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Identification and Characterization of Terpene Synthases Potentially Involved in the Formation of Volatile Terpenes in Carrot (*Daucus carota* L.) Roots

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Supporting Information

ABSTRACT: Plants produce an excess of volatile organic compounds, which are important in determining the quality and nutraceutical properties of fruit and root crops, including the taste and aroma of carrots (*Daucus carota* L.). A combined chemical, biochemical, and molecular study was conducted to evaluate the differential accumulation of volatile terpenes in a diverse collection of fresh carrots (*D. carota* L.). Here, we report on a transcriptome-based identification and functional characterization of two carrot terpene synthases, the sesquiterpene synthase, *Dc*TPS1, and the monoterpene synthase, *Dc*TPS2. Recombinant *Dc*TPS1 protein produces mainly (*E*)-β-caryophyllene, the predominant sesquiterpene in carrot roots, and α-humulene, while recombinant *Dc*TPS2 functions as a monoterpene synthase with geraniol as the main product. Both genes are differentially transcribed in different cultivars and during carrot root development. Our results suggest a role for *DcTPS* genes in carrot aroma biosynthesis.

KEYWORDS: *Daucus carota, terpenes, (E)-β-caryophyllene, geraniol, terpene synthase*

INTRODUCTION

Carrots (*Daucus carota* L.) are popular vegetables because of their health benefits and characteristic flavor.[1,2] Carrot quality is determined by several traits that affect taste, aroma, and nutritional value.[3] In particular, terpene secondary metabolites, which are synthesized during carrot root development, have a direct effect on root quality and flavor.

Terpenes constitute the largest class of plant secondary metabolites, represent major components of floral scents and essential oils of herbs,[4] and are important in determining the quality and nutraceutical properties of horticultural food products, including the taste and aroma of wine and melon.[5,6] Plants produce terpenoids that function in primary metabolism, such as phytohormones (absisic acid, gibberellins, and brassinosteroids), and are part of photosynthetic pigments (phytol and carotenoids).[7] Terpenes can serve as phytoalexins in defense against plant pathogens[8] or direct defense against herbivores.[9] In addition, volatile terpenoids can function as indirect defensive compounds by attracting predators or parasitoids of the attracting insect.[10]

All plant terpenes are made from the 5-carbon precursor, isopentenyl diphosphate (IPP), and its isomer, dimethylallyl diphosphate (DMAPP), both of which are derived from two alternative pathways, the mevalonate (MVA) pathway in the cytosol or the methylerythritol phosphate (MEP) pathway in plastids. Condensation of the C_5 precursors leads to the formation of C_{10}, C_{15}, and C_{20} *trans*- or *cis*-prenyl diphosphate intermediates, such as geranyl diphosphate (GPP), neryl diphosphate (NPP), *trans,trans*-farnesyl diphosphate or *cis,cis*-farnesyl diphosphate [(*E,E*)-FPP or (*Z,Z*)-FPP], and all-*trans*-geranylgeranyl diphosphate (GGPP) that are converted into monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), respectively, by the activity of terpene synthase (TPS) enzymes.[11] GGPP also functions as a precursor in the carotenoid biosynthetic pathway by condensation of two GGPP units to C_{40}-phytoene.[12] TPSs are the primary enzymes in the formation of low-molecular-weight terpene metabolites. To date, TPSs have been identified and characterized in many species,[13,14] including *Arabidopsis thaliana*,[15] *Cucumis melo* L.,[6] *Gossypium hirsutum* L.,[16] *Solanum lycopersicum*,[17] *Vitis vinifera* L.,[18] *Coriandrum sativum*,[19] and *Thapsia garganica*. TPSs are encoded by large gene families and have the ability to produce multiple terpene products from a single substrate. The formation of mixtures of structurally diverse compounds contributes to the specific aroma and flavor characteristics of

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Table 1. Quantification of of Mono- and Sesquiterpene Volatile Compounds in Different Raw Colored Carrot Varieties at 10 Weeks after Sowing[a]

compounds	level of mono- and sesquiterpene volatile compounds [ng/g of fresh weight (FW)]					IC[b]
	Nairobi	Rothild	Purple Haze	Yellowstone	Crème de Lite	
α-pinene	110.72 ± 0.74	214.22 ± 1.25	227.15 ± 1.63	110.93 ± 0.97	89.55 ± 0.27	RI, MS, STD
camphene	17.26 ± 0.25	22.91 ± 0.25	27.00 ± 0.19	12.44 ± 0.28	17.02 ± 0.23	RI, MS, STD
sabinene	16.56 ± 0.16	348.87 ± 0.67	30.67 ± 0.28	604.89 ± 1.51	168.92 ± 1.58	RI, MS, STD
β-pinene	60.51 ± 0.63	100.50 ± 0.94	182.31 ± 1.44	181.36 ± 0.94	57.83 ± 0.45	RI, MS, STD
β-myrcene	168.21 ± 0.51	688.81 ± 1.02	219.81 ± 0.45	440.44 ± 0.94	233.10 ± 1.1	RI, MS, STD
α-phellandrene	47.18 ± 0.20	47.44 ± 0.31	22.39 ± 0.29	71.69 ± 0.20	26.98 ± 0.433	RI, MS, STD
α-terpinene	35.51 ± 0.55	129.32 ± 0.12	21.75 ± 0.16	223.01 ± 0.61	63.21 ± 0.75	RI, MS,STD
p-cymene	194.21 ± 0.97	186.31 ± 0.73	156.70 ± 1.01	109.27 ± 0.47	85.73 ± 0.87	RI, MS, STD
limonene	68.85 ± 0.55	99.17 ± 0.52	47.21 ± 0.44	82.02 ± 0.29	55.55 ± 0.846	RI, MS, STD
Z-β-ocimene	35.12 ± 0.33	28.49 ± 0.22	35.38 ± 0.05	49.33 ± 0.31	18.91 ± 0.12	RI, MS, STD
E-β-ocimene	9.81 ± 0.26	15.98 ± 0.42	8.46 ± 0.24	26.00 ± 0.42	6.77 ± 0.28	RI, MS, STD
γ-terpinene	348.11 ± 1.32	496.98 ± 0.76	417.69 ± 1.31	361.53 ± 1.38	160.71 ± 0.77	RI, MS, STD
terpinolene	566.80 ± 1.23	516.53 ± 0.9	80 ± 0.16	744.23 ± 0.65	304.92 ± 0.99	RI, MS, STD
borneol	1.72 ± 0.12	1.38 ± 0.05	T	1.88 ± 0.05	2.42 ± 0.07	RI, MS, STD
geraniol	0.78 ± 0.17	5.10 ± 0.39	T	6.28 ± 0.89	1.88 ± 0.02	RI, MS, STD
thymol	3.12 ± 0.25	T	T	5.98 ± 0.29	T	RI, MS, STD
β-elemene	T	8.18 ± 1.3	T	2.51 ± 0.22	T	RI, MS, STD
β-caryophyllene	622.66 ± 2.85	696.40 ± 1.08	993.55 ± 0.75	412.84 ± 1.09	1048.32 ± 0.93	RI, MS, STD
α-humulene	66.80 ± 0.76	83.71 ± 0.64	103.30 ± 1.24	88.07 ± 0.18	124.47 ± 0.55	RI, MS, STD

