Effects of Gibberellic Acid on Gene Expression of Menthol Biosynthesis Pathway in Peppermint (*Mentha piperita* L.)

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***Abstract*—**Peppermint is the major source of menthol rich essential oil and widely cultivated for pharmaceutical purposes. In this study, the effect of exogenous applications of gibberellic acid (GA3) was investigated in the main genes of menthol biosynthesis pathways within a 72 h time period using qRT-PCR. Transcript accumulation for the respective enzymes of early pathway steps relatively remained unaffected whereas expression levels of genes in later stages of oil biosynthesis, including *neo-red* and *m-deh* were down-regulated by GA3 treatment which is likely related to the negative feedback of rising GA levels following elicitor application in transcript level of genes involved in isoprenoid biosynthesis.

***Keywords*—** Mentha x piperita, Menthol, qRT-PCR, Gibberellic acid.

I. Introduction

MONOTERPENES belong to C_{10} isoprenoids that constitute the major components of the essential oils of mint family, including peppermint (*Mentha piperita* L.) and spearmint (*Mentha spicata*) [1]. Biosynthesis of monoterpens including menthol are restricted to highly specialized nonphotosynthetic secretory cells derived from the epidermal layer called peltate glandular trichomes. The major pathway for biosynthesis of menthol is conducted by eight enzymatic reactions. Menthol formed from the assembly of C_5 isoperne units, namely IPP and its allylic isomer DMAPP to produce acyclic precursor geranyl diphosphate by GPPS [2]. The second specific step of the pathway is cyclization of GPP by plastidial (-)-(4S) limonene synthase and thereby establishes limonene [3]. Following hydroxylation at C3 and a sequence of four redox reactions on cyclohexanoid ring affords (-) – (1R, 3R, 4S) menthol [4]. This valuable natural product has considerable economic importance due to its multitude aromatherapy and industrial applications (5). An important menthol biosynthesis pathway side-product is menthofuran. This undesirable monoterpen is derived from C9 hydroxylation, cyclization and dehydration of (+)- pulegone by an endoplasmic reticulum- localized enzyme called menthofuran synthase. It's worth mentioning that abiotic stress, promotes the accumulation of menthofuran and pulegone metabolites during leaf expansion period [6]. Metabolic engineering approaches have been undertaken to improve quantitative and compositional changes in commercially distilled peppermint oil [7-9].

It is worth noting that many pharmaceutical metabolites produce when plants subjected to stresses, including various elicitors or signal molecules like hormones [10, 11]. Gibberellins are recognized compounds that play an important role in the eliciting the biosynthesis of secondary metabolites in plant cells. Gibberellins are also reported to increase plant biomass and menthol content In *M. piperita* [12]. In spite of the fact that isoprenoids are a structurally and functionally diverse group of natural products, all of them are derived from two C_5 units provided by cytosolic MVA and plastidial MEP pathways. Sequential elongation reactions with the addition of one, two or three IPP units lead to the biosynthesis of GPP (C_{10}), FPP (C_{15}) and GGPP (C_{20}) which are the starting points of downstream pathways for the production of monoterpenes, sesquiterpenes and diterpens respectively [13]. The MEP pathway provides IPP for the synthesis of monoterpenes, carotenoids, abscisic acid and gibberellin. Gibberellins are usually produced by plastidial GGPP derived from MEP pathway. In the MEP pathway, eight enzymatic reactions are used to yield GA from GGPP. Analysis of the expression of genes involved in GA biosynthesis revealed new insights into the regulation of GA concentration in Plants [14, 15].

This study was performed to evaluate the effect of time-course exogenous application of gibberellic acid (GA_3) on transcript level alterations of genes involved in monoterpene biosynthesis under this treatment.

II. Materials and Methods

2-1. Plant Materials, GA_3 Treatment and Samplings

This experiment was carried out under natural light conditions in the greenhouse of Ramin Agriculture and Natural Resources University of khouzestan (IRAN). The rhizomes of peppermints were collected from Pakanbazr Company, Esfahan, Iran. Then 10-15 cm rhizomes were transferred into pots. They were watered every day. Two months-old uniform plants were selected for sampling.

In order to treat the plants 50 mg/L GA_3 sprayed on the surface of the peppermint. The untreated peppermint plants (control) were sprayed with only distilled water. Leaves from the untreated (control) and treated peppermint plants were randomly sampled at 12, 24 and 72 h after treatment. For each sampling, 4 leaves under the second visible leaf from the apex were harvested, frozen in liquid nitrogen, stored at -80 °C

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immediately for RNA extraction. In order to more accuracy of experiments, and separate associated control was individually considered for each treatment.

