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Divergence in tissue-specific expression patterns of genes associated with the terpeniod biosynthesis in two oregano species *Origanum vulgare* L., and *Origanum majorana*

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ABSTRACT

Oregano (*Origanum vulgare*) and marjoram (*O. majorana*) are two discrete (in terms of sensory characteristics) species within the genus *Origanum* (Lamiaceae). Their aroma, flavor, and pharmaceutical value are a result of their essential oils, which comprise mainly of monoterpenes and sesquiterpenes. Marjoram is rich in bicyclic monoterpene cis-sabinene hydrate derived from the biosynthetic "sabinyl" pathway while the phenolic monoterpene carvacrol, arising from the "cymyl" pathway, is the distinctive feature of oregano. To investigate differences between the terpene biosynthetic pathways of both species, we identified key enzymes of terpene biosynthesis from the two species of genus *Origanum*. The heterologous expression of these enzymes showed that each formed multiple mono- or sesquiterpene products and, in combination, were responsible for the direct production of almost all terpenes found in *Origanum* essential oils. The correlation between essential oil composition and relative terpene synthase transcript concentrations in *O. vulgare* and *O. majorana* demonstrated that monoterpene synthase activity is predominantly regulated at the level of transcription and that the phenolic monoterpene alcohol thymol is derived from α-terpinene, the product of a single monoterpene synthase. The combination of heterologously expressed terpene synthases for in vitro assays resulted in blends of mono- and sesquiterpene products that strongly resembled those found *in vivo*, indicating that terpene synthase expression levels directly control the composition of the essential oils. These results will facilitate the metabolic engineering, directed breeding of Origanum cultivars with higher quantities of essential oils, and improved oil compositions.

1. Introduction

The genus *Origanum* comprises two distinct, aromatic species, namely marjoram (*Origanum majorana*) and oregano (*Origanum vulgare*), exhibiting discrete, sensorial characteristics (Baricevic and Bartol, 2002; Singletary, 2010). The aromatic quality of marjoram is characteristic of the species *O. majorana* (Lukas et al., 2013a,b). However, the most exceptional aromatic essential oils are procured from different species of the genus *Origanum*, including *O. vulgare*, *O. onites,* and *O. syriacum* (Arcila-Lozano et al., 2004; Busatta et al., 2008). The essential oils from marjoram and oregano contain mono- and sesquiterpenes; however, bicyclic monoterpene *cis*-sabinene hydrates are characteristically derived from the "sabinyl" pathway responsible for marjoram scent, whereas oregano owes its aroma to phenolic monoterpene carvacrol, originating from the "cymyl" pathway (Skoula et al., 1999; Lukas et al., 2010). The biosynthetic pathway of terpenes and terpenoids is quite intricate; however, in the past decade there has been an increasing focus on deciphering the pathways resulting in the aroma of essential oils (Crocoll, 2011a, 2011b; Zeng et al., 2016; Padovan et al., 2017; Reed and Osbourn, 2018). Crocoll et al. (2010) first illustrated key enzymes of terpene and terpenoid biosynthesis through the development of an expressed sequence tag (EST) library from epidermal gland cells. Terpene synthases catalyze the oxidation and cyclization of precursors implicated in the biosynthesis of monoterpenes and sesquiterpenes (Tholl, 2006). Numerous terpene synthase genes were characterized from various members of Lamiaceae, including *Salvia*

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officinalis L. (Wise et al., 1998), *Rosmarinus officinalis* L., (Tan et al., 2007), *O. vulgare* (Crocoll et al., 2010), Tomato (Falara et al., 2011), *Thymus caespititius* Brot. (Lima et al., 2013), *Mentha spicata* (Wang et al., 2008); *Murraya koenigii* (Meena et al., 2017), *Zanthoxylum piperitum* (Fujita et al., 2017), *Nicotiana benthamiana* (Vattekkatte et al., 2018), *Piper nigrum* (Jin et al., 2018). As our perceptive of plant secondary metabolism increases, expression of these pathways in heterologous hosts offers potential prospect to tackle these constraints to meet persistent requirement for both presently utilized and novel natural products.

Terpene synthase is the main gene responsible for polymorphism in essential oil components (Franz and Novak, 2009). The enzyme γ-terpinene synthase is significant, in terms of polymorphism in essential oil components, which gives rise to two different pathways yielding "cymyl" and "sabinyl" compounds resulting in diverse chemotypes in *Origanum* (Novak et al., 2008; Lukas et al., 2013a,b). The molecular characterization of the terpene synthase gene in different chemotypes may explain the disparity between different genotypes (Padovan et al., 2017; Bustos-Segura and Foley, 2018). Several studies have shown that the structural diversity of plant mono- and sesquiterpene synthases originate from diverse reaction mechanisms (Degenhardt et al., 2009; Chen et al., 2011; Vattekkatte et al., 2018). Furthermore, the oxidation and conjugation of the first terpene synthase products is carried out by an important class of enzymes, Cytochrome P450s monooxygenases (P450s), which are implicated in the downstream modification of mono- and sesquiterpenes (Jung et al., 2011; Weitzel and Simonsen, 2015). For cytochrome P450s, all terpenes, such as mono-, sesqui-, and triterpenes, are substrates and these enzymes further add to the complexity of their structural diversity (Grogan, 2011). The major diversity of monoterpene hydroxylation via cytochrome P450s is well documented in limonene (Luo et al., 2001). Numerous limonene-using P450s have been discovered in mint, caraway, and perilla (Mau et al., 2010). Cytochrome P450 monooxygenases catalyze the biosynthesis of menthol and carvone via hydroxylation in *Mentha* (Mau and Croteau, 2006; Haudenschild et al., 2000). However, the site of hydroxylation varies and this determines the fate of the products in the biosynthetic pathways via a downstream process, suggesting that cytochromes P450s are highly regiospecific (Cankar et al., 2011). According to previous research, most monoterpenes are derived from γ-terpinene, through the up-regulation of *CYP71D180* and *CYP71D181* whereas *CYP71D178*, *CYP71D179,* and *CYP71D182* are presumably thymol synthases (Crocoll, 2011b; Morshedloo et al., 2017). Secondary metabolites are necessary for plant defense as well as pharmaceutical and nutraceutical functions. Hence, comprehending the mechanisms underlying the generation of secondary metabolites is vital to further investigate their function in plant defense and expand novel approaches for enhancing their quantities for pharmaceutical purposes (Cheng et al., 2007). In addition to polymorphism in different genotypes, there is an urgent need to elucidate variations in mono- and sesquiterpenes at different developmental stages as well as their accumulation in different tissues (Hakola et al., 2006). The invariable accumulation of different mono- and sesquiterpenes result in different chemotypes even in related species like oregano and marjoram (Bisht et al., 2009; Lukas et al., 2013a,b). This variability could be associated to the invariable effects of genetic factors, diverse geographical origins, plant parts used, harvesting time, methods of extraction and ecological factors (Lukas et al., 2015; Morshedloo et al., 2018a, 2018b). Morshedloo et al., 2018b demonstrated discrepancy in accumulation of essential oil in different plant parts with flowers exhibiting highest essential oil content (79.2%) followed by shoot (70%) and early vegetative growth (67.34%). Depending on the oxidation and cyclization of different substrates via terpene synthase and the site of hydroxylation of the basic terpene structure by cytochrome P450s, diverse volatiles are produced (Chang et al., 2007). Thus, in order to understand disparities among chemotypes and variations in mono- and sesquiterpenes at and in different developmental stages and tissues, we isolated and characterized three cultivars of each species, *O. vulgare* and *O. majorana*. The aim of our study was to investigate molecular and metabolite variations in mono- and sesquiterpene biosynthesis in two species of the genus *Origanum* to emphasize the function of terpene synthase in producing the fundamental terpene carbon skeleton. The correlation between terpene synthase transcript levels, determined by real time PCR, and essential oil components in the cultivars were utilized to investigate the function of these terpene synthase genes in the biosynthesis of various terpenes. Five new P450 s and 14 terpene synthase genes from oregano and marjoram chemotypes were described and their relative expression was determined in the different cultivars (Supplementary Table 1). Furthermore, we investigated the relative gene expression at different developmental stages and in different tissues and estimated that of major volatiles along with their correlation to concentrations of metabolites.

