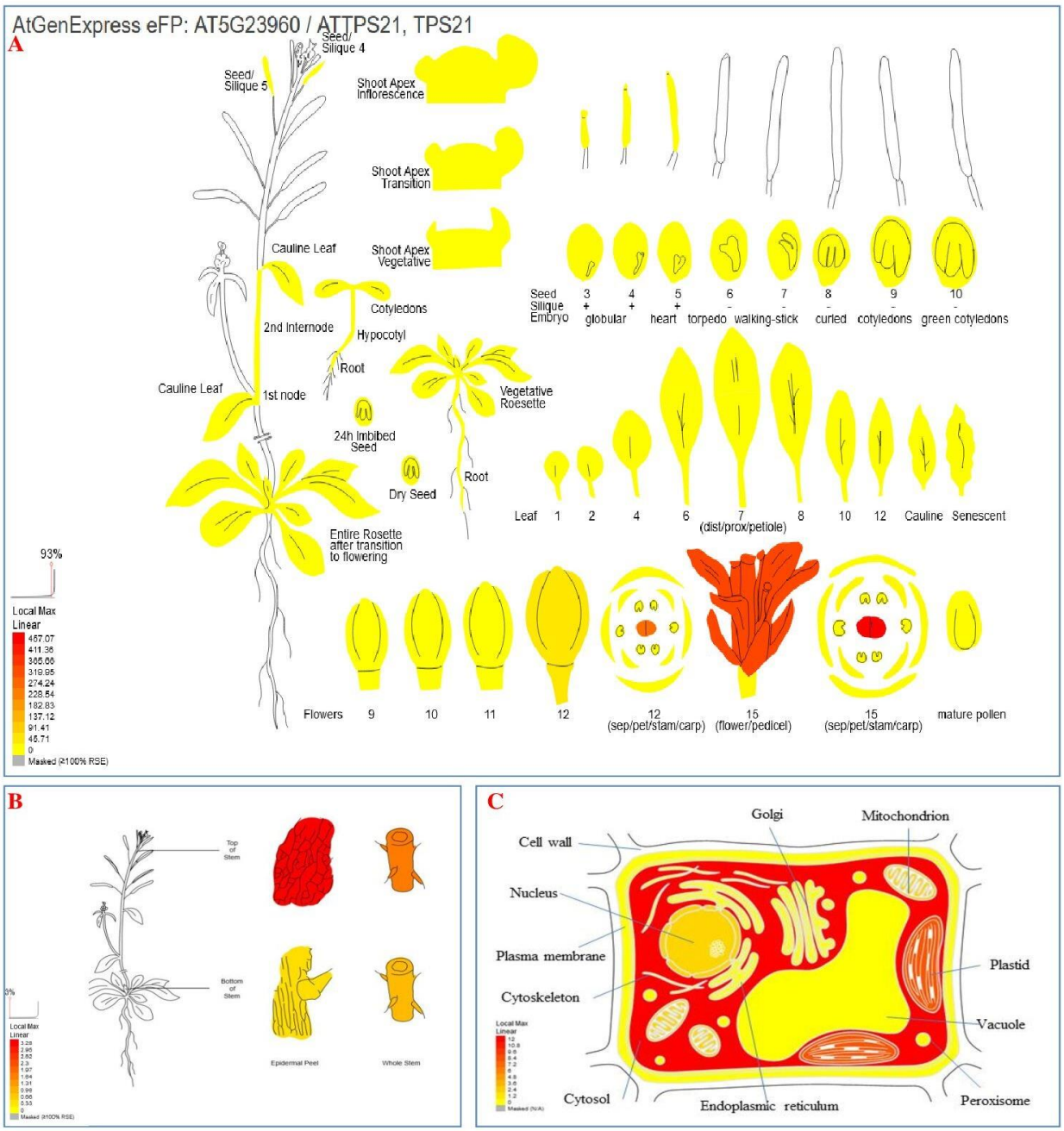

Figure 5. Visualization of the putative tissue expression and cell localization of *SoTPS3* (AT5G23960.1) gene using "electronic fluorescent pictograph" browsers, based on known Arabidopsis gene expression and protein localization. **a** Expression data at different tissues ranging from seedling to flowering stages. **b** Expression data of tissue specific stem epidermis at top and bottom. **c** Expression data at different cell organelles. The color box points the expression scale (the more intense red color, the more gene expression).