2-2. RNA Extraction,cDNA Synthesis, Primer DesignandqRT-PCR Reaction

Total RNA was isolate from mentha leaves using GeneAll® RiboEx ™kit (BioFrontier, Korea) based on manufacturer's protocol. The quality of extracted RNA was checked by 1%agarose gel electrophoresis. The first strand cDNA was synthesized from 500 ng of total RNA, using Fermentas kit (RevertAid ™ First Strand cDNA Synthesis Kit) according to the manufacturer's instructions.

Primer pairs of *pr, mfs, ls,l3h, iso-red, neo-red, m-deh* and *gds* genes were designed with online Primer Quest software according to the cDNA sequences (pr (AY300163.1), mfs (AF346833.1), *ls* (EU108697.1), *l3h* (AF124817), *iso-red* (AY300162), *neo-red* (DQ362936), *m-deh* (AY288138) and *gds* (AF182828.1) of *M. x piperita*, and actin (KM044035.1) of *M. spicata* that was employed as an internal standard (table 1). To ensure the specific amplification of designed primers for these genes, PCR reaction was performed using cDNA.

The qRT-PCR was performed using HIFI SYBR® Green kit (Iran) Master mix and Step One Plus® (ABI, America) machine under the following conditions: 95°C for 30 s followed by 40 cycles 95°C for 15 s, 52°C for 20 s and 72°C for 20 s. Relative expression levels of the genes were calculated by ΔΔCT. Relative Expression Software Tool (REST)® software [16] was used to analyses of data. This experiment was carried out with two biological and two technical repeats.

III. Results and Discussion

3.1 Expression of Main Genes in Menthol Biosynthetic Pathway

To investigate whether exogenous application of GA_3 at different time (12, 24 and 72 h) after treatment affects transcription of the main genes in menthol biosynthesis, we used qRT-PCR to evaluate the responses of the genes *gds*, *ls*, *l3h*, *iso-red*, *pr*, *mfs*, *neo-red* and *m-deh*. Transcript levels changes of genes in plants exposed to GA_3 were slightly variable. The transcript level of *gds* was increased 6 fold than in the untreated control at 12 h, whereas thereafter did not significantly change. The expression level of *ls*, *iso-red*, *mfs* and *pr* genes didn't show significant changes at all mentioned times after treatment. The *l3h* mRNA level increased whithin 12 h of GA_3 elicitor treatment (5.2 fold higher than that in the control) while at 24 and 72 h expression changes were not remarkable. The transcript levels of *neo-red* gene (Catalysing the conversion menthone to neomenthol or isomenthone to isomenthol) decreased markedly to below the control at 12 h (approximately 1.4 times lower than those in the control) as well as the expression of *m-deh* (catalysing the conversion of menthone to menthol or isomenthone to neoisomenthol) notably reduced (3 fold lower than those in the control) during 24h following GA_3 application. (Figure 2).

A regulatory role of DXS (Catalyzing the first reaction of the MEP pathway) in controlling the synthesis of MEP-derived isoprenoids came from the analysis of transgenic plants in which DXS was upregulated. A distinct positive correlation between DXS transcript levels and the synthesis of plastidic isoprenoids has been reported in Arabidopsis [36] and in *M. piperita* [8]. In vegetative cannabis plants, reduction in DXS activity was parallel with the reduction in chlorophyll and carotenoid contents by GA_3 application. Furthermore, the number and percentage of mono and sesquiterpens - derived MEP pathway declined in treated plants. This finding tightly has confirmed new insights into limiting the role of the MEP pathway in the synthesis of plastidic isoprenoids by exogenous application of GA_3 [17]. The current study also demonstrated that transcript accumulation for the respective enzymes of early pathway steps remained unaffected under GA_3 treatment. One other interesting result is elucidated that expression levels of genes in later stages of oil biosynthesis, including *neo-red* and *m-deh* was down-regulated by GA_3 treatment. This result is consistent with finding in former study, which observed no substantial alterations in transcript levels of genes dedicated to the early steps of oil biosynthesis (*gds*, *lh*,*ls*) in response to GA_3 in *M. arvensis* [18]. A recent study also has reported the effect of GA_3 on the stimulation of trichome formation and increases in its density and diameter on *M. arvensis* eventually resulted in increase oil yield. Since the biosynthesis of gibberellins are derived from MEP pathway, Most of genes involved in the formation of bioactive GA, downregulated by applying GA. Existing evidences confirmed that GA biosynthesis is regulated by feedback control [19, 20]. This self–regulation mechanism may interfere with biosynthesis of monoterpens and other isoprenoids derived from MEP pathway.

IV. Conclusions

A set of genes involved in the menthol biosynthetic pathway was selected for qRT-PCR to analyze their expression in phyto-hormonal GA_3 treated and control plants. On the whole, these results showed that with the few exceptions noted above, most transcript levels of genes were either unaffected or downregulated. Since exogenous application of GA_3 downregulate transcript levels of several genes involved in GA biosynthesis and other isoprenoids, there is this expectance that GA_3 treatment might not have a prominent role in enhancing menthol yield. However to understand the role of GA_3 negative feedback mechanism in menthol biosynthesis, we need further investigation. More profound studies on genes affecting trichomes formation and development, as well as menthol production, could be the next step for a further improvement yield of this valuable therapeutic.