2. Material and methods

2.1. Plant material

Oregano (*O. vulgare* L.) and marjoram (*O. vulgare* L.) were propagated from stem cuttings procured from different cultivars and grown in the field station of the Central Institute of temperate horticulture (ICAR, Srinagar). The field was irrigated after three or four days and deweeding was done every week. Three cultivars of each species were selected from the collection of *O. majorana* and *O. vulgare* designated as *OM1, OM2,* and *OM3* and *OV1*, *OV2,* and *OV3,* respectively in the Indian Institute of Integrative Medicine (IIIM, Srinagar) and some from wild habitats, which were selected for the presence of both sabinyl- and *p*-cymyl compounds in their essential oils.

2.2. Terpene extraction from leaves

For terpene extraction, leaves were harvested from three cultivars of each species in July. Fully expanded leaves from ten plants of each cultivar were pooled, macerated in frozen liquid nitrogen, and ground into a fine powder with a mortar and pestle. The powder (100 mg) was then dissolved in 1 ml of ethyl (acetate: pentane [2:1]), containing the internal standard (menthol, 50 ng μl^{-1}), for 24 h at 25 °C with stirring at constant speed. This solution was cleared with activated charcoal for 5 min and dried over a column of 500 mg water-free Na_2SO_4. For terpene extraction, samples were taken in triplicates.

2.3. GC–MS analysis of plant volatiles

The extraction of plant material (leaves, stems, and flowers) harvested at full bloom were subjected to GC and GC–MS analyses estimated as per the Liu et al. (2011) method. For GC and GC–MS analyses, 2 µl of ethyl acetate: pentane (2:1) extracts were injected at a temperature of 230 °C. Solid phase micro extraction fibers subjected to leaf volatiles (30 min, 30 °C) were injected at 180 °C. The terpenes were isolated on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 µm film (J&W Scientific, Santa Clara, CA, USA); GC-program 40 °C for 2 min, first ramp 5 °C min^{-1} to 175 °C, second ramp 90 °C min^{-1} to 250 °C, final 3 min hold). We used the GC–MS carrier gas, helium, at 1 ml min^{-1} and GC-FID carrier gas, hydrogen, at 2 ml min^{-1}. All volatiles were identified via Agilent Technologies software with the Wiley 275.L and NIST98.L MS libraries via a comparison of mass spectra and retention times with those of authentic standards (Sigma-Aldrich Chemicals, Steinheim, Germany). The quantity of individual terpenes was determined by GC-FID using monoterpene standards. A Spearman's rank correlation coefficient was calculated between the terpene quantity and transcript levels.

2.4. Reverse Transcription PCR analysis

Frozen stems, leaves, and flowers were ground into a fine powder in

a pre-chilled, DEPC-treated and sterilized mortar, pestle, and RNA was extracted using an RNA isolation kit (Roche Applied Science) following the manufacturer's protocol. First strand cDNA was transcribed from an initial RNA concentration of 1000 µg μl^{-1} to obtain a higher copy number of mRNA. The amplification of genes as represented in supplementary Table 1, using the Ov*TUB* gene as an internal control, was conducted using the AMVRT cDNA kit (Roche Applied Science, Penzberg, Germany) according to the user manual. PCR reactions were performed in a thermocycler (Takara, Japan) with 3–6 µg of cDNA. The steps were as follows: initial denaturing at 95 °C for 7 min followed by 40 cycles of amplification with denaturation for 1 min at 94 °C, annealing at 56.2 °C for 30 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 10 min. To check the amplification results 6–8 µl of the PCR products were used on 1.2% (w/v) agarose (Sigma-Aldrich, St Louis, MO, USA).

