Enhancement of valerenic acid production in *Valeriana officinalis* roots by methyl jasmonate-mediated transcriptional changes of sesquiterpene synthase genes

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Abstract

Valeriana officinalis (valerian), as a nutraceutical herb, is widely used for its sedative and hypnotic properties. It is known that C15 sesquiterpenoid valerenic acid (VA) is active ingredient responsible for pharmacological effects of *V. officinalis*. To evaluate the effect of methyl jasmonate (MeJA) concentrations (50 and 100 µM) in the modulation of expression patterns of the genes involved in valerenic acid (VA) biosynthesis, transcript abundance of the identified sesquiterpene synthase (Sesqui-TPS) genes in the root of *V. officinalis* was monitored by quantitative real time PCR (qRT-PCR) within a 144 h time period. In addition, valerenic acid contents were measured by high-performance liquid chromatography (HPLC). The highest amount of VA (12.45 mg/g dry weight (DW)) was found at 100 µM MeJA with a 12 fold increase over control culture (1.03 mg/g DW) at exposure time of 72 h. Moreover, MeJA in a concentration dependent manner, enhanced transcription rate of *VoTPS1* and *VoTPS7* genes. Accordingly, exposure to 100 µM MeJA for 24 h can be more effective on induction of these genes than observed for 50 µM. Such enhancement correlated with increased VA accumulation suggesting that these genes may be responsible for the biosynthesis of intermediates involved in the VA-biosynthetic pathway. However, MeJA treatment seemed to have a less significant effect on *VoTPS3* expression than *VoTPS1* and *VoTPS7* genes. These results provide insights for more effective biosynthesis of VA by MeJA-mediated transcriptional changes of putative sesqui-TPS.

Keywords: *Valeriana officinalis*; Sesquiterpenes synthase; Expression pattern; Quantitative real time PCR

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Introduction

Valeriana officinalis (valerian) is a well-known medicinal plant that has many chemical constituents in its essential oil extract including monoterpenoids, sesquiterpenoids, iridoid derivatives (known as valepotriates) and alkaloids (Letchamo et al., 2004; Wang et al., 2010). It is known that the C15 sesquiterpenoid valerenic acid (VA) is a pharmacologically-active ingredient responsible for sedative and hypnotic properties of *Valeriana officinalis* (Bent et al., 2006; Takemoto et al., 2009). Valerian extract increases release of gamma aminobutyric acid (GABA) from

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brain nerve ending and then inhibits this inhibitory neurotransmitter from being re-absorbed back into nerve cells (Santos et al., 1994; Beaubru and Gray, 2000). Furthermore, VA decreases GABA degradation by its inhibitory effect on an enzyme that destroys GABA and thereby increases GABA availability in the synaptic cleft (Morazzoni and Bombardelli, 1995). Farnesyl diphosphate (FPP, C15) as a precursor for the biosynthesis of numerous structurally diverse sesquiterpenes (e.g. germacrene B/C/D and valerenadiene) is synthesized by the condensation of the 3 isomeric isoprene C5 units namely, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Hampel et al. 2005). Yeo et al. (2013) revealed seven terpene synthase gene (VoTPSs) candidates. Among them, VoTPS1 and VoTPS7, which were expressed more highly in root tissue, utilized significant amount of FPP rather than geranyl diphosphate (GPP) as substrate. These pieces of information suggest that these terpene synthases may be sesquiterpene synthase (sesqui-TPS). In contrast, *VoTPS3* mRNA was present in all tissue types examined (leaves, stems and roots) and used GPP rather than FPP as substrate. VoTPS1 was functionally characterized as a valeranadiene synthase, whereas VoTPS7 encoded germacrene synthase. It is thought that valerenic acid might be derived from valerenadien (Fig **I**). However, biochemical mechanism of their biosynthesis has been remained enigmatic. As well as, it was also proposed that germacrene intermediate might contribute to valernadien biosynthesis (Yeo et al., 2013).

Effective strategies such as metabolic engineering, tissue culture, and recruitment of phytohormones as the signaling molecules regulating metabolite pathways have been considered for enhancing the yield of pharmaceutical metabolites (Pandey et al., 2016). MeJA is involved in enhancement of bioactive compounds production through the regulations of expression of a series of genes involved in the synthesis of secondary metabolits (Zhang et al., 2004).

In order to increase understanding of how the elicitor modulates VA metabolism in *V. officinalis*, MeJA-mediated dynamic responses of some identified *VoTPS* genes probably involved in VA biosynthesis were measured by quantitative real time PCR (qRT-PCR).