The Arabidopsis eFP Browsers clearly showed that (*SoTPS3* gene: AT5G23960) was present in most of the tissues with highly expressed in flower-stage-15-carpels (457.06), flower-stage-15 (332.31), flower-stage-12-carpels (263.98) and flower-stage-12 (43.26) (Figure 5a and Supplementary Table S1). Moreover, putative subcellular localization built using ePlant and cell eFP (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) for (*SoTPS3:* AT5G23960) gene was present with different expression levels at the fourteen cell organelles see (Figure 5c).

3.4. Screening the expression of SoTPS3 gene using qRT-PCR.

QPCR-PCR was used to inspect the transcription levels of *SoTPS3* at various tissues of *S. officinalis* (e.g. flowers, bud flowers, stems, young leaves, old leaves and roots) (Figure 6). And from our qPCR-PCR analysis results, we found the highest expression levels were observed in flowers, then in bud flowers, young leaves, stems, old leaves and roots (Figure 6).

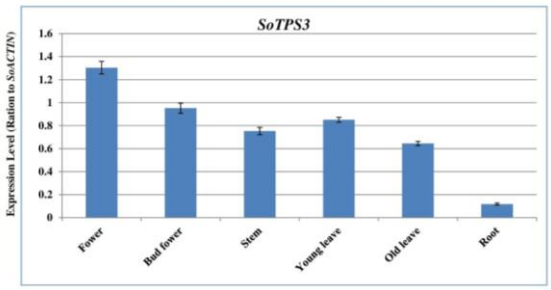

Figure 6. Quantitative RT-PCR validation of *SoTPS3* gene expression from *S. officinalis*. Total RNAs were extracted from flower, bud flower, stem, young leaves, old leaves and bud flower roots samples and the expression of *SoTPS3* gene was analysed using quantitative real-time. We used *SoACTIN* as an internal reference. The values are means±SE of three biological replicates.

3.5. Functional expression of SoTPS3 gene in non- and transgenic A. thaliana plants

To study the effect of *SoTPS3* gene on *A. thaliana* plants phenotypes after 33 days of growth, this gene was cloned from *S. officinalis*. Thereafter, *A. thaliana* was utilized as a transient expression system to overexpress the isolated *SoTPS3* gene. We used *A. tumefaciens* strain GV101 harbouring the vector pB2GW7-*SoTPS3* controlled by 35S promoter to generate transgenic *A. thaliana* plants overexpressing *SoTPS3* gene constitutively (Figure 7a). The positive transformants were further verified using BASTA reagent and sqRT-PCR of the genomic cDNA (Figure 7b). Transgenic *A. thaliana* plants showed a decrease in leaf diameter, while the flowering stems start growth earlier when compared to the GUS control