2.5. Real time PCR analysis

The Real Time PCR reaction was done in 96-well plates in a LightCycler 480 real-time PCR instrument (Roche Diagnostics) using the LightCycler 480 SYBR Green I Master kit. All the reactions were conducted in triplicates using 5 µl SYBR Green I Master, 2 µl PCR-grade water, 2 µl cDNA, and 0.5 µl each of the 10 µM forward and reverse gene-specific primers in a final volume of 10 µl. The tubulin gene was used as the reference gene for each primer. Gene expression in the stigma was taken as a positive calibrator for relative quantification analyses. The plate was incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 56.2 °C for 15 s, and 72 °C for 20 s. The LightCycler 480 software (version 1.5; Roche Diagnostics) was used to collect the fluorescence data. Advanced relative quantification of both genes, terpene synthase (14 primers) and cytochrome P450 (5 primers), in leaves, flowers, stems, and roots were done using the $2^{-\Delta\Delta cp}$ method (Livak and Schmittgen, 2001). Primers for the RT-PCR were selected from previous studies (Crocoll et al., 2010; Patricelli et al., 2015) or designed using the integrate DNA Technologies (Coralville, USA) qPCR primer design web tool for the amplification of 14 selected genes from two pathways in terpenoid biosynthesis (Thornton and Basu, 2011) as represented in supplementary table Table S1. Elongation factor 1 alpha (*OvEF1alpha*) served as a housekeeping gene for relative qRT-PCR, quantitatively comparing the Ct values of the samples with a calibrator. The housekeeping gene was used as an internal control to ensure that equal amounts of RNA were used for each sample. The Ct values of the calibrator and samples were normalized to the endogenous housekeeping gene. Relative gene expression was determined according to the $\Delta\Delta C_t$ method, using the formula:

$2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = [\Delta]\ Ct_{\ sample} - [\Delta]\ Ct_{\ reference,}$

Where, $[\Delta]C_{t\ sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene, and $[\Delta]C_{t\ reference}$ is the Ct value for the reference sample normalized to the endogenous housekeeping gene (Livak and Schmittgen, 2001).

2.6. Statistical analysis

All observations were determined using a factorial experiment in a complete randomized block design with ten replicates. Each real time PCR experiment was carried out in three independent biological replicates and determined in three technical replicates. All data obtained were subjected to an analysis of variance (ANOVA) followed by the LSD test at a $P < 0.05$ probability level. Spearman's rank correlation coefficient was calculated between the major compositions of essential oil and transcript levels.

Table 1
Proportion (%) changes of essential oil components in *Origanum vulgare* and *Origanum majorana*.

Compounds	*Origanum marjorana* L.,			*Origanum vulgare* L.,		
	OM1	OM2	OM3	OV1	OV2	OV3
α-Thujene	0.01	0.04	–	0.02	0.03	0.03
α-Pinene	0.01	0.04	–	0.02	0.03	0.03
Camphene	0.01	0.04	–	0.02	0.03	0.03
Sabinene	**5.8**	**2.1**	**5.1**	**0.23**	**0.17**	**0.32**
β-Pinene	0.01	0.02	–	0.01	0.03	0.01
1-Octen-3-ol	–	0.01	–	–	0.03	0.03
3-Octanone	–	0.02	–	–	0.01	0.02
Myrcene	0.02	0.07	0.01	0.02	0.12	0.13
α-Phellandrene	–	0.03	–	–	0.09	0.08
3-Carene	–	0.01	–	–	0.14	0.01
α-Terpinen	–	0.08	–	–	0.04	0.02
p-Cymene	–	**0.03**	–	–	**1.23**	**0.08**
Limonene	0.02	0.14	0.07	0.04	0.19	0.09
β-Phellandrene	–	0.02	–	–	0.06	0.03
(Z)-β Ocimene	**0.21**	**0.30**	**0.40**	**1.22**	**2.39**	**2.87**
β-Terpinen	–	–	–	–	0.09	–
γ-Terpinene	**10.84**	**11.8**	**8.48**	**15.10**	**14.09**	**13.2**
trans-Sabinene hydrate	0.01	0.9	0.04	0.02	1.43	1.01
Borneol	–	0.01	–	–	0.03	0.03
Terpinen-4-ol	**0.29**	**0.19**	**0.32**	**0.22**	**0.12**	**0.10**
α-Terpineol	–	0.02	–	–	0.03	0.01
trans-Dihydrocarvone	0.01	0.04	–	0.02	0.03	0.03
Carvacrol methylether	0.07	0.02	0.01	0.04	0.08	0.02
Thymoquinone	0.02	0.14	0.07	0.04	0.19	0.09
Thymol	**0.48**	**0.08**	**1.09**	**1.53**	**0.04**	**0.13**
Carvacrol	**78.27**	**79.46**	**75.23**	**84.54**	**79.89**	**80.12**
β- Caryophyllene	**1.97**	**1.21**	**3.4**	**0.2**	**0.19**	**0.16**
α-Humulene	0.04	0.08	0.02	00.3	0.07	0.09
Allo-Aromadendrene	0.01	0.04	–	0.02	0.03	0.03
α-Muurolol	0.01	0.04	–	0.02	0.03	0.03
β-Bisabolene	0.07	0.02	0.01	0.04	0.08	0.02
γ-Cadinene	0.02	0.14	0.07	0.04	0.19	0.09
δ-Cadinene	0.01	0.04	–	0.02	0.03	0.03
Spathulenol	0.07	0.02	0.01	0.04	0.08	0.02
Caryophyllene oxide	0.01	0.02	–	0.01	0.03	0.01
Epi-α-Muurolol	0.07	0.02	0.01	0.04	0.08	0.02
α-Eudesmol	0.02	0.14	0.07	0.04	0.19	0.09
Total	**98.38**	**97.38**	**94.39**	**103.76**	**101.61**	**99.08**

Bold values represent the metabolites present in appreciable concentration.

3. Results

3.1. High variability in the essential oil composition and content of Origanum vulgare and O. majorana

The GC–MS analysis identified 37 major volatile compounds containing mono- and sesquiterpenes in both species, Oregano (*O. vulgare* L.) and marjoram (*O. majorana* L.), at the full blossom stage (Table 1). GC analysis using internal standards revealed that carvacrol and thymol were accumulated at higher concentrations in both species; however, concentration of thymol was significantly higher in marjoram (0.48–1.53%) than oregano (0.04–0.13%). Marjoram accumulated significantly higher levels of sabinene (2.1–5.8%) than oregano (0.17–0.32%). Moreover, sesquiterpene β-caryophyllene was accumulated at a higher concentration (0.97–3.4%) than oregano (0.16–0.20%) (Table 1). In contrast, oregano accumulated significantly higher concentrations of carvacrol with a maximum value of 84.54% and as much as 15.1% of γ-terpinene was accumulated followed by p-cymene (1.23%) and ocimene (2.87%) (Fig. 1). Sabinene levels significantly varied between both species of *Origanum,* while γ-terpinene displayed minimum variation (Table 1). Total essential oil content was more abundant in all cultivars of oregano (102.54%) than in marjoram (81.41%). The remaining essential oil components included myrcene, β-bisabolene, limonene, and spathulenol.