Materials and Methods

Plant growth and phytohormone treatment

Seeds of *V. officinalis* were obtained from Pakanbazr Company, Esfahan, Iran. The seeds were surface sterilized with 70 % (v/v) ethanol for a min and rinsed with sterile distilled water. Seeds were then treated with 2.5 % sodium hypochlorite (NaClO) for 10 min, and then rinsed 3 times in distilled water. Surface-sterilized *V. officinalis* seeds were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 30 g l^{-1} sucrose and solidified with 8 g/l agar. The pH of medium was adjusted to 5.8. The culture media were maintained under controlled conditions in tissue culture room (16/8 h light/dark cycle at 25±2 °C).

MeJA was purchased from Sigma Co. (Sigma Aldrich, Germany). A stock solution of MeJA was prepared by dissolving it in 96% ethanol. Two months after growth of seedlings on half-strength MS media, plants were subcultured on the media containing 50 and 100 µM MeJA. The control cultures were treated with only ethanol. The root sampling was carried out 12, 24, 48, 72, and 144 h after treatment to evaluate terpene synthase genes expression and VA content.

Determination of VA content

For preparation of VA extraction, 200 mg of dry powder of roots of *V. officinalis* were extracted three times with 5 ml of 70 % Me OH, sonicated for 10 min and then diluted to a final volume of 15 mL with Me OH. All samples were filtered through a 0.45 µM micro-filter (Merck, Germany) subjected to reverse-phase high-performance liquid chromatography (HPLC) analysis as described in previous studies (Tania and Brian, 2004).

HPLC analysis was carried out using a Knauer HPLC system (Germany). VA was separated by reverse-phase isocratic C18 column (250 × 3 mm) with a pre-column (25 × 4.6 mm, particle size 5 µm) and detection by UV was conducted at 225

nm. The mobile-phase consisted of 0.5 % (v/v) phosphoric acid (A) and 0.5 % phosphoric acid: methanol mixture (27:73) (B) delivered at a flow rate of 1 ml min^{-1} and an injection volume of 20 µl. VA was provided by Sigma-Aldrich Co. (USA). Standard solution was prepared by dissolving VA in methanol to obtain solutions with 5 different concentrations in the range of 5 to 25 mg L^{-1}. The calibration curves were constructed by measuring their respective peak areas. This experiment was carried out with three replicates. Results were expressed as mean values ± SD. The statistical significance of VA contents was analyzed using a *t* test with Excel software for significant differences between treated and control plants.

RNA extraction, cDNA synthesis, primers design and qRT-PCR reaction

For expression analysis of terpene synthase genes, 100 mg roots were collected from the treated and control valerian and ground to powder in liquid nitrogen. Total RNAs were extracted from the roots using GeneAll® RiboEx ™ kit (BioFrontier, Korea) based on manufacturer protocol. RNA quantification was performed using NanoDrop 2000C (Thermo Fisher Scientific Inc., MA, USA). First strand cDNA synthesis was done from 500 ng of total RNA using Fermentas kit (Revert Aid ™ First Strand cDNA Synthesis Kit) according to the manufacturer instructions .

Primer pairs of the terpene synthase genes including *VoTPS1, VoTPS3*, and *VoTPS7* were designed using online IDT Primer Quest™ Software of Integrated DNA according to the predicted coding DNA sequence (CDS). The primers sequences designed for qRT-PCR amplification of target genes are listed in Table 1.

The quantitative real time PCR (qRT-PCR) was performed using HIFI SYBR® Green kit (Iran) Master Mix and Step One Plus® (ABI, America) machine. PCR conditions were an initial denaturation of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 20 s, and extension at 72 °C for 20 s. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ comparative method (Livak and Schmittgen, 2001). The threshold cycle (Ct) for each gene was normalized against the Ct for *18S rRNA* gene (AJ236003) of *V. officinalis*, which was used as the internal reference. Relative Expression Software Tool (REST)® software (Pfaffl (2001) was used to analyze the obtained data. This experiment was carried out with three biological and two technical repeatitions.

Results

Effect of MeJA on valerenic acid production

To evaluate the effects of MeJA concentrations on stimulating VA production 24, 48, 72, and 144 h after elicitation, HPLC analysis of root cultures of valerian was performed. Efficiency of MeJA in promoting VA accumulation at both concentrations are shown in Fig. II. The maximum increments of 4.33 times (9.14 mg/g DW) in VA content, in comparison to control culture (2.11 mg/g DW) were obtained after 144 h of exposure to 50 µM MeJA. However, maximum accumulation of VA (12.45 mg/g DW) was recorded at 100 µM