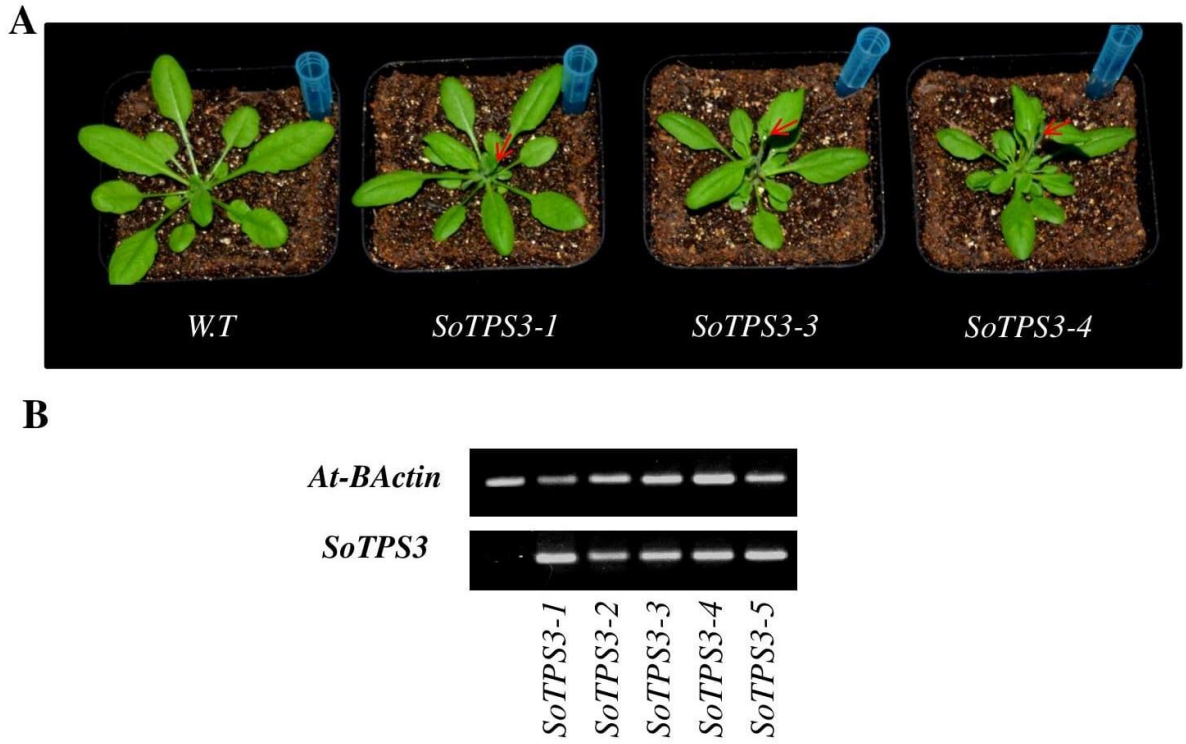

Figure 7. Overexpression of *SoTPS3* gene in transgenic Arabidopsis. (A) The phenotypes of the transgenic *A. thaliana* compared with the wild type *A. thaliana*. (B) Confirmation of the expression of terpenoid genes via Semiquantitative RT-PCR.

3.6. Overexpression of SoTPS3 gene altered the terpene profiles in transgenic A. thaliana leaves.

To examine the effect of overexpressing *SoTPS3* gene in *A. thaliana* leaves, we analysed the changes of terpene metabolic in transgenic A. thaliana leaves using GC-Mass. The results revealed that a variety of terpenes significantly increased in transgenic *A. thaliana* leaves overexpressing *SoTPS3* gene in parallel with control as we notificated in Table 2 and Supplementary figure S1. In leaves of *A. thaliana* plants overexpressing *SoTPS3*, Germacrene D-4α-ol appeared as the main sesquiterpene compound (75.25%), followed by Cis-Caryophyllene epoxide (1.31%), and Topanol (0.97%), whereas 3-Thujen-2-one (1.57%) and Phytol compound (2.05%) were observed as the major monoterpene and diterpene compounds, respectively.

4. Discussion

4.1. Cloning and sequence analysis of SoTPS3 gene from S. officinalis.

The full-length cDNA of *SoTPS3* gene was recognized and isolated from leaves of *S. officinalis*, based on the highly conserved sequence similarity between our query sequence and the other sequences detected in different plant species such as (*Salvia splendens*, *Origanum vulgare*, *Ocimum basilicum* and *Vitis vinifera*). When compared to other sesquiterpene synthases, SoTPS3 protein has three domains, which were specified by the InterPro database. The first domain is the N-terminal-domain (IPR001906: from 32-207 aa), and the second is the C-terminal-domain (IPR005630: from 238-499 aa), while the third is the metal-binding-domain (IPR034741: from 233-554aa) (Figs 1 and 2).