[a]Volatile terpenes were measured by auto-HS−SPME−GC−MS. T = traces. The results shown are an average of three biological replicates. [b]IC = identification criteria. The identification criteria were based on mass spectra matching with the standard NIST-98.L and Wiley 7N.I libraries (MS), comparison of the retention index (RI), and comparison to the authentic standard (STD).

plant tissues.[17] Transcriptional regulation plays an important role in controlling volatile organic compound biosynthesis, but it is not the only mechanism involved. While little is known about post-transcriptional regulation of volatile organic compound formation, recent comprehensive reviews provide a thorough discussion of the regulation of the MVA and MEP pathways.[7,20] Recently, it has been reported that the progressive biosynthesis of (*E*)-β-caryophyllene in *Arabidopsis* inflorescence after flowering is regulated by miR156-targeted SQUAMOSA promoter binding protein-like (SPL), which directly activates TPS21 expression.[21]

Carrots produce a large number of different mono- and sesquiterpene volatiles in leaf and root tissues.[22] In carrot roots, terpenes are more abundant in mature tissues and primarily synthesized in an interconnected network of oil ducts located in the phloem.[23] Labeling experiments with stable isotope precursors also indicated *de novo* biosynthesis of terpenes in the xylem.[24]

Despite the characterization of a large number of plant TPSs, surprisingly, no carrot TPSs have been described to date. Here, we report on the isolation and functional characterization of two carrot terpene synthase genes, *Daucus carota* terpene synthase 1 (*DcTPS1*) and *D. carota* terpene synthase 2 (*DcTPS2*), whose encoded enzymes catalyze the formation of the sesquiterpene, (*E*)-β-caryophyllene, and the monoterpene alcohol, geraniol, respectively. We show that *DcTPS1* is expressed in all five cultivars investigated in this study and is most likely associated with the production of high amounts of (*E*)-β-caryophyllene in these cultivars. *DcTPS1* gene transcript levels peak at week 12 post-germination in correlation with highest concentrations of terpene compounds at this developmental stage.

MATERIALS AND METHODS

Chemicals. Unlabeled FPP and GPP (1 mg/mL), terpene standards, other chemicals, and reagents were purchased from Sigma-Aldrich, unless noted otherwise.

Plant Material. Commercial colored carrot cultivars, orange "Nairobi", orange "Rothild", purple "Purple Haze", yellow "Yellowstone", and white "Crème de Lite" (Kiepenkerl Profi-Line, www.kiepenkerl.com), were grown in the "Newe Ya'ar" Research Center in northern Israel, under standard field irrigation and fertigation conditions. Freshly harvested 9−12-week-old carrot roots were crushed in liquid nitrogen and stored at −80 °C for terpene and transcript analysis.

Statistical Analysis. Amounts of terpenes are presented as means ± standard error (SE). Chemical terpene data were analyzed by multivariate data analysis [principle component analysis (PCA)] using the JMP software (SAS Institute, Inc.).

Extraction of Volatile Compounds from Fresh Carrot Roots. Three replicates of fresh 10-week-old root carrots (1 g) of each cultivar were ground into a uniform powder under liquid nitrogen with a mortar and pestle. The fine powder was placed in a 20 mL DuPont autosampler vial (DuPont Performance Elastomers, http://www.dupontelastomers.com) with a white solid-top polypropylene cap (Alltech, http://www.alltech.com). Samples were overlaid with 5 mL of NaCl (25%) solution and 1 g of NaCl (for inhibition of enzyme activity). Each sample was supplemented with 1 mg/kg [1 part per million (ppm)] of 2-heptenone as an internal standard. Samples were incubated at room temperature (25 °C) for 1 h, and thereafter, volatile compounds were collected with a solid-phase microextraction (SPME) device PDMS-100 with a polydimethylsiloxane fiber (Supelco, http://www.sigmaaldrich.com) by inserting the fiber into the tube and leaving it in place for 20 min at room temperature. After this incubation step, the SPME fiber was injected directly into gas chromatography−mass spectrometry (GC−MS).

Auto-Head Space (HS)−SPME−GC−MS Analysis of Carrot Root Volatile Compounds. Volatile compounds were analyzed on a GC−MS apparatus (Agilent Technologies, Santa Clara, CA) equipped with an Rtx-5SIL MS (30 m × 0.25 mm × 0.25 μm) fused-silica capillary column. He (1 mL min^{-1}) was used as a carrier gas. The

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injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 220 °C at a rate of 5 °C min^{-1}. For SPME analysis, thermal desorption was allowed for 40 min. The detector temperature was 280 °C. The mass range was recorded from m/z 41 to 450, with an electron energy of 70 eV. Identification of the main components was performed by comparison of mass spectra and retention times to those of authentic standards and supplemented with a Wiley GC−MS library. Quantification of absolute amounts was performed for (*E*)-*β*-caryophyllene, *α*-humulene, and the monoterpenes listed in Table 1 by comparing their retention time and mass spectra to those of authentic standards.

For the construction of the calibration curves, a mixture of straight-chain alkanes (C_7−C_{23}) was run under the above-mentioned conditions to determine retention indices. The amount of each compound in the sample was calculated as (peak area × internal standard response factor) divided by (response factor × internal standard peak area).[25] For compounds for which no standards were available, only normalized peak areas are shown. Total terpene levels were based on normalized peak areas of all compounds.