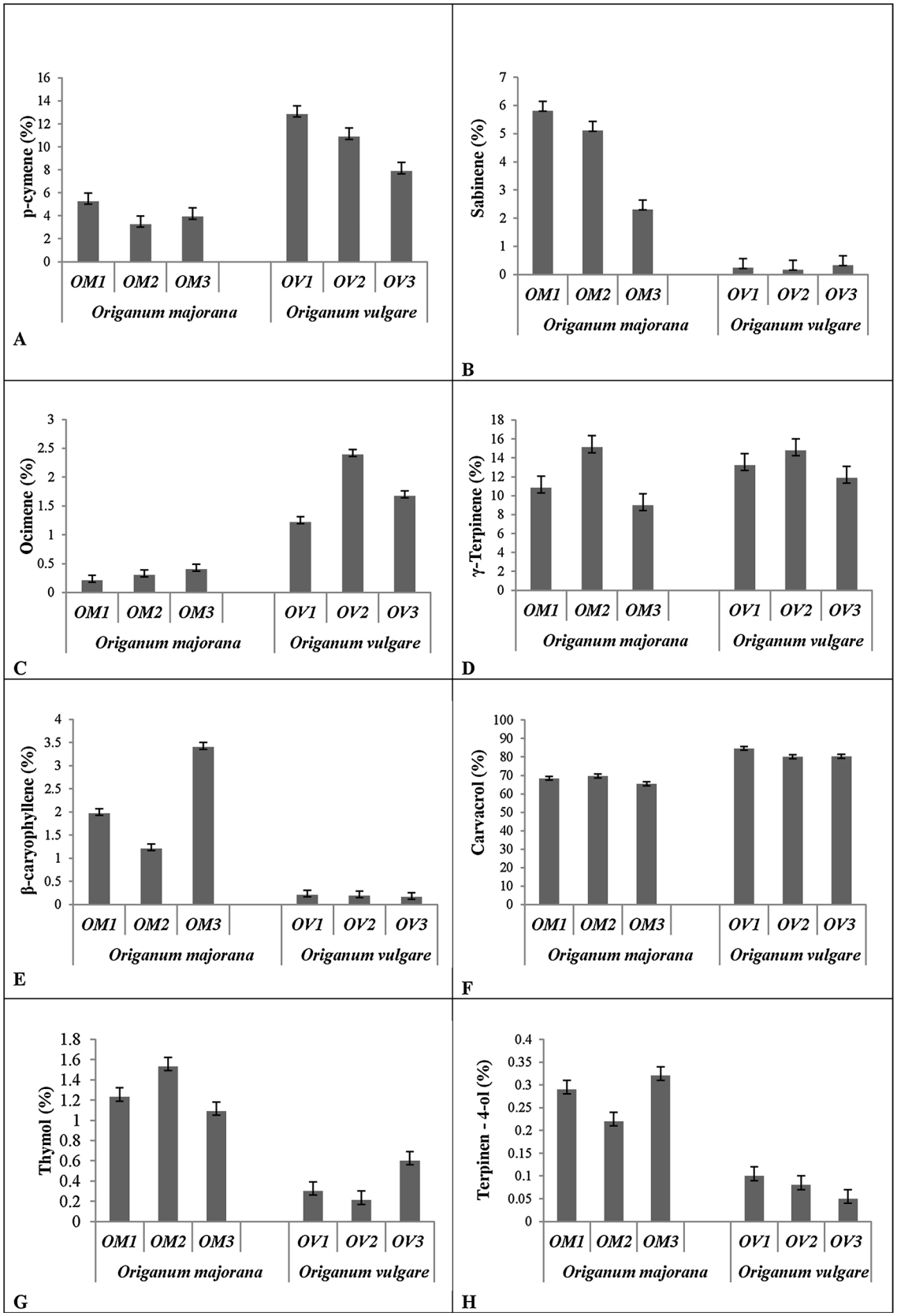

Fig. 1. Variation in p-cymene (A), sabinene (B), (Z)-b-ocimene (C), γ-terpinene (D), β-caryophyllene (E), carvacrol (F), thymol (G) and terpene 4-ol (H) in different cultivars of *O. vulgare* and *O. majorana*. Data shown are mean values of n = 10 and the error bars represent standard errors of the means. Different letters indicate significant differences ($P < 0.05$).

Table 2
Variation in phytochemical accumulation in different plant tissues of *Origanum vulgare* and *Origanum majorana*.

Compounds	*Origanum majorana* L.,									*Origanum vulgare* L.,								
	OM1				OM2			OM3					OV1				OV2			OV3			
Tissues	Root	stem	leaf	flower	root	stem	leaf	flower	root	stem	leaf	flower	root	stem	leaf	flower	root	stem	leaf	flower	root	stem	leaf	flower
Sabinene	0.43	1.26	1.4	2.0	0.067	0.78	0.92	1.09	0.07	1.23	1.32	2.43	–	0.08	0.012	0.13	–	0.017	0.024	0.12	–	0.60	0.10	0.16
p-Cymene	–	–	–	–	–	0.007	0.19	0.097	–	–	–	–	–	–	–	–	0.004	0.26	0.81	0.039	–	0.020	0.04	0.02
(Z)-β Ocimene	0.021	0.042	0.07	0.10	0.03	0.06	0.1	0.15	0.04	0.08	0.13	0.20	0.122	0.24	0.40	0.61	0.239	0..478	0.79	1.19	0.267	0.574	0.956	1.39
γ-Terpinene	0.94	1.97	3.01	5.42	1.12	2.36	3.93	5.19	0.75	1.54	2.56	4.05	1.23	2.96	4.67	6.98	1.13	2.52	4.57	6.78	1.09	2.34	4.21	6.23
Terpinen-4-ol	0.022	0.050	0.096	0.145	–	0.038	0.063	0.095	–	0.064	0.10	0.16	–	0.044	0.073	0.90	–	0.024	0.04	0.02	–	0.02	0.03	0.05
β-caryophyllene	0.186	0.394	0.656	0.905	0.09	0.240	0.40	0.60	0.34	0.68	1.13	1.64	0.02	0.04	0.06	0.10	0.017	0.030	0.063	0.090	0.012	0.029	0.053	0.08
Thymol	–	0.096	0.16	0.24	–	0.008	0.026	0.04	0.98	0.190	0.363	0.556	0.149	0.34	0.48	0.745	–	0.01	0.01	0.02	–	0.013	0.026	0.065
Carvacrol	6.52	9.78	26.09	39.12	7.02	15.89	26.48	39.73	7.02	15.04	25.07	37.45	8.23	16.90	28.18	40.89	7.67	15.89	26.34	39.89	8.01	16.02	26.70	40.06
Essential Oil	7.933	13.19	30.81	47.025	8.23	19.14	31.70	46.39	8.86	18.50	29.54	44.84	9.73	20.56	33.81	50.25	9.043	19.19	32.58	48.05	9.36	19.58	32.06	47.97