Table 2. The major terpenoid composition in transgenic *A. thaliana* leave over-expressing of *SoTPS3*.

N	Compound name	R.T (min.)	Formula	Molecular Mass (g mol-1)	Terpene Type	% Peak area	
						W.T	*SoTPS-3*
1	Acetic acid, [o-(trimethylsiloxy) phenyl]-, trimethylsilyl ester	29.591	C14H24O3Si2	296.5096		-	0.42
2	Topanol	31.135	C15H24O	220.3505	Sesqui	-	0.97
3	Cyclooctasiloxane, hexadecamethyl-	34.467	C16H48O8Si8	593.2315		-	0.31
4	Lauryl ethoxylate	35.598	C14H30O2	230.3868		1.52	-
5	3-Thujen-2-one	36.075	C10H14O	150.2176	Mono	-	1.57
6	Narceol	36.955	C9H10O2	150.1745		1.57	-
7	2-Methyldecane	38.48	C11H24	156.3083		1.48	-
8	Cyclohexasiloxane, dodecamethyl-	38.649	C12H36O6Si6	444.9236		-	0.38
9	6-Octen-1-ol, 3,7-dimethyl-, acetate	40.625	C12H22O2	198.3019		-	0.82
10	Phytan	41.196	C20H42	282.5475	Diter	2.39	-
11	Dodecamethylcyclohexasiloxane	42.353	C12H36O6Si6	444.9236		-	0.37
12	Oleic Acid	43.799	C18H34O2	282.468		3.29	-
13	Pentadecylic acid	44.721	C15H30O2	242.3975		-	0.86
14	Palmitic acid	45.31	C16H32O2	256.4241		27.86	-
15	Cis-Caryophyllene epoxide	46.055	C15H24O	220.3505	Sesqui	-	1.31
16	2-Methyldodecane	46.257	C13H28	184.3614		3.37	-
17	Palmitic acid, trimethylsilyl ester	47.316	C19H40O2Si	328.6052		6.33	-
18	Phytol	47.572	C20H40O	296.531	Diter	-	2.05
19	Heneicosane	48.625	C21H44	296.5741		3.02	
20	4,8,13-Duvatriene-1,3-Diol	49.158	C20H34O2	306.4828		-	5.34
21	Trans-Elaidic acid	49.488	C18H34O2	282.4614		26.79	
22	Germacrene D-4α-ol	49.898	C15H26O	222.3663	Sesqui	-	**75.25**
23	Heptadecane, 8-methyl-	50.911	C17H36	240.4677		2.41	-
24	Hexadecamethylcyclooctasiloxane	51.877	C16H48O8Si8	593.2315		-	0.72
25	Cadinane	51.916	C20H41Cl	316.993		10.23	
26	8,11,14-Eicosatrienoic acid, methyl ester	53.502	C21H36O2	320.5093		-	1.51
27	Oleamide	54.178	C18H35NO	281.4766		-	0.42
28	Hexasiloxane, tetradecamethyl-	56.117	C14H42O5Si6	458.9933		-	1.3
29	N-Pentatriacontane	58.321	C35H72	492.9462		-	0.61
30	Phthalic acid dioctyl ester;	59.765	C24H38O4	390.5561		-	0.57
31	Mandelic acid di(tert butyldimethylsilyl)-	60.91	C20H36O3Si2	380.669		-	0.29
32	Octadeamethyl-cyclononasiloxane	62.357	C18H54O9Si9	667.3855		-	0.75
33	9-Methylnonadecane	63.294	C20H42	282.5475		-	0.46
34	N-Nonacosane	68.714	C29H60	408.7867		-	1.03
35	Hexasiloxane, tetradecamethyl-	69.697	C14H42O5Si6	458.9933		-	0.57
36	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	74.561	C15H26O	222.3663		-	0.75
37	Octadeamethyl-cyclononasiloxane	77.334	C18H54O9Si9	667.3855		-	0.6
38	N-Nonacosane	78.147	C29H60	408.7867		-	0.77
39	Tetrapentacontane	78.973	C54H110	759.4512		9.74	-
	Total % Peak area					% 100	% 100