Isolation and Characterization of Carrot Terpene Synthases. Putative carrot *TPS* encoding genes were identified using Blastx to compare known *TPS* genes in *Arabidopsis* (*At2g24210* and *At5g23960*) to the *de novo* assembled RNA-Seq carrot transcriptome contigs from Iorizzo et al.[26] The expression levels of these candidate genes in the purple (B7262) cultivar and an orange cultivar (B6274) were estimated by mapping the Illumina GAII reads for each sample from Iorizzo et al.,[26] (SRA accessions SRR187758−SRR187763) using the "Map to Reference" feature of the Geneious (BioMatters, Ltd.) alignment software.

Two specific primers corresponding to the 5′ and 3′ ends of the *DcTPS1* coding sequence (P1: 5′-ATG TCT CTC AAT GTT CTG GC-3′) and (P2: 5′-TGA TGG AAC CCG ATC AAT GA-3′) were designed. *DcTPS2* was obtained using the forward primer P3 (5′-ATG GCC CTC CCA GCT CTG TTT T-3′) and the reverse primer P4 (5′-CTG GCT AAG AGT AAA GGG TTC GAC C-3′).

RNA from carrot roots of all colored cultivar was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich). To produce a cDNA clone, 5 *μ*g of total RNA from the Nairobi (orange) cultivar was reverse-transcribed using the SuperScript one-step reverse transcriptase polymerase chain reaction (RT-PCR). The DNA molecule was then amplified using Platinum *Taq* DNA polymerase (Invitrogen), yielding a 1683 bp specific fragment for *DcTPS1* and a 1782 pb fragment for *DcTPS2*, respectively. The cDNAs were ligated into the pEXP5-CT/TOPO TA expression vector (Invitrogen Corporation, Carlsbad, CA), producing pEXP-*DcTPS1*, and pEXP-*DcTPS2*, respectively, in which the *DcTPS1* and *DcTPS2* coding sequences were fused with a His-tag-coding extension at the C terminus and transformed into *Escherichia coli* TOP10 cells. The constructs were verified by DNA sequencing.

Preparation of Bacterial Lysates. A 3 mL preculture of *E. coli* BL21 (DE3) was grown overnight at 37 °C in lysogeny broth (LB) medium containing 100 g/mL ampicillin. The culture was used to inoculate 500 mL of fresh medium to which 500 *μ*M isopropyl-1-thio-*β*-D-galactopyranoside (IPTG) was added after 3 h to induce protein expression. Cells were further grown for 12 h at 30 °C. After centrifugation for 10 min at 11000*g*, the bacteria were resuspended in 50 mM Bis-Tris at pH 6.9, containing 10% (v/v) glycerol, 10 mM dithiothreitol (DTT), and 5 mM $Na_2S_2O_5$. Cells were lysed by a combination of a 30 min lysozyme treatment (1 mg/500 mL of culture) and subsequent ultrasonication (five pulses of 20 s at 20 W, at 4 °C). After the cells lysed, the suspensions were centrifuged (20000*g* for 30 min at 4 °C). The supernatant containing the soluble recombinant *Dc*TPS1 and *Dc*TPS2 proteins, respectively, were purified by metal (nickel) affinity chromatography (Qiagen, Valencia, CA) with a stepwise gradient of increasing imidazole concentrations using standard procedures. The *Dc*TPS1- and *Dc*TPS2-containing fractions were pooled and desalted with 50 mM Bis-Tris at pH 7.0 containing 10% (v/v) glycerol and 10 mM DTT, using Vivaspin 20 [GE Healthcare, molecular weight cutoff (MWCO) of 10 kDa]. Proteins were checked for purity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). Protein concentrations were determined by the method of Bradford[27] using reagent obtained from Bio-Rad. Bovine serum albumin was used for calibration.

Assay for Terpene Synthase Activity. Enzyme activity assays were performed in screw-capped 2 mL GC glass vials, using 1 to 500 ng of purified recombinant proteins, 10 *μ*M substrate (GPP or FPP), 10 mM $MgCl_2$, 10 *μ*M $MnCl_2$, and 50 mM Bis-Tris at pH 7.0 assay buffer in a total volume of 100 *μ*L. The reactions were incubated for 30 min at 30 °C. After incubation, the samples were analyzed by auto-HS−SPME−GC−MS for the identification of volatile terpenes generated during the 30 °C incubation. As controls, *E. coli* cells transformed with control plasmids devoid of the *DcTPS1* and *DcTPS2* genes, and heat-inactivated DcTPS1 and DcTPS2 proteins or assays without GPP/FPP as a substrate were used.

***DcTPS* Transcript Analysis.** For quantitative RT-PCR (qRT-PCR) analysis of *DcTPS1* and *DcTPS2*, total RNA (5 *μ*g) from the orange carrot cultivar Nairobi was extracted (Spectrum Plant Total RNA Kit, Sigma-Aldrich) and reverse-transcribed using an oligo-dT primer and the SuperScript II first-strand system (Invitrogen).

qRT-PCR was performed on an Applied Biosystems StepOnePlus real-time PCR system (Life Technologies) using ABsolute Blue qPCR SYBR Green ROX Mix (Tamar Laboratory Supplies, Ltd., Israel), 5 ng of reverse-translated total RNA, and 100 ng of each primers. Primers for *DcTPS1* were *DcTPS1*-qRT-F (5′- CCTCTACTGTGTTCCG-CAAATA-3′) and *DcTPS1*-qRT-R (5′-CTGAAGTGCTTCCTC-CAGTATC-3′), and primers for *DcTPS2* were *DcTPS2*-qRT-F (5′-AAAGATGACACAGCGGGTAAA-3′), and *DcTPS2*-qRT-R (5′-GCCTCTGAAACCGAAGAAAGA-3′). A relative quantification of gene expression was performed using the housekeeping gene tubulin from carrot as a reference gene. The primers used for tubulin were Tubulin_F_qPCR (5′-TCTTGGAGGTGGCACAGGAT-3′) and Tubulin_R_qPCR (5′-ACCTTAGGAGACGGGAACACAGA-3′).

The difference in relative expression levels of *DcTPS1* and *DcTPS2* were calculated from the $2^{-\Delta\Delta C_t}$ value after normalization of *DcTPS1* and *DcTPS2* data to tubulin. All analyses were performed using at least three biological replicates.

