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 C10 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 monoterpenes 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 C5 isoprene 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, an endoplasmic reticulum- localized enzyme called menthofuran synthase. It’s worth mentioning that abiotic stress, including negative feedback of rising GA levels following elicitor application, is 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.

II. MATERIALS AND METHODS

II-1. Plant Materials, GA3 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 GA3 sprayed on the surface of the peppermint. The untreated peppermint plants (control) were sprayed with only distilled water. Leaves from the untreated (control) 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.
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 Design and qRT-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 GA3 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 GA3 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 within 12 h of GA3 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 isomenthol) notably reduced (3 fold lower than those in the control) during 24 h following GA3 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 plastic 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 GA3 application. Furthermore, the number and percentage of mono and sesquiterpenes - 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 plastic isoprenoids by exogenous application of GA3 [17]. The current study also demonstrated that transcript accumulation for the respective enzymes of early pathway steps remained unaffected under GA3 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 GA3 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 GA3 in M. arvensis [18]. A recent study also has reported the effect of GA3 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 GA3 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 GA3 downregulate transcript levels of several genes involved in GA biosynthesis and other isoprenoids, there is this expectance that GA3 treatment might not have a prominent role in enhancing menthol yield. However to understand the role of GA3 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

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REFERENCES


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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>TM (°C)</th>
<th>Product size (bp)</th>
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<tr>
<td>act</td>
<td>F</td>
<td>TCCTGAGAGGAAGTACAGTGTC</td>
<td>62</td>
<td>108</td>
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<tr>
<td></td>
<td>R</td>
<td>GACGGCCCGAGATTCATCATAC</td>
<td>62</td>
<td></td>
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<tr>
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<td>TGACAGAGGTGGGGAAGAG</td>
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<td>113</td>
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<tr>
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<tr>
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<td>115</td>
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<tr>
<td></td>
<td>R</td>
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Fig. 1.: The relative expression rate of ls, gds, l3h, iso-red, mfs, pr, neo-red and m-deh genes in times after applying GA3 treatment. * and “ns” indicate significant differences respectively at (P<0.05) and non-significant differences between treat and itself control.