A comparison between our putative SoTPS3 protein sequence and other plant sesquiterpene synthase revealed various highly conserved motifs. Two of these motifs are called aspartate-rich-DDxxD motif (residues 313–317) and NSE/DTE motif (residues 457–465); they were reported to be surrounding the doorway to the active site position, and most likely responsible for coordination of divalent metal ion co-factors and substrate binding (Rebecca *et.al*.2020; Lima *et al*. 2013; L´opez-Gallego *et al*. 2010; Abbas *ettal*. 2019; Su-Fang *et.al*. 2014; Degenhard *et al*. 2009; Ali *et al*. 2017; Ali *et al*. 2018) (Figure 2). Moreover, they are also known for their role in binding a tri-nuclear-magnesium cluster, two-magnesium-ions and one-magnesium-ion (Christianson, 2006; Lima *et al*. 2013; Abbas *et al*. 2019). This magnesium cluster binds and interacts with the diphosphate moiety of farnesyl diphosphate (FPP), hence catalyzing the C15-substrate-FPP formation at the hydrophobic substrate binding pocket (Davis and Croteau, 2000; Degenhardt *et al*. 2009; Abbas *et al*. 2019). Moreover, we detected another conserved region RXR motif (residues 276–278) in the SoTPS3 protein, which is needed for product cyclization in class-III-TPS proteins (Rebecca *et al*. 2020; Su-Fang *et al*. 2014; Whittington *et al*. 2002; Hyatt *et al*. 2007) (Figure 2). Our analysis revealed further conserved region motifs in the SoTPS3 protein that are dominant in other sesquiterpene synthases, namely, RR (X8) W (residues 22–32) and GTLxEL (residues 320–325) region. Eventually, each protein sequence that belongs to the family of terpene synthase has one or two of these conserved domains and

motifs (Ali *et al*., 2017, 2018, 2022, 2022a). A phylogenetic tree was generated to analyze the evolutionary relationship between SoTPS3 and other plant sesquiterpene synthase genes, and the evolutionary tree was constructed in a good way by the neighbor-joining method parameters. Based on our classification results, the SoTPS3 protein was classified into the TPS-a subfamily that can encode mono- and sesquiterpene, which explains the ability of SoTPS3 to produce various types of terpenes as previously mentioned by (Ali *et al*., 2017 and 2018) (Figure 3).

4.2. 3D molecular modeling of SoTPS3

The 3D protein model for SoTPS3 was constructed depending on the crystal structure of (+)-δ-cadinene synthase from *G. arboreum* as depicted in Figure 4. The sequence similarities between our target sequence and (+)-δ -cadinene synthase were about 79% with 2.65Å as a root-mean-square deviation values, which indicates that the SoTPS3 sequence is very similar and well compatible with the (+)-δ-cadinene synthase X-ray template from *G. arboreum*. Moreover, our target sequence has a C-terminal-domain, a N-terminal-domain and the active site to catalyse the substrate FPP as shown in the active site pockets. These results suggest that SoTPS3 possesses similar mechanisms to that (+)-δ-cadinene as a sesquiterpene synthase gene from *G. arboreum*.