RESULTS AND DISCUSSION

Terpene Profiles in Colored Carrot Cultivars. We previously demonstrated that variation in norisoprenoid accumulation in different colored carrot cultivars is genotype-dependent.[2] Also, variation in the total content of carrot terpenes has been reported.[28−31] However, to the best of our knowledge, the role of TPSs in the formation of mono- and sesquiterpene volatiles and their flavor properties in carrots has not been reported. Our primary goal is to decipher the role of individual TPSs in terpene flavor variation in different carrot cultivars. To this end, we first examined the volatile compound composition of freshly harvested (10 week old) tissue of five colored cultivars (orange "Nairobi", orange "Rothild", purple "Purple Haze", yellow "Yellowstone", and white "Crème de Lite") using SPME−GC−MS. More than 41 volatile compounds were detected, among which 23 were identified or predicted to be monoterpenes and 17 were found or predicted to be sesquiterpenes (Table 1 and Supplemental Table 1 of the Supporting Information). The orange carrot cultivar, Rothild, accumulated the highest levels of total volatile compounds, and the yellow carrot cultivar, Yellowstone, accumulated the lowest level of total terpenes (see Supplemental Table 1 of the Supporting Information). Our analyses unveiled the presence of different terpenes in carrot roots according to the variety analyzed (Table 1 and Supplemental Table 1 of the Supporting Information). The main sesquiterpene compounds found in all cultivars were (*E*)-*β*-caryophyllene, (*E*)-*γ*-bisabolene, and *α*-humulene, and the main monoterpenes were terpinolene, *γ*-

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terpinene, myrcene, *p*-cymene, and α-pinene (Table 1 and Supplemental Table 1 of the Supporting Information).

To determine whether the terpene volatile compositions in carrot cultivars of diverse color are influenced by the genotypes, PCA was applied. The results are visualized by mean values and as bi-plot (PC1 versus PC2) (Figure 1), showing the first two

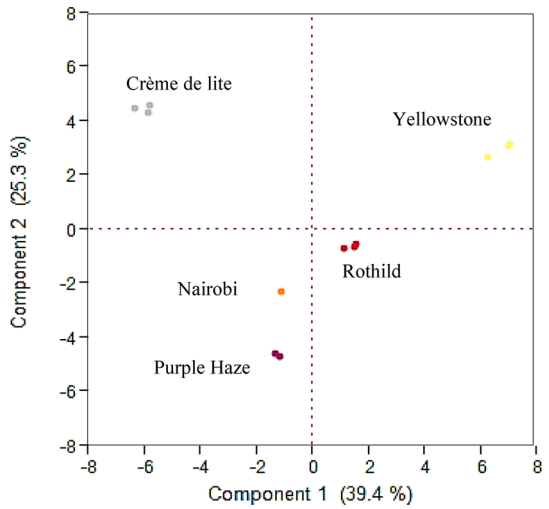

Figure 1. PCA analysis of 41 volatile compounds in five different colored carrot cultivars: orange "Nairobi", orange "Rothild", purple "Purple Haze", yellow "Yellowstone", and white "Crème de Lite". All analyses were performed using three biological replicates.

PCs to explain 64% of the variation. A large proportion of the variation in the terpene volatiles is found in PC1 (39.4%), whereas PC2 (25.3%) mainly explains a chemical variation in the terpene content. Our analysis showed that mono- and sesquiterpene profiles varied widely among different genetic stocks (Figure 1). These findings of genotypic differences are supported by other previous analyses of diverse carrot varieties.[28−31]

Analysis of Terpene Levels during Carrot Root Development. To better understand the pattern of terpene accumulation during carrot root development, we determined the total terpene content of the orange carrot cultivar Nairobi between 9 and 12 weeks after sowing (Figure 2). This time period of root maturation overlaps with the time of harvest 10−14 weeks post-seeding.[32]

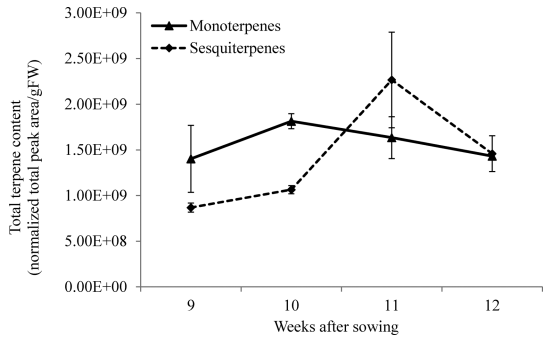

Figure 2. Terpene volatile accumulation during carrot root development in the orange cultivar Nairobi. The authenticity of mono- and sesquiterpenes was confirmed by comparing their retention time and mass spectra to those of authentic standards. Values are the mean ± standard error (n = 4).

Auto-HS−SPME−GC−MS analysis of total terpene volatile accumulation demonstrated that mono- and sesquiterpene concentrations consistently increased between the 9th and 11th week of carrot root development prior to a slight decrease in week 12 (Figure 2). The accumulation of sesquiterpenes was more rapid than that of monoterpenes between weeks 10 and 11. The reduced concentration observed after week 11 may be attributable to a period where root growth outpaced terpene accumulation. The composition of compounds during carrot root development further depends upon the different soils, climates, and genetic variability of the carrot cultivars.[32,33]

The chemical profile of terpenes found in carrot roots was first assessed to facilitate the design of PCR primers for the isolation of *TPS* genes catalyzing the biosynthesis of particular classes of terpenes. Interestingly, (*E*)-β-caryophyllene account as the main sesquiterpene compounds found in all cultivars (Table 1). A possible rationale would be to identify the TPSs that are responsible for producing the predominant terpene products in carrot roots. Therefore, two candidate *DcTPS1* and *DcTPS2* genes were chosen on the basis of sequence homology to other known plant TPSs for further characterization (see below).

