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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