4.3. Putative tissue expression pattern and subcellular localizations of SoTPS3 gene

To identify the physiological roles of *SoTPS3*, we explored its putative expression in forty-seven tissues. This was aided by the high resemblance between *SoTPS3* and AT5G23960 gene from *A. thaliana*. *SoTPS3* gene was recognized in the tested tissues, and this result lines up with Ali et al., 2017, 2018, 2022a, who reported that most TPS genes (e.g. *SoFLDH*, *SgTPSV*, *SgGERIS*, *SoLINS2*, *GmTPS21*, *SgFARD*, *SoNEOD* and *SoHUMS*) from *S. officinalis*, *Glycine max*, and *S. guaranitica* showed an increased expression in leaves, roots and seeds. Furthermore, the putative subcellular localization for SoTPS3 protein revealed that our gene is present at most in the Cytosol, then Plastid, Mitochondria and Nucleus. Our gained results are similar with Ali *et al*., Taniguchi *et al*., Chen *et al*., and Wang *et al*., (Ali *et al*., 2017, 2018, 2022a; Wang *et al*., 2015b; Taniguchie*t al*., 2014; Chen *et al*., 2018; Makhadmeh et al., 2022a and 2022b) who found that various TPSs genes were localized in the plastid, mitochondria and nucleus (Figure 5).

4.4. Effectiveness of qRT-PCR for analysis the SoTPS3 gene expression.

We used qRT-PCR to measure the expressed levels of *SoTPS3*. Therefore, qPCR data revealed that *SoTPS3* is highly expressed in flowers then at bud flowers, young leaves and stems. This fits with the information provided previously that found various TPS syntheses genes were highly expressed in bud flowers, young leaves, stems, and old leaves (Sabin *et al*., 2012; Croteau *et al*., 1981; Ali *et al*., 2017; Ali *et al*., 2018). The low expression of *SoTPS3* gene in old leaves and roots is probably due to many reasons such as: gene-regulatory mechanisms (possible posttranscriptional and/or post-translational) (Figure 6). It is also possible that the expression levels of *SoTPS3* is associated with tissue-developmental-stages, which strongly influences the expression of terpene synthase in salvia (Sabin *et al*., 2012; Croteau *et al*., 1981; Ali *et al*., 2017; Ali *et al*., 2018)

4.5. Overexpression of SoTPS3 gene changed the A. thaliana plants phenotypes

To evaluate the function of *SoTPS3* in *A. thaliana* (Col-0: Columbia-0) plants, we overexpressed *SoTPS3* in *A. thaliana* through the use of Agrobacterium bacteria harboring the overexpression vector pB2GW7-*SoTPS3*. Thereafter, sqRT-PCR confirmed the expression of our target gene in positive transgenic lines (Figure 7). The transgenic lines showed higher expression level of the *SoTPS3* gene in parallel with the wild-type, which indicated the existence of our target gene in the transgenic plants. After that, we chose three transgenic plants (named; OE-SoTPS3-1, OE-SoTPS3-3 and OE-SoTPS3-4) for terpene analysis. The morphological analysis showed that the previous transgenic plants had an accelerated rate of flowering stem formation unlike the wild type plants **(**Figure 7). Our earned positive results are in line with Ali *et al*. 2017, 2018 and 2022, where the overexpression of TPS synthesis and terpenoids genes, such as *SgGPS*, *SgLINS*, *SgFPPS*, *SoTPS6*, *SoCINS*, *SoLINS*, *SoFLDH*, *SoSABS*, *SgGPS* and *SoNEOD* from *S. guaranitica* and *S. officinalis* in *A. thaliana* and *Nicotiana tabacum*, accelerated the growth and flower formation when compared to wild type plants. In previous results, numerous TPSs family genes played a key role in different cell-specific processes, such as: 1, 8-cineole, Z-γ-bisabolene, Rhizathalene, β-amyrin and thalianol synthesis as a mono-,sesqui-, di- and triterpene, respectively **(**Field and Osbourn, 2008; Field *et al*. 2011; Kampranis *et al*. 2007; Wang *et al*. 2016; Ro *et al*. 2006; Vaughan *et al*. 2013; Chen *et al*. 2011; Chen *et al*. 2004**).** This indicates that these genes can initially co-express in a variety of cells, tissues, and organs to produce distinct plant phenotypes, confirming the function of TPSs-genes in plant development, growth, and blooming. **(**Field and Osbourn, 2008; Field *et al*. 2011; Kampranis *et al*. 2007; Wang *et al*. 2016; Ro *et al*. 2006; Vaughan *et al*. 2013; Chen *et al*. 2011; Chen *et al*. 2004**).** The metabolites were analyzed by GC-MS-system to recognize the specific terpenes synthesized after introducing the *SoTPS3* gene into Arabidopsis plants and generating over expressing transformant lines. The mono-, sesqui- and diterpene peaks were easily visible; the percentage of peak area (% peak area) demonstrated the type and amount of the compounds. To identify these terpenes in our transgenic Arabidopsis plants, we used the libraries of mass spectra, the previous extracts of wild-type Arabidopsis as a reference, which produce different quantitative and qualitative of terpenoids. Per the results shown in Table 2 and Supplementary figure S1, a clear alteration was observed in the transgenic plants, and a new peak at retention time (49.898) was detected. This peak was identified as Germacrene D-4α-ol, based on the matched mass with the Wiley GC/MS, NIST Library and VOC Analysis S/W software. The production of Germacrene D-4α-ol as a sesquiterpene by the overexpression of *SoTPS3* gene in *A. thaliana* was described formerly by Ali *et al*. 2018; Su-Fang *et al*. 2014. Various terpene synthase genes are known to synthesize various products in unison, *e.g.* carene, (±)-linalool, cineole, myrcene, β-amyrin and