Isolation and Functional Characterization of Carrot TPSs. Despite the characterization of a large number of plant TPSs, surprisingly no TPS enzymes or their encoding genes have been isolated and characterized from *D. carota*. Moreover, the role of TPSs in the formation of mono- and sesquiterpene volatiles and their flavor properties in carrots have not been reported. To identify the genes responsible for mono- and sesquiterpene volatile formation in carrot roots, we performed a homology search of RNA-Seq carrot transcriptome sequences, which had previously been obtained by Iorizzo et al.[26] Blastx searches of the assembled contigs in the "additional file 2" (1471-2164-12-389-S2.FASTA) from Iorizzo et al.,[26] against *TPS* genes in Genbank identified more than a dozen candidate *TPS* genes. At least six were full-length carrot *TPS* cDNAs, of which three are predicted to be monoterpene synthases and three may be sesquiterpene synthases (Table 2). The wide variation observed in the number of mapped reads for these genes in the purple (B7262) and orange (B6275) cultivars suggests substantial differences in gene expression between the two cultivars (Table 2).

Table 2. Detection of Carrot *TPS* Genes Based on *de Novo* Assembly of Illumina Sequences of Two Carrot Genotypes[26] [a]

	number of mapped reads per *TPS* contig		
carrot EST contig	cultivar B6274 (orange)	cultivar B7262 (purple)	predicted TPS function according to best Blast Match (Genbank) >50% amino acid
1324	1079	2160	monoterpene synthase
21245	2392	6925	monoterpene synthase
43814	3119	940	monoterpene synthase
4929	5726	304	sesquiterpene synthase
52846	1045	3478	sesquiterpene synthase
58617	602	598	sesquiterpene synthase
	total RNA-Seq reads per cultivar = 28363561	total RNA-Seq reads per cultivar = 33029462	

[a] Six contigs correspond to full-length *TPS*.

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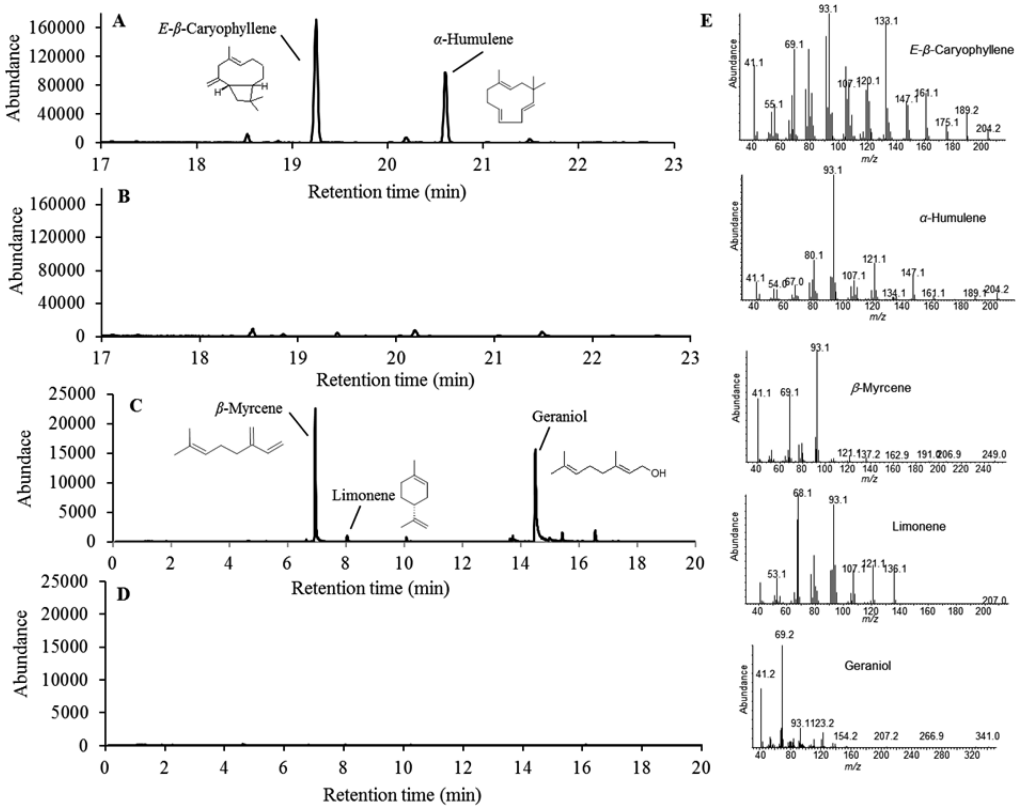

Figure 3. GC−MS of the products generated *in vitro* by nickel−nitrilotriacetic acid (Ni−NTA)-purified recombinant *Dc*TPS1 protein. (A) GC−MS analysis of *Dc*TPS1 with FPP as a substrate and measured by auto-HS−SPME−GC−MS. (B) GC−MS analysis of boiled *Dc*TPS1 with FPP as a substrate and measured by auto-HS−SPME−GC−MS. (C) GC−MS analysis of *Dc*TPS1 with GPP as a substrate and measured by auto-HS−SPME−GC−MS. (B) GC−MS analysis of boiled *Dc*TPS1 with GPP as a substrate. Identification of the products was performed by GC−MS comparing to authentic standards and according to the retention time and by mass spectral library comparison. (E) Mass spectra of enzymatic reaction products of *E*-*β*-caryophyllene, *α*-humulene, *β*-myrcene, limonene, and geraniol. *m*/*z* = mass-to-charge ratio. The inset shows the structure of the products *E*-*β*-caryophyllene, *α*-humulene, *β*-myrcene, limonene, and geraniol.

