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The Effect of Methyl Jasmonate on the Expression of Phenylalanine Ammonia Lyase and Eugenol-o-Methyl Transferase Genes in Basil

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Basil (*Ocimum basilicum* L.) is one of the important medicinal plants belonging to the Lamiaceae family, used as fresh herb. Methyl jasmonate (MeJa) is a hormone signal and endogenous growth regulator involved in the regulation of defense responses, which induces a broad range of physiological pathways in many plant species. In this study, a completely randomized design (CRD) with three replications was conducted in a greenhouse to evaluate the effect of MeJa on the expression of eugenol-o-methyl transferase (*EOMT*) and phenylalanine ammonia lyase (*PAL*) genes (key genes involved in the biosynthesis of phenylpropanoids) in basil. Two concentrations of MeJa (0 and 0.5 mM) were sprayed on healthy plants at pre-flowering stage. Plant leaves were sampled at 0, 24, 48 and 72 h after MeJa application, and the expression of the *EOMT* and *PAL* genes was studied using real time polymerase chain reaction (PCR). Results showed that MeJa with 0.5 mM concentration significantly increased the expression of both genes. The expression of both genes reached its maximum amount 48 h after MeJa application, but the expression of the *PAL* gene significantly declined after that. In conclusion, it was demonstrated that the external application of MeJa could significantly induce the expression of *EOMT* and *PAL* genes in basil.

Key Words: *Ocimum basilicum* L., phenylalanine ammonia lyase, pre-flowering, real time PCR

Abbreviations: cDNA – complementary deoxyribonucleic acid, *EOMT* – eugenol-o-methyl transferase, MeJa – methyl jasmonate, PAL – phenylalanine ammonia lyase, PCR – polymerase chain reaction

INTRODUCTION

The Lamiaceae family is well recognized for the diversity of its secondary compounds (Iijima et al. 2004). *Ocimum basilicum* L. (2n = 48), which belongs to this family, is used in the treatment of headaches, diarrhea, coughs, warts, worms and kidney malfunctions (Bais et al. 2002; Rai et al. 2004). Basil leaves contain essential oils of distinctive aroma (Grayer et al. 2004; Ozcan et al. 2005) with high proportions of phenylpropanoid derivatives, such as methyleugenol and methylchavicol often combined with various proportions of monoterpenes, and sesquiterpenes (Politeo et al. 2009). Many studies have been conducted to increase the production of these essential oils in plants (Wasternack et al. 2007). It has been reported that various elicitors, such as chitosan, glucan, yeast extracts, and plant hormonal chemicals, such as jasmonic acid (JA) and methyl jasmonate (MeJA), can increase the content of secondary metabolites in various plants (Turner et al. 2002; Radman et al. 2003). JA and its methyl ester (MeJa) are widely distributed in plants and they affect a variety of physiological processes including fruit ripening, root growth, production of viable pollen, senescence, defense response against pathogens and insect attack, plant response to wounding, and abiotic stresses (Pieterse et al. 2009).

Phenylalanine amonia-lyase (*PAL*) catalyzes the first step of the phenylpropanoid pathway.

Conflicting reports have suggested that eugenol is formed from the monolignol precursor coniferyl alcohol (Achnine et al. 2004) or is instead formed via an undefined mechanism involving methylation and decarboxylation of the hydroxycinnamic acids (Manitto et al. 1975). Confirmation of the last step in the formation of methylchavicol and methyleugenol, the addition of the methyl group to the 4-OH, has been reported recently (Wang et al. 1997; Lewinsohn et al. 2001).

In recent studies, it has been reported that MeJA could induce the expression of the genes involved in phenylpropanoid biosynthesis as well as the amount of the phenylpropanoids. The amount of eugenol and L-linalool has been increased in sweet basil treated with 0.5 mM MeJA 4 d after application by 56% and 43%, respectively (Johnson et al. 1999). Zare Mehrjerdi et al. (2013) reported that MeJa increases the expression of the genes involved in artemisin in biosynthesis in *Artemisia annua*. Ellard-Ivey and Douglas (1996) stated that jasmonates and a-linolenic acid strongly induce the expression of 4-coumarate coA ligase (4CL) in a dose-dependent manner in parsley cells; methyl jasmonate also activated the coordinate expression of other phenylpropanoid genes and the accumulation of furanocoumarin phytoalexins.

To our knowledge, the effect of MeJA on the expression of genes encoding *PAL* and *EOMT* enzymes has not been investigated yet, thus the main objective of the current study was to survey the effect of MeJa on the expression pattern of the above-mentioned genes in basil.