Table 1
Primer sequences designed for qRT-PCR

Target gene	Accession number	sequence (5'-3')	Product size (bp)
VoTPS1	JX494699	F: GTAGGCATGGGAGTAACTACAG R:TCGTGTCCAACCTTATCATCC	119
VoTPS3	JX494701	F: CGTGCGTAAAGTCTTACG R: CTACTCACTCCGTGTTCTTTC	114
VoTPS7	JX494705	F: GCTAGGCTCGACAATGATAAAG R: TGAACTCTACAACCACTTCTTC	124
18S rRNA	AJ236003	F: AGGATGGCAAGCGTATTGG R: TGCATTTCCCAGAGTGTCTTC	118

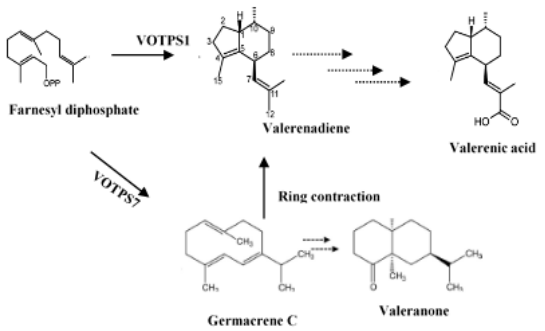
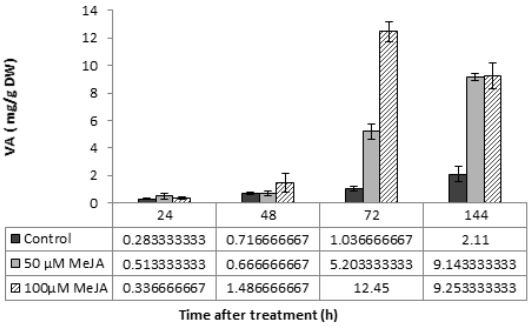


Fig. I. Proposed pathway for the biosynthesis of the sesquiterpene valerenic acid (Yeo et al., 2013)



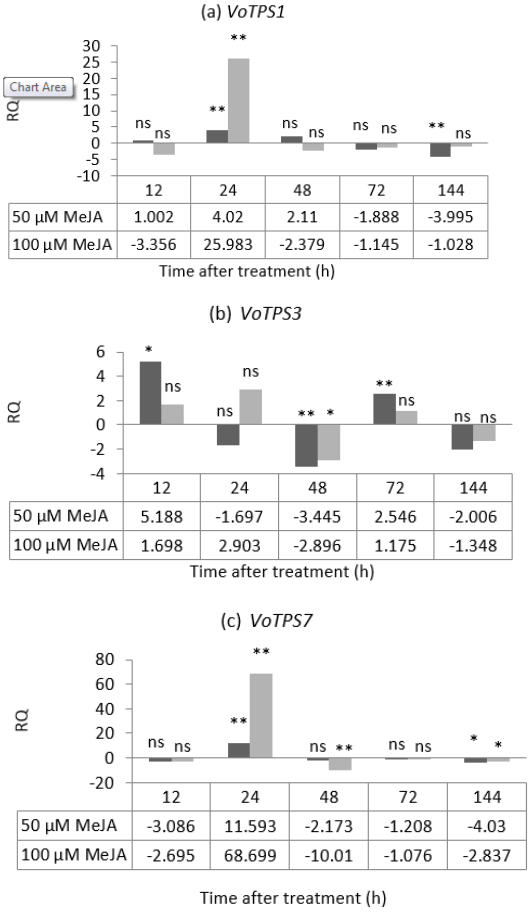
	24	48	72	144
Control	0.283333333	0.716666667	1.036666667	2.11
50 µM MeJA	0.513333333	0.666666667	5.203333333	9.143333333
100µM MeJA	0.336666667	1.486666667	12.45	9.253333333

Fig. II. Time-course effects of 50 and 100 µM MeJA on VA production in the root of V. officinalis plants; values are mean ± SD. Data analyzed using t test on VA contents in treated plants compared to the control ($p<0.05$).

MeJA with a 12 fold increase over control culture (1.03 mg/g DW) at 72 h. Accordingly, increasing MeJA concentration from 50 to 100 µM more effectively enhanced VA accumulation at shorter exposure time (72 h).