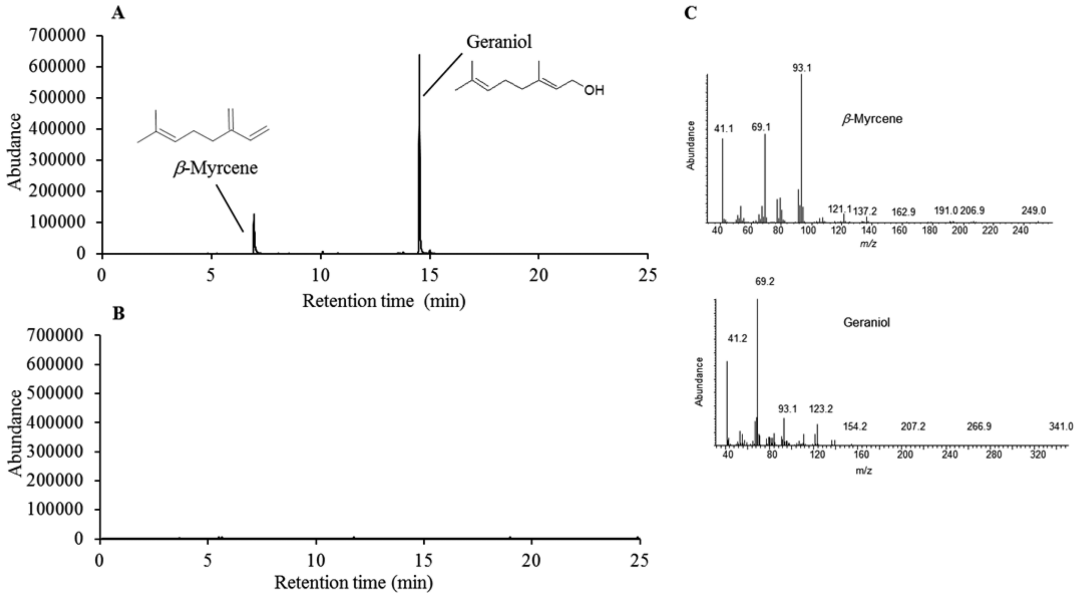

Figure 4. GC−MS of the products generated *in vitro* by Ni−NTA-purified recombinant *Dc*TPS2 protein. (A) GC−MS analysis of *Dc*TPS2 with GPP as a substrate and measured by auto-HS−SPME−GC−MS. (B) GC−MS analysis of boiled *Dc*TPS2 with GPP as a substrate and measured by auto-HS−SPME−GC−MS. Identification of the products was performed by GC−MS comparing to authentic standards and according to the retention time and by mass spectral library comparison. (C) Mass spectrum of enzymatic reaction products of *β*-myrcene and geraniol. *m*/*z* = mass-to-charge ratio. The inset shows the structure of the products *β*-myrcene and geraniol.

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We then generated cDNAs for the *DcTPS* contig 4929 (called *DcTPS1* from here on) and *DcTPS* contig 43814 (called *DcTPS2* from here on) from RNA isolated from the orange cultivar Nairobi in our cultivar collection. Amino acid sequences encoded by *DcTPS1* and *DcTPS2* were 90% identical to those identified from the B7262 and B6274 cultivars. Recombinant *Dc*TPS1 protein expressed in *E. coli* converted (*E*,*E*)-FPP to (*E*)-β-caryophyllene, the predominant sesquiterpene in all cultivars (Table 1), as the primary product, along with α-humulene (Figure 3A). Incubation of *Dc*TPS1 with GPP led to the production of several monoterpenes, including β-myrcene, limonene, and geraniol (Figure 3C). *E. coli* cells transformed with control plasmids devoid of the *DcTPS1* gene, and heat-inactivated DcTPS1 protein or assays without GPP/FPP as a substrate were used as a control (panels B and D of Figure 3).

Our results confirm previous findings in other plants indicating that sesquiterpene synthases, e.g., β-caryophyllene synthase from *Artemisia annua*, produced monoterpenes[34] and 9-*epi*-caryophyllene from *Lavandula* × *interminda*,[35] and *trans*-α-bergamotene synthase from *Lavandula angustifolia*,[36] produced monoterpenes when assayed with GPP as a substrate. The mono- and sesquiterpenes formed by *Dc*TPS1 were found as constituents of the carrot volatile blend in all carrot cultivars (Table 1). However, it can be assumed that *Dc*TPS1 most likely functions as a sesquiterpene synthase *in vivo* because of the lack of a plastidial targeting sequence (Figures 3A and 7). TPS enzymes producing (*E*)-β-caryophyllene and α-humulene have been reported primarily from aboveground tissues of a variety of plants, where these sesquiterpenes are assumed to function in mutualistic interactions and in plant defense.[37,38] Functions of root-specific (*E*)-β-caryophyllene synthases have been less well-described, with the exception of a maize (*E*)-β-caryophyllene synthase that is involved in indirect defense against root herbivores.[39]

Analysis of reaction products formed after incubation of the *Dc*TPS2 protein with GPP showed the presence of two monoterpene compounds, geraniol and β-myrcene (Figure 4A). As control experiments, heat-inactivated DcTPS2 protein and *E. coli* cells transformed with control plasmids devoid of the *DcTPS2* gene or assays without GPP as a substrate were used (Figure 4B). Geraniol and β-myrcene are not actually the major monoterpenes accumulated. Besides terpinolene, some monoterpene hydrocarbons, e.g. *E*-β-ocimene, β-myrcene, and sabinene, are the major compounds in all carrot cultivars investigated in this study (Table 1 and Supplemental Table 1 of the Supporting Information).

Volatile mono- and sesquiterpenes have been reported to be synthetized and accumulated in roots and rhizomes of various plant species.[40,41] It has been reported that root-emitted volatile compounds can be involved in the plant defense by directly repelling herbivores or pathogens or recruiting enemies of their aggressors to limit or eliminate further damage.[42,43] Besides their ecological benefits to plants, terpene-specialized compounds are widely used by humans as flavors, fragrances, or pharmaceuticals.[6] However, terpene oils produced in carrot root tissues are assumed to contribute significantly to the aroma and flavor of carrots,[1,28,29] and they could also play interesting defensive roles in belowground biotic stress. The terpene products found for *Dc*TPS1 and *Dc*TPS2 represent only a fraction of the terpene constituents detected in carrot roots. Additional *TPS* gene transcript analysis, enzyme characterization, and subsequent targeted gene silencing will provide a better understanding of the individual or overlapping functions of root carrot *TPS* genes in terpene biosynthesis.

Cultivar-Specific and Developmental Expression Patterns of *DcTPS1* and *DcTPS2* Genes. To examine whether terpene volatile compound accumulation during carrot root development and differences in terpene composition between carrot cultivars could be related to the expression of terpene biosynthetic genes, transcript levels of *DcTPS1* and *DcTPS2* were analyzed by qRT-PCR (Figure 5) in the same samples as used for auto-HS−SPME−GC−MS analysis.