3.2. Tissue-specific accumulation of Terpenoids among species of Origanum

GC-FID and GC–MS analysis identified 37 compounds that accounted for 94.37–98.85% of the total compositions (Table 2). Carvacrol (6.52–49.39%), p-cymene (0.004–0.81%), and γ-terpinene (0.75–6.98%) were the most prominent constituents in all plant parts at full bloom stage. The maximum level of carvacrol was observed in flowers of *Origanum vulgare* cultivars *OV1* (40.89%), *OV3* (40.06%) and *OV2* (39.89%). Significant levels of carvacrol were also present in the plant leaves *OV1* (28.18%), *OV3* (26.70%) and *OV2* (26.34%) and stems *OV1* (16.90%), *OV3* (16.02%) and *OV2* (15.89%). Following carvacrol, γ-terpinene accumulated at highest level in flowers of *Origanum vulgare* cultivars *OV1* (6.98%), *OV2* (6.78%) and *OV3* (6.23%). Minimum concentration among the terpenoids was p-cymene it was absent in two cultivars of *Origanum marjorana* (*OM1*) and (*OM3*) in all the plants parts at flowering stage while as *OM2* exhibit accumulation of p-cymene in stem (0.007%), leaf (0.19%) and flower (0.097%). Pronounced accumulation of sabinene was found in all cultivars of *Origanum marjorana* with maximum levels in flowers of *OM3* (2.43%), OM1 (2.0%) and OM2 (0.097%). On the contrary, cultivars of *Origanum vulgare* exhibited comparatively lesser amount of sabinene than *Origanum marjorana* with maximum accumulation in leaves of *OV3* (0.16%) followed by *OV1* (0.13%) and *OV2* (0.12%). The concentration of β-caryopllene, sabinene and terpene 4-ol was comparatively higher in cultivars of *Origanum marjorana* than in cultivars of *Origanum vulgare.* Carvacrol, p-cymene and γ-terpinene accumulated at consistent level in all the cultivars of both species (Table 2).

3.3. Tissue-specific gene expression and variability among species of Origanum

Variations in gene expression were determined in the different cultivars of both species as well as in their tissues at the full bloom stage when essential oil content was at its maximum. Gene expression was evaluated via real time PCR analysis on RNA isolated from different cultivars of both species. We determined the relative expression levels of 14 terpene synthase genes in cultivars of both species. The results, represented in Fig. 2(A, B, D, and E) demonstrate that the expression of *Ovtps1*, *Ovtps3*, *Ovtps5,* and *Ovtps6* was significantly higher in cultivars of *O. vulgare* L., than in *O. majorana* cultivars, whereas *Ovtps4* and *Ovtps7* (Fig. 2C and F) exhibited relative expression levels that were analogous in both species. In contrast, the relative expression of *CYP1D178* was significantly higher in majorana than in oregano, which had significantly higher levels of *CYP1D180* and *CYP1D181*. However, the relative gene expression of *CYP1D179* and *CYP1D180* was comparable in both species (Fig. 2F). The maximum tissue specific gene expression of both *Ovtps* genes and CYP sequences occurred in flowers, then the leaves and finally, stems in both species. In contrast, roots exhibited minimum relative gene expression as displayed by *Ovtps7*, *Ovtps3*, *CYP1D178, CYP1D179,* and *CYP1D180* in both species (Figs. 3 and 4). However, gene expression of *Ovtps2* was completely absent in the cultivars of both species, in all tissues.

3.4. Correlations between gene expression patterns and essential oil compositions

Correlations between the relative gene expression of 14 terpene synthase genes and five CYP sequences, essential oil content and compositions in cultivars of both oregano and marjoram were utilized to determine the functional role of terpene synthase and cytochrome mono-oxygenase enzymes in the biosynthesis of various terpenes (Table 3). Spearman's correlation analyses revealed significant correlations between the relative expression of *Ovtps7*, *Ovtps5,* and *CYP71D180* and carvacrol content (0.73 and 0.81 respectively, $P < 0.005$) in oregano. Conversely, the over-expression of these genes was correlated to higher phenolic monoterpene levels. Furthermore, we