Acknowledgments

The experiment was performed in the central laboratory of Ramin University, Khouzestan, Iran. The authors are thankful to the laboratory personals for putting all the means at our disposal.

Nomenclature: *gds*: Geranyl diphosphate synthase, *ls*: Limonene synthase, *l3h*: Cytochrome P450 (2)-limonene-3-hydroxylase, *iso-red*: Isopiperitenone reductase, *pr*: Pulegone reductase , *mfs*: Menthofuran synthases , *neo-red*: Neomenthol reductase, *m-deh*: Menthol dehydrogenase, IPP: Isopentenyl

diphosphate, DMAPP: Dimethylallyl diphosphate, ROS: Reactive oxygen species, MVA: Mevalonic acid, MEP: methyl erythritol phosphate, DXS: 1-deoxy-D-xylulose 5-phosphate synthase, GGPP: geranyl geranyl diphosphate, FPP: Farnesyl pyrophosphate.

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TABLE I
SEQUENCE, TM AND PRODUCT SIZE OF *ACT, GDS, LS, L3H, ISO-RED, PR, MFS, NEO-RED* AND *M-DEH* PRIMERS.

Gene	primer	sequence (5'-3')	TM (°C)	Product size (bp)
act	F	TCCTGAGAGGAAGTACAGTGTC	62	108
	R	GACGGCCCAGATTCATCATAC	62	
ls	F	TGACAGAGGTGTGGAAGAAG	62	113
	R	GTACATCAACTGCGCCATC	62	
pr	F	GAAGCTGTGATCAACAACATGAG	62	126
	R	ACGAATTTGCTTTGGGATTAGC	62	
mfs	F	TGACTGAAGCTCCTGGATTTG	62	111
	R	CCTTCCCTTCCGTGTGTATATG	62	
Neo-red	F	CAGAGGAGAAACTGGAGGAAG	62	115
	R	GCTGCTTTCGACACTTTGTAG	62	
M-deh	F	TCGGATCATAGCGCGAAAG	62	112
	R	AGCACCTTCAGCTTCACTTAG	62	
gds	F	TAGGGCAGCTCCATTGATTG	62	119
	R	AGAAAGGAGCATCATGTTTGTG	62	
L3h	F	ATTTCGAGTTCGTCCCGTTC	62	129
	R	TCATTCCTTCCGCCAACTTC	62	
Iso- red	F	CGAAGAAGTACCCGAGTTTCC	62	115
	R	TTCACCGGAACTTGAGCAG	62	

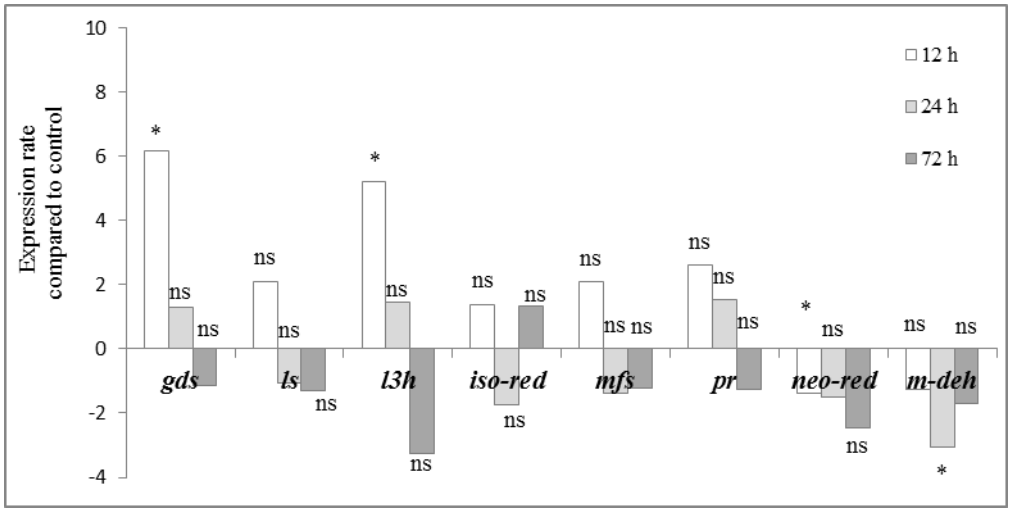

Fig. 1:, The relative expression rate of *ls, gds, l3h, iso red, mfs, pr, neo-red* and *m-deh* genes in times after applying GA_3 treatment. * and "ns" indicate significant differences respectively at ($P<0.05$) and non- significant differences between treat and itself control.