Effect of MeJA on expression pattern of Sesqui-TPS genes

To examine how dynamic transcriptional responses of some identified *VoTPS* genes probably involved in VA biosynthesis to MeJA, transcript abundance *of VoTPS1*, *VoTPS3* and *VoTPS7* genes were monitored by qRT-PCR in the root of *V. officinalis* plants (Fig. III). At both MeJA concentrations, the highest level of *VoTPS1* and *VoTPS7* transcripts were detectable 24 h after elicitation. Nevertheless, transcript abundance of these genes increased with increasing MeJA concentration. Compared with the control, mRNA levels of *VoTPS1* and *VoTPS7* were 4 and 11.5-fold higher after exposure to 50 µM MeJA, respectively. However, after exposure to 100 µM



	12	24	48	72	144
50 µM MeJA	1.002	4.02	2.11	-1.888	-3.995
100 µM MeJA	-3.356	25.983	-2.379	-1.145	-1.028

	12	24	48	72	144
50 µM MeJA	5.188	-1.697	-3.445	2.546	-2.006
100 µM MeJA	1.698	2.903	-2.896	1.175	-1.348

	12	24	48	72	144
50 µM MeJA	-3.086	11.593	-2.173	-1.208	-4.03
100 µM MeJA	-2.695	68.699	-10.01	-1.076	-2.837

Fig. III. Time-course transcriptional changes of *VoTPS1*, *VoTPS3*, and *VoTPS7* induced by 50 and 100 µM MeJA in the root of *V. officinalis* plants; for normalization, *18S rRNA* gene was used as the endogenous gene. The Y-axis represents relative quantity, * represents a significant difference at $p<0.05$, ** represents a highly significant difference at $p<0.01$, and "ns" indicates non-significant difference between the control and treated plants.

MeJA, transcript expression of these genes reached the peak at 24 h (approximately 25 and 68 times more compared to the control plants, respectively). Whereas, transcription rate of *VoTPS3* was mostly unaffected or down-regulated by MeJA at both concentrations.

Discussion

Our study showed that MeJA elicitation at both concentrations (50 and 100 µM) substantially promoted VA content in the root of *V. officinalis*. However, exposure to 100 µM of MeJA more effectively enhanced VA accumulation than observed for 50 µM. Moreover, a shorter period (72 h) was required for obtaining the highest

increase in VA content than observed in roots of *V. officinalis* elicited with 50 µM. High levels of MeJA may trigger further activation of downstream signaling components which results in increased expression of JA target genes and in enhanced accumulation of corresponding protein expression (Endt et al., 2002). Such dose- dependent effect of MeJA on the biosynthesis of secondary metabolites has been supported by the finding of Uddin et al. (2013) and Dini Torkamani et al. (2014).

The mechanism underlying JAs in triggering signal transduction pathways leading to transcriptional reprogramming of secondary metabolism pathway and enhancement of desired metabolites has been studied extensively (Afrin et al., 2015). It is found that JA-ZIM domain (JAZ) acts as an on/off switch in JA signaling. In the absence of the JA and its bioactive derivatives, JAZ recruits a set of transcription factors (TFs) acting in modulation of biosynthesis of secondary metabolites pathways, thereby limiting their activity (Chini et al., 2007). Upon perception of JA signaling, JAZ repressor is targeted for degradation by 26S proteasome-dependent proteolysis and ultimately releases MYC2 (Memelink 2009). It has been recently revealed that regulators of terpenoid indole alkaloid (TIA) biosynthesis in *Catharanthus roseus* is regulated by a JA-responsive TFs cascade (Yang et al., 2013). These finding suggest that coordinated induction of *VoTPS1* and *VoTPS7* can be conducted by such regulatory network. However, expression pattern of *VoTPS3* was more or less unaffected by MeJA at both concentrations. It may be concluded that *VoTPS3*, as a putative terpene synthase, does not appear to have any significant influence on the biosynthesis of VA.

It is well known that MYC2 as a master switch activates early JA-responsive genes transcription including JAZ. Formation of complex MYC2- JAZ repressor deactivates MYC2 and subsequently weakens JA-dependent responses (Chico et al., 2008). Our data indicated MeJA at both concentrations increased transcription levels of *VoTPS1* and *VoTPS7* for up to 24 h after elicitation. Further increase of exposure time decreased transcription rate of both genes probably due to attenuated JA signaling. Herein we also showed that substantial VA content enhancement was associated with simultaneous increased expression of *VoTPS1* and *VoTPS7* suggesting that intermediates valerenadiene and germacrene B/C/D (encoded by *VoTPS1* and *VoTPS7*, respectively) may possibly be involved in the VA biosynthesis and this was also proposed previously (Yeo et al., 2013).

Overall, these results illustrated that induction of *VoTPS1* and *VoTPS7* expression and increase in VA content were more effectively enhanced with increasing concentration of MeJA from 50 to 100 µM. Therefore, it is concluded that VA biosynthesis is probably regulated at the transcriptional level of *VoTPS1* and *VoTPS7* in roots of *V. officinalis*. However, the molecular mechanism underlying MeJA-mediated transcriptional regulation of these putative terpene synthases needs to be further studied.

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