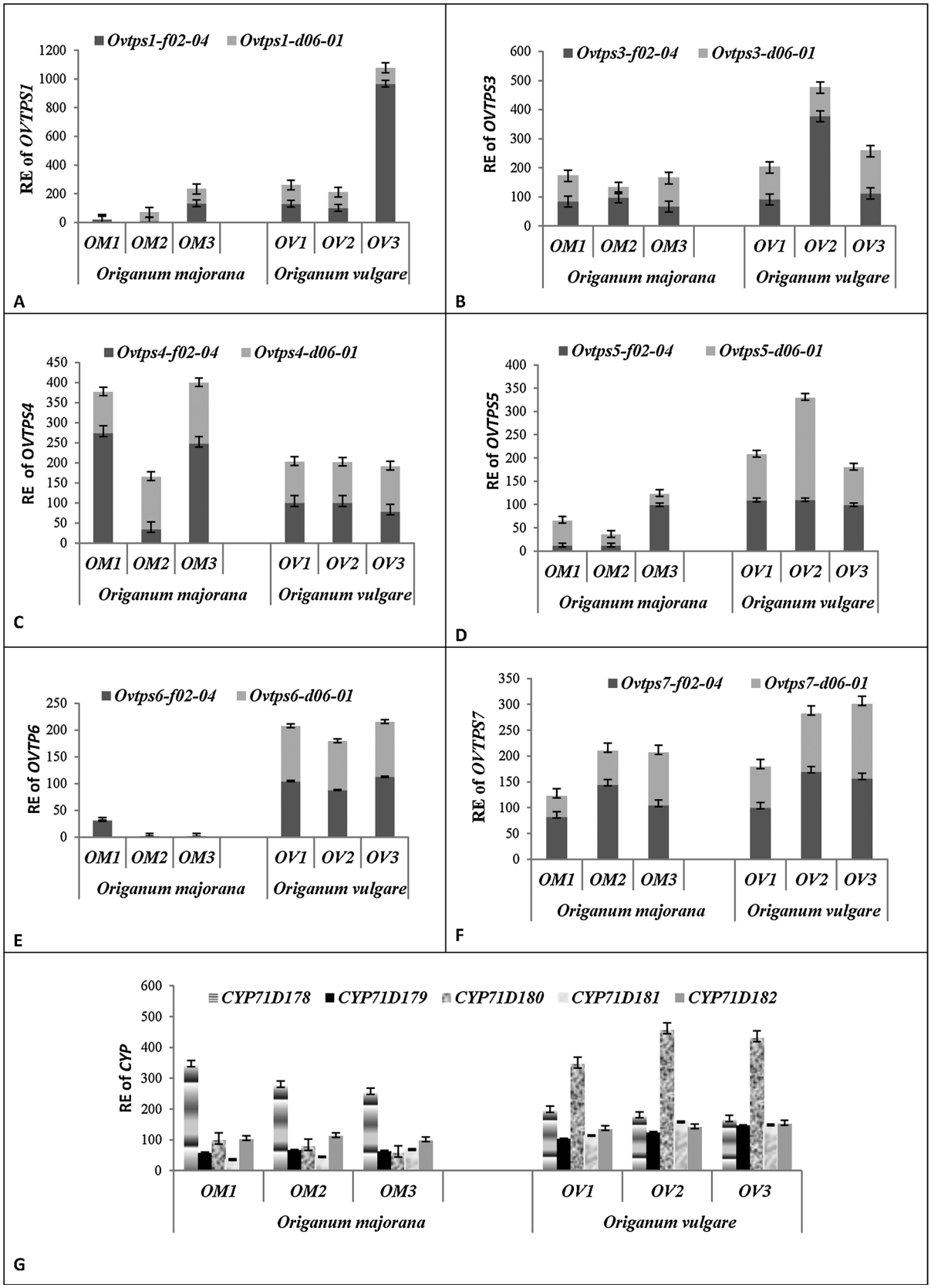

Fig. 2. Relative expression (RE) of Ovtps1 (A),Ovtps3(B),Ovtps4 (C), Ovtps5 (D), Ovtps6 (E), Ovtps7 (F) and CYP71D178, CYP71D179, CYP71D180, CYP71D181 (G) genes in *O. vulgare* and *O. majorana*. Data represent mean RE values. Error bars indicate standard error of the mean (n = 15). Different letters indicate significant differences ($P < 0.05$) among the cultivars within each genotype (LSD test at 5% level).

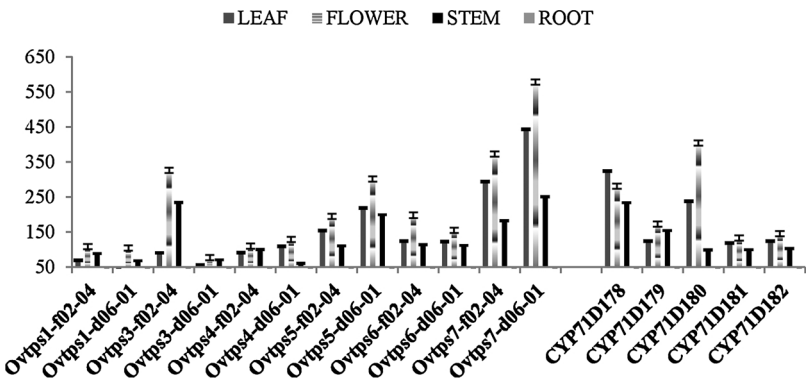

Fig. 3. Tissue specific relative expression (RE) of Ovtps1, OVtps3, Ovtps4, Ovtps5, Ovtps6, Ovtps7 and CYP71D178, CYP71D179, CYP71D180, CYP71D181 genes in *O. vulgare*. Data represent mean RE values. Error bars indicate standard error of the mean (n = 15). Different letters indicate significant differences ($P < 0.05$) among the cultivars within each genotype (LSD test at 5% level).

found a positive correlation between *Ovtps7*, *Ovtps5,* and *CYP71D180* (0.83, $P < 0.001$) in both species. In addition, there was positive correlation between *Ovtps4, CYP71D178,* and sabinene in marjoram. However, there was strong, negative correlation between β-caryophyllene and sabinene, but a positive correlation between *Ovtps6* and β-caryophyllene content (0.83, $P < 0.001$).

4. Discussion

4.1. High variability in essential oil composition and phytochemical content of Origanum vulgare and O. majorana

Approximately half of the volatile fraction in oregano included carvacrol, γ-terpinene, terpinene 4-ol, and thymol, identifiable by its characteristic “cymyl” flavor, while in case of marjoram there was pronounced accumulation of sabinene resulting in a sweet “sabinyl” aroma (Teixeira et al., 2013, Lukas et al., 2015; De Mastro et al., 2017). Among the phytochemicals p-cymene accumulated at lesser levels, which may be due to conversion of p-cymene to carvacrol and thymol at flowering stage (Crocoll, 2011a,b; Majdi et al., 2017). Pronounced accumulation of sabinene in all cultivars of *Origanum marjorana,* explains sweet marjorana smell originating from sabinyl compounds (Novak et al., 2000; Larkov et al., 2005; Kintzios, 2012). Higher accumulation of thymol in cultivars of *Origanum marjorana* may be due to higher influx of p-cymene towards thymol biosynthesis.