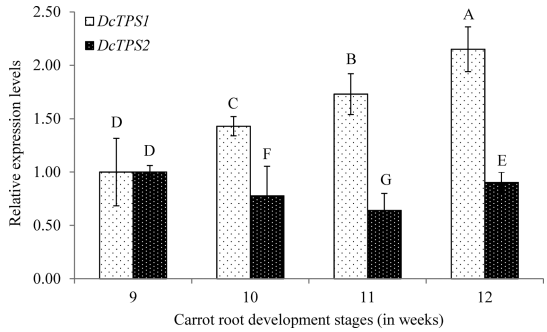

Figure 5. Expression patterns of *DcTPS1* and *DcTPS2* during carrot root development from 9 to 12 weeks after sowing in the orange carrot cultivar Nairobi. Quantification of *DcTPS1* and *DcTPS2* transcript levels by real-time RT-PCR analysis normalized to equal levels of tubulin transcripts. All analyses were performed using three biological replicates. Bars labeled with different letters indicate significant differences as determined by JMP statistic software [$F < 0.0001$; Tukey−Kramer honestly significant difference (HSD) test].

Transcripts of *DcTPS1* and *DcTPS2* were observed in all four stages in the Nairobi cultivar, demonstrating that these two genes were expressed at all developmental stages. Furthermore, transcript abundance increased during root maturation for the *DcTPS1* gene, with maximum transcript levels occurring in 12-week-old roots (Figure 5), which is consistent with the accumulation of high terpene levels in this time period (Figure 2). The expression level of *DcTPS1* and *DcTPS2* varied statistically between carrot tissues at all developmental stages (Figure 5).

We also examined possible differences in the expression of *DcTPS1* and *DcTPS2* in the five different carrot cultivars (Figure 6). Transcript abundance was highest for both genes in the Rothild cultivar, consistent with high levels of total terpenes in this cultivar (see Supplemental Table 1 of the Supporting Information). The lowest level of both gene transcripts was observed in the cultivar Yellowstone (Figure 6), correlating with lowest terpene concentrations in this cultivar (see Supplemental Table 1 of the Supporting Information). Also, the expression level varied statistically between *DcTPS1* and *DcTPS2* in the five different carrot cultivars investigated in this study (Figure 6). However, *DcTPS1* and *DcTPS2* expression levels did not directly correlate with the amounts of (*E*)-β-caryophyllene and geraniol in the different cultivars, which indicates the presence of other TPSs producing the same compounds or possible post-transcriptional/translational regulatory mechanisms. Post-transcriptional regulation of TPS enzymes[44] as well as light-dependent substrate availability[45] are also discussed as regulatory steps in terpene formation.

***Dc*TPS1 and *Dc*TPS2 Are Members of the TPS-a and TPS-b Families.** The predicted *Dc*TPS1 protein sequence

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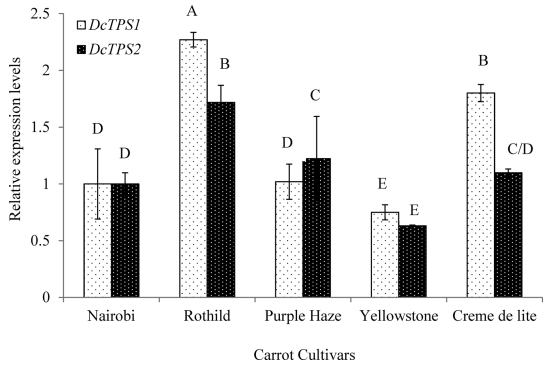

Figure 6. Quantitative RT-PCR analysis of *DcTPS1* and *DcTPS2* expression in roots of five different colored carrot varieties. Bars labeled with different letters indicate the significant differences as determined by JMP statistic software ($F < 0.0001$; Tukey–Kramer HSD test).

consists of 560 amino acids, with a calculated molecular mass of 64.7 kDa (see Supplemental Figure 1 of the Supporting Information). No chloroplast-targeting sequence was identified for *Dc*TPS1, which is indicative for the function of this enzyme as a cytosolic sesquiterpene synthase. The *Dc*TPS2 protein consists of 593 amino acids with a calculated molecular mass of 68.4 kDa. In contrast to *Dc*TPS1, *Dc*TPS2 appears to carry a 53 amino acid N-terminal plastidial transit peptide (see Supplemental Figure 1 of the Supporting Information), as predicted by the ChloroP 1.1 software (http://www.cbs.dtu.dk/services/ChloroP/), suggesting a function of *Dc*TPS-2 as a monoterpene synthase *in vivo*.

Both *Dc*TPS1 and *Dc*TPS2 sequences contain the conserved features of plant TPSs, including the asparate-rich motif DDxxD and the RxR motif, both of which are involved in catalysis (see Supplemental Figure 1 of the Supporting Information).[13,1446] A RR(x)8W motif is present in the N-terminal region of *Dc*TPS1 and downstream of the N-terminal transit peptide of *Dc*TPS2 (see Supplemental Figure 1 of the Supporting Information). The motif is assumed to participate in the ionization of the substrate[47] and is characteristic of most TPS members of the subfamilies TPS-a and TPS-b.[4849]

To analyze the phylogenetic relationship of the carrot TPS proteins (*Dc*TPS1, *Dc*TPS58617, *Dc*TPS52846, *Dc*TPS2, *Dc*TPS1324, and *Dc*TPS21245) with known TPSs of other plants, a phylogenetic tree was constructed using the neighbor-joining method (Figure 7). On the basis of protein sequence and function, plant TPSs were recently organized into seven subfamilies. These include three angiosperm-specific clades (TPS-a, TPS-b, and TPS-g), a gymnosperm-specific subfamily (TPS-d), a subfamily most conserved among land plants (TPS-c), two subfamilies most conserved among vascular plants (TPS-e and TPS-f), and a subfamily specific to *Selaginella moellendorffii* (TPS-h).[13]

*Dc*TPS1, *Dc*TPS58617, and *Dc*TPS52846, along with some TPSs from Apiaceae, the same plant family as *D. carota*, e.g., the sesquiterpene cyclase from *Centella asiatica* (55% identity of amino acids), STS1 and STS2 from *Thapsia garganica* (51 and 55% identity of amino acids, respectively),[50] β-caryophyllene synthases from *Solanum lycopersicom*[37] and *Vitis vinifera*, and pinene synthase from *Malus* × *domestica*,[51] belong to the TPS-a group of the terpene synthase superfamily (Figure 7). Furthermore, the *Dc*TPS1 protein displays a high degree of sequence similarity to the α-copaene synthase (62% identity)