terpinolene synthases (Yoko *et al*., 2004; Shimada *et al*. 2005; Abbas *et al*. 2019; Lucker *et al*. 2002; Fahnrich *et al*. 2011; Xi *et al*. 2016; Faldt *et al*. 2003). We believe that *SoTPS3* was responsible for the production of Germacrene D-4α-ol via isoprenoid pathway which is common in sesquiterpene biosynthesis.

5. Conclusions

S. officinalis is a significant Egyptian medicinal herb with unique pharmacological properties. Hence, the cloning and characterization of many genes related to secondary metabolic pathway will aid the success of metabolic engineering in *S. officinalis* and other medicinal plants**.** In this study, we have cloned a plant Germacrene D-4α-ol biosynthesis gene from *S. officinalis* and named it SoTPS3. Overexpression of *SoTPS3* in *A. thaliana* accelerated the flowering in *OE-SoTPS3-1*, *OE-SoTPS3*-3 and *OE-SoTPS3-4* transgenic lines. These previous lines showed a high production of Germacrene D-4α-ol compared with control. The Germacrene D-4α-ol produced in these transgenic lines reveals that the *A. thaliana* plants have the ability to synthesize the same product through the common mevalonate-pathway (MVK) of sesquiterpene biosynthesis. Moreover, the putative expression patterns and subcellular localization results revealed that the *SoTPS3* gene was highly expressed in various flower tissues and mainly localized in the cytosol, which underscores the possible role of this gene in yielding various types of terpenes, especially the sesquiterpene Germacrene D-4α-ol. This study revealed that the *A. thaliana* as a model plant can robustly use as suitable model for study the sesquiterpene gene that can be used for improving essential oil composition in *S. officinalis* and other plant species through metabolic engineering.

6. Ethics approval and consent to participate

No investigations were undertaken using humans/human samples in this study. No experimental animals were used to conduct any of the experiments reported in this manuscript. Our study did not involve endangered or protected species.

7. Competing interests

The authors declare that they have no competing interests.

8. Funding

This study was supported by the Department of Genetic Resources, Desert Research Center (DRC), Egypt.

9. Author's contributions

MA conceived and designed the study; MA, FAE, EAE, MNSS and MSR performed experiments, MA wrote the paper. All authors discussed the results and commented on the manuscript and participated in the analysis of the data. All authors participated in reading and approving the final manuscript.

10. Availability of data and materials

All data supporting my findings can be available and found in the supplementary data.

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