4.2. Variability in gene expression among species of Origanum

Gene expression of 14 terpene synthase genes, of which *Ovtps5*, *Ovtps1*, *Ovtps2*, and *Ovtps7* are known to regulate biosynthesis of monoterpenes, was estimated (Tholl, 2006; Irmisch et al., 2012). However, in this study, we found no expression of *Ovtps2* in both species of *Origanum,* which suggests that there was some monoterpene fraction not synthesized in either species. Several studies reported remarkable similarities between these terpene synthase genes and other monoterpene synthases (Crocoll, 2011b). Terpene synthases illustrate the biosynthesis of most terpene constituents in *Origanum*. Crocoll et al. characterized seven terpene synthases, which are implicated in the biosynthesis of monoterpenes such as thymol and carvacrol. However, there is no report documenting the functional role of terpene synthase in the biosynthesis of sabinene. Surprisingly, in this study we found a positive correlation between the relative expression of *Ovtps4* and sabinene levels. Previous studies predicted a direct relationship between γ-terpinene, *Ovtps2* and the synthesis of thymol (Lukas et al., 2010; Markus Lange and Turner, 2013). Nevertheless, in this study, we found no expression of *Ovtps2* in either species, but thymol concentrations were similar to those of previous studies. Moreover, we found a direct correlation between thymol and *CYP71D178;* previous research described the conversion of γ-terpinene to thymol by cytochrome P450 oxidases analogous to the hydroxylation of S-limonene in menthol biosynthesis in Mentha sp. (Weitzel and Simonsen, 2015). Some studies described the biosynthesis of thymol from γ-terpinene via the intermediate, p-cymene, which may be the source of the lower levels of p-cymene in Majorana (Poulose and Croteau, 1978; Skoula et al., 1999). Previous research studies reported *Ovtps2* as a major terpene synthase activity producing 48.4% of the total terpene content, in this study the expression of *Ovtps2* was completely absent even at tissue level, signifying that *Ovtps2* is not major enzyme implicated in the biosynthesis of mono- and sesquiterpenes (Crocoll et al., 2010; Morshedloo et al., 2016). Nevertheless, the over-expression of *Ovtps5* and *Ovtps7* in *O. vulgare* and *Ovtps4* in *O. majorana* is likely to contribute to the conversion of γ-terpinene to the major monoterpene fraction. There was a direct correlation between the relative gene expression of *Ovtps7* in all cultivars, as well as in their different tissues, and the monoterpene fraction in both species suggesting that *Ovtps7* is a major contributing factor in terpene synthase activity. Though we estimated the sabinene

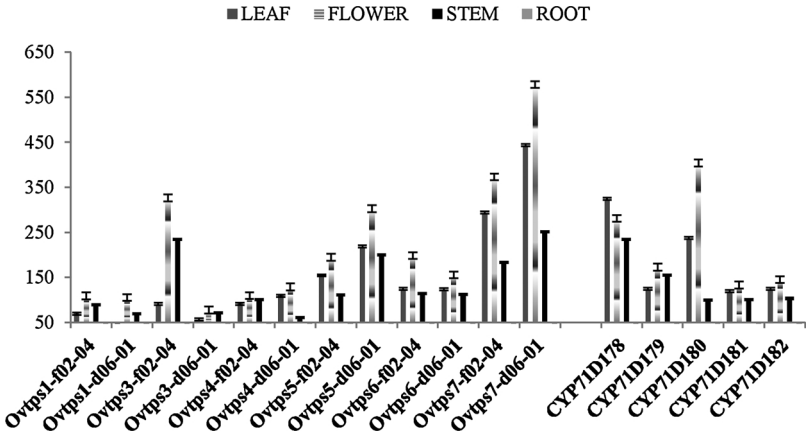

Fig. 4. Tissue specific relative expression (RE) of Ovtps1, OVtps3, Ovtps4, Ovtps5, Ovtps6, Ovtps7 and CYP71D178, CYP71D179, CYP71D180, CYP71D181 genes in *O. majorana*. Data represent mean RE values. Error bars indicate standard error of the mean (n = 15). Different letters indicate significant differences ($P < 0.05$) among the cultivars within each genotype (LSD test at 5% level).

Table 3
Spearman's correlation coefficients among essential oil content, compositions and relative gene expression of mono- and sesquiterpene synthesis in *O. majorana and Origanum vulgare* L.

Ovtps1	Ovtps3	Ovtps4	Ovtps5	Ovtps6	Ovtps7	*CYP71D178*	*CYP71D179*	*CYP71D180*	*CYP71D181*	*CYP71D182*
Ovtps1 1	0.85									
Ovtps3		−0.54								1
Ovtps4	−0.82		0.94							
Ovtps5	0.94	0.6		0.94						
Ovtps6	0.94	0.77	0.54		−0.71					
Ovtps7	0.71	0.82	0.71	0.65		0.77				
CYP71D178	−0.82	−0.71	−0.82	−0.88	0.82		0.11			
CYP71D179	0.94	0.77	0.94	0.94	−0.77	−0.71		0.77		
CYP71D180	0.77	0.77	0.94	0.94	−0.94	−0.82	0.04		0.20	
CYP71D181	0.94	0.77	0.71	0.77	−0.54	−0.54	0.26	0.6		0.6
CYP71D182	0.71	0.69	0.82	0.82	−1	−0.94	0.004	1	< .0001	
p−cymene	0.82	0.54	−0.77	0.71	0.77	−0.6	0.20	0.54	0.26	0.94
Sabinene	−0.77	−0.77	0.77	−0.94	−0.94	0.82	0.04	−0.77	0.07	−0.71
Ocimene	0.77	0.6	0.88	0.77	−0.88	−0.71	0.11	0.6	0.20	0.82
γ−terpinene	0.88	0.65	0.64	0.82	−0.77	−0.6	0.20	0.65	0.15	0.94
β−caryophyllene	0.64	−0.65	0.94	0.66	−0.94	−0.82	0.04	0.77	0.07	0.71
Carvacrol	0.94	−0.71	−0.88	0.77	0.88	−0.54	0.26	0.6	0.2	1
Thymol	−0.88	0.77	−0.82	−0.82	0.82	0.69	0.20	−0.65	0.15	−0.94
Terpene 4−ol	−0.82	0.94	0.94	−0.88	−0.77	0.77	0.07	−0.71	0.11	−0.77
Essential Oil content	0.94	0.56	0.94	0.94	0.86	−0.71	0.11	0.77	0.07	0.82