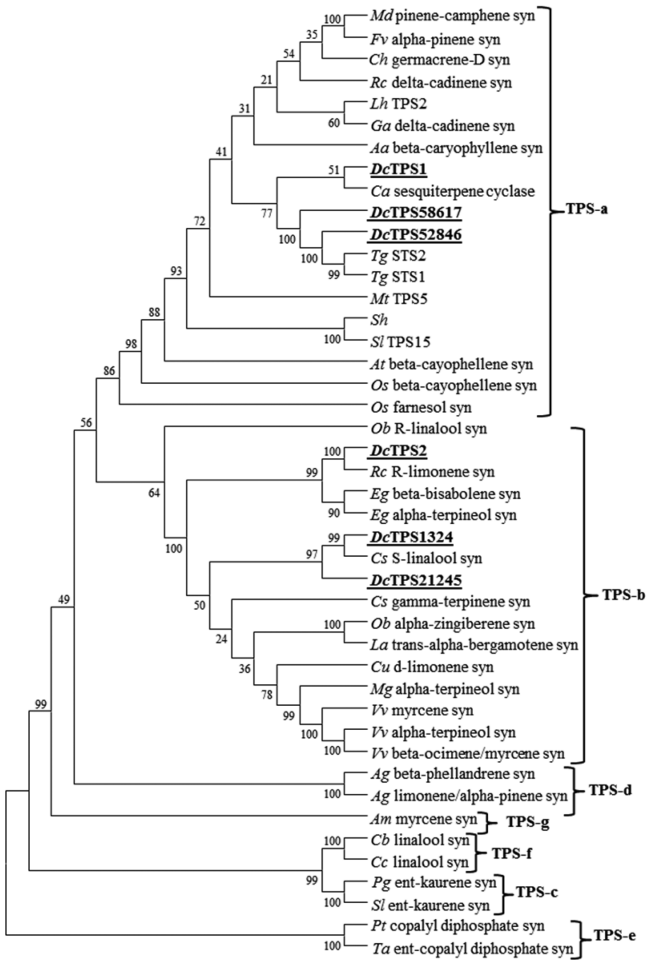

Figure 7. Phylogenetic tree of deduced amino acid sequences of plant sesquiterpene synthases. The sequences were aligned using phylogeny analysis (http://www.phylogeny.fr). The evolutionary history was inferred using the neighbor-joining methods and drawn by TreeView. The TPS family was subdivided into six subfamilies, designated TPS-a–TPS-f, each distinguished by sharing a minimum of 40% identity among members.[49] Cu is *Citrus unshiu*; Vv is *Vitis vinifera*; Md is *Malus* × *domestica*; Rc is *Ricinus communis*; Ch is *Citrus hystrix*; Sh is *Solanum habrochaites*; Sl is *Solanum lycopersicum*; Fv is *Fragaria vesca*; Ga is *Gossypium arboretum*; Lh is *Lycopersicon hirsutum*; Mt is *Medicago truncatula*; At is *Artemisia annua*; Cb is *Clarkia breweri*; Cc is *Clarkia concinna*; Pg is *Picea glauca*; Pt is *Populus trichocarpa*; Ta is *Triticum aestivum*; Ag is *Abies grandis*; Ob is *Ocimum basilicum*; Am is *Antirrhinum majus*; La is *Lavandula angustifolia*; Os is *Oryza sativa*; Aa is *Artemisia annua*; Mg is *Magnolia grandiflora*; Eg is *Eucalyptus grandis*; Cs is *Coriandrum sativum*; Ca is *Centella asiatica*; and Tg is *Thapsia garganica*. The black bold underline indicates the carrot TPSs, *Dc*TPS1, *Dc*TPS2, *Dc*TPS58617, *Dc*TPS52846, *Dc*TPS1324, and *Dc*TPS21245 proteins, identified in this study.

from *Eleutherococcuc trifoliatus*, a sesquiterpene synthase (60% identity) from *Panax ginseng*, and (*E*)-β-caryophyllene synthase (54% identity) from *Vitis vinifera*.

*Dc*TPS2, *Dc*TPS1324, and *Dc*TPS21245 cluster in the TPS-b clade, together with *S*-linalool synthase (39% identity of amino acids) and γ-terpinene synthase (41% identity of amino acids) from *Coriandrum sativum* (Apiaceae family),[19] α-terpinol synthase and myrcene synthase from *Vitis vinifera*,[48] and D-limonene synthase from *Citrus* (Figure 7).[52] *Dc*TPS2 showed the highest amino acid similarity to terpene synthase (63% identity) from *Jatropha curcas*, (*R*)-limonene synthase (59%

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identity) from *Ricinus communis*, and α-terpineol synthase (54% identity) and β-bisabolene synthase (52% identity) from *Eucalyptus grandis*.

In conclusion, several terpene volatile compounds and the total volatile terpene content varied widely among different colored carrot genotypes. Two TPSs (*Dc*TPS1 and *Dc*TPS2) of a larger carrot TPS family were found to produce (*E*)-β-caryophyllene, the predominant sesquiterpene constituent of carrot roots, and the monoterpene component, geraniol, respectively. The identification of carrot *TPS* cDNAs will facilitate the cloning of additional genes of the *TPS* family in *D. carota* and will allow for future molecular, physiological, and biochemical studies of the regulation of flavor and aroma formation during carrot root development. The *TPS* genes described here and potential other *TPS* genes from *D. carota* may be developed into molecular markers to aid in breeding and improvement of cultivars with superior carrot flavor and aroma.

■ ASSOCIATED CONTENT

Supporting Information

Levels of mono- and sesquiterpene volatile compounds, for which no authentic standards are available, and total content of all terpene compounds in different carrot varieties at 10 weeks (Supplemental Table 1) (PDF) and comparison of deduced amino acid sequences of *Dc*TPS1 and *Dc*TPS2 (Supplemental Figure 1) (PDF). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b00546.

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Notes

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■ ABBREVIATIONS USED

*Dc*TPS, *Daucus carota* terpene synthase; DMAPP, dimethylallyl diphosphate; (*E*,*E*)-FPP, *trans*,*trans*-farnesyl diphosphate; (*Z*,*Z*)-FPP, *cis*,*cis*-farnesyl diphosphate; FW, fresh weight; GC−MS, gas chromatography−mass spectrometry; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HS, head space; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IPP, isopentenyl diphosphate; MEP, methylerythritol phosphate; MVA, mevalonate; *m*/*z*, mass-to-charge ratio; NPP, neryl diphosphate; ppm, part per million; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SPME, solid-phase microextraction; TPS, terpene synthase

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