Ovtps1	p−cymene	Sabinene	Ocimene	γ−terpinene	β−caryo-phyllene	Carvacrol	Thymol	Terpene 4−ol	Essential Oil content
Ovtps1 1									
Ovtps3									
Ovtps4									
Ovtps5									
Ovtps6									
Ovtps7									
CYP71D178									
CYP71D179									
CYP71D180									
CYP71D181									
CYP71D182	0.004								
p−cymene		−0.65							
Sabinene	0.11		0.11						
Ocimene	0.04	0.71		0.06					
γ−terpinene	0.004	0.77	0.07		0.04				
β−caryophyllene	0.11	1	< .0001	0.54		0.63			
Carvacrol	< .0001	0.71	0.11	0.82	0.07		0.26		
Thymol	0.004	−0.77	0.07	−0.77	0.004	−0.82			
Terpene 4−ol	0.072	−0.6	0.20	−0.94	< .0001	0.34	0.20	1	0.01
Essential Oil content	0.04	0.54	0.26	1	0.34	−0.54	0.56	0.88	1

content and its correlation to *Ovtps4*, it is necessary to validate the relationship using different assays in future studies. The pronounced accumulation of sabinene was also correlated to *CYP71D178* and *CYP71D179,* which displayed a higher expression in *O. majorana*. Furthermore, we found a direct correlation between *OvTPS6* and β-caryophyllene, which were accumulated predominantly in *O. majorana.*

Previous studies describe a low similarity between the *Ovtps6* sequence, (E)-b-caryophyllene and sesquiterpene synthase genes from other plant species like *Artemisia* and *Cucumis sativa*. Crocoll et al. (2010) which demonstrated that the expression of *Ovtps6* led to the production of the enzyme producing β-caryophyllene obtained similar results. In contrast, monoterpene synthase in Lamiaceace was very similar to monoterpene synthase in other species, irrespective of their catalytic functions. However, terpene synthase genes outside the Lamiaceace family are more divergent irrespective of their similar catalytic action (Iijima et al., 2004; Deguerry et al., 2006). Crocoll et al. also showed the phylogenetic diversity of terpene synthases, which originated from *Ovtps1* and *Ovtps7* resulting from gene duplication and neofunctionalization. Further, similarities between *Ovtps2* and *Ovtps5* resulted from gene duplication tagged along the functional loss of *Ovtps5* due to inactivation followed by the loss of transient peptides (Martin et al., 2010; Falara et al., 2011). The high level of gene diversification implicated in mono- and sesquiterpene biosynthesis in both species might contribute to the diversity in essential oil composition leading to discrete terpene expression patterns. The expression of terpene synthase genes from both species played a significant role in regulating the terpene composition in these species as the relative expression levels of individual genes were closely correlated to levels of enzyme products observed in essential oils. Our study describes significant positive correlation between relative gene expression of *Ovtps4* with sesquiterpene levels in all the cultivars of Origanum species, which has been earlier demonstrated in closely related species of genus Origanum (Novak et al., 2002; Dambolena et al., 2009). This suggests that terpene synthase expression levels are directly implicated in the regulation of essential oil compositions and no other regulatory pathway, possibly in the same way compartmentalization or metabolite channeling are involved in the biosynthesis of mono- and sesquiterpenoids (Tissier, 2012). Discrepancies in gene expression can further be explained by disparities in the activity of major enzymes implicated in mono- and sesquiterpene biosynthesis (Vranová et al., 2012; Pribat et al., 2013).

4.3. Tissue-specific gene expression

Tissue-specific gene expression further confirmed the biosynthesis of mono- and sesquiterpenes by both species. Higher essential oil content in Oregano may be due to the relatively higher abundance of glandular trichomes, which is the main site for mono- and sesquiterpene biosynthesis (Wang et al., 2008, Wölwer-Rieck et al., 2014). Terpene synthases exhibited significantly high activity in flowers followed by leaves, and stems. Minimum terpene synthase activity was recorded in the roots of both species. In addition, *Ovtps7* and *Ovtps3* exhibited insignificant levels of expression even in the roots of both species. Gene expression patterns of Cytochrome monooxygenases were quite comparable in both species particularly *CYP71D179* and *CYP71D182,* whereas CYP71D178 and *CYP71D180* were predominantly expressed in *O. majorana* and *O. vulgare,* respectively. Tissue-specific gene expression revealed maximum expression in flowers followed by leaves and stems. However, in comparison to terpene synthases, CYP450 s were expressed even in the roots. Crocoll (2011a,b) and Cankar et al. (2011) demonstrated a single step conversion of γ-terpinene to thymol and carvacrol via CYP450. Crocoll (2011b) reported *Ovtps2* was the main determining factor in the biosynthesis of γ-terpinene. In contrast, we found no relationship between *Ovtps2* and γ- terpinene, but *CYP71D180* was positively correlation to γ-terpinene. Hence, it can be concluded that the biosynthesis of γ-terpinene is highly intricate with multifaceted gene homologues.

5. Conclusion

Using regulatory pathways of terpene synthases, we can alter or modify a single terpene synthase gene to generate desired products. We found that a lack of Ovtps2 did not affect the accumulation of mono- and sesquiterpenes. However, the positive correlation and high expression of Ovtps4 represented the accumulation of sabinene in *O. majorana*. Some monooxygenase CYP450 genes, such as CYP71D180, were expressed even in the roots of both species. Moreover, terpene synthase genes could be employed as markers for the marker assisted selection of *Origanum* varieties with high essential oil yields.

Author contribution

Sumira Jan and Parvaiz Ahmad designed the experimental work and Sumira Jan, Javid Iqbal Mir, Wajida Shafi performed the experimental work. Shafia Zaffer Faktoo and MN Alyemeni performed the statistical analysis of the data. All the authors have read the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.indcrop.2018.07.006.

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