MATERIALS AND METHODS

Plant Materials and MeJa Treatment

Seeds of sweet basil (*Ocimum basilicum* L.) var. Keshkeni Luvelou, (kindly provided by Dr. A. Hassani, Department of Horticulture, Urmia University) were grown in plastic pots containing a mixture of soil and sand (1:1), under natural light condition in a greenhouse. The plants were irrigated every day and maintained at day/night temperature of 27–30 and 18–20 °C, respectively. Whole basil plants were sprayed with 0.5 mM of MeJA dissolved in 2% ethanol at the pre-flowering stage (Naderi et al. 2014). The untreated (control) plants were sprayed with double-distilled water. Plant leaves were sampled 0, 24, 48 and 72 h after MeJa application and immediately frozen in liquid nitrogen and stored at −80 °C for gene expression studies.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the plant leaves using RNX™ Plus solution (SinaClon, Iran) according to the manufacturer's recommendations. The integrity and quantity of the RNA were determined using 1% agarose gel electrophoresis and spectrophotometer. Complementary deoxyribonucleic acids (cDNAs) were synthesized using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions. Negative control reactions including reverse transcriptase minus (RT-) negative control and no template control (NTC) were performed during cDNA synthesis to assess for genomic DNA contamination of the RNA sample and for reagent contamination, respectively. To test the proper working of the cDNA synthesis kit, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control RNA (1.3 kb), supplied with the kit, was also converted to cDNA and PCR was carried out for this cDNA using gene-specific primers, and then, a fragment of 496 bp was observed in 1.5% agarose gel. All control reactions were carried out based on the instructions provided in the kit.

Primer Design and Real Time PCR Reactions

Coding sequences of the 18s-rRNA, *PAL* and *EOMT* genes were downloaded from the National Center for Biotechnology Information (NCBI). Specific primers (Table 1) were designed using Fast PCR 4.0 and Gen runner 3.05 softwares. Primers were blasted against nucleotide sequences in NCBI to ensure their specificity. Annealing temperature of the genes was optimized using PCR in a volume of 20 μL containing 3 μL diluted cDNA (1:5), 1× PCR buffer (10 mM Tris–HCl, 50 mM KCl, pH = 8.3), 1.5 mM $MgCl_2$, 0.2 μM dNTP, 0.5 unit of Taq DNA polymerase, and 10 pmol of primer. PCR conditions were as follows: an initial denaturation step of 2 min at 95 °C, 35 cycles of 40 s at 95 °C, 30 s at 56–58.8 °C (Table 1) and 50 s at 72 °C, followed by a final extension step of 5 min at 72 °C. PCR reactions were run in an Eppendorf thermal cycler. Amplified products were separated on 1.8% agarose gel and stained with ethidium bromide. Images were captured and documented using a Gel documentation system

Table 1. Primer sequences, annealing temperature (Ta) and amplicon length of 18s rRNA, phenylalanine ammonia lyase (*PAL*) and eugenol-o-methyl transferase (*EOMT*) genes in basil.

Gene	Accession No.	Primer Sequence	Ta (°C)	Amplicon Length (bp)
18sRNA	AK059783	CTACGTCCCTGCCCTTTGTACA (F) ACACTTCACCGGACCATTCAA (R)	58.8	65
PAL	AB436791	CATTGCTGGTGTCCTCTTAG (F) CCACTGCTGTCCCATTTACT (R)	56	158
EOMT	AF435008	ATCAAGAGGTGTGCTACTGGC (F) CCTTGCTTGGCTCATTTGC (R)	58.2	219

(Gel Logic 212 PRO, Carestream, USA).

Real-time PCRs were performed in a volume of 12.5 µL in Rotor-Gene Q (QIAGEN, USA) using Maxima SYBER Green/Flourescein qPCR Master Mix (Fermentas, Germany) according to the manufacturer's recommendations. Temperature conditions were as follows: holding for 10 min at 95 °C and 40 cycles of 95 °C for 15 s, 56 to 58.8 °C (Table 1) for 30 s and 72 °C for 40 s. Two biological replicates for each sample were used for real-time PCR analysis. The specificity of amplicons was verified by melting curve analysis (45 to 95°) and 1.8 % agarose gel electrophoresis. An 18s-rRNA gene was used as reference gene.

Statistical Analysis

The experiment was conducted in a completely randomized design with three replications. The relative expression levels of the genes were calculated using $2^{-\Delta\Delta Ct}$ value (Bustin 2002; Klein 2002; Bustin and Nolan 2004). In order to apply the $2^{-\Delta\Delta Ct}$ method, the results of real-time PCRs were represented as cycle threshold (Ct) values. Normality test of the data and residuals was performed using software MINITAB 16. Data were subjected to analysis of variance, then means were compared by Duncan's method in SAS program ver. 9.1.

RESULTS AND DISCUSSION

Significant differences ($p<0.01$) were found among sampling times after 0.5 mM MeJa application for both genes (Table 2). Relative expression of *EOMT* gene was initially low (24 h after MeJa application) and significantly increased 48 h after MeJa application. It was 10.29 and 8.32 times more than that of the control sample, 48 and 72 h after 0.5 mM MeJa application, respectively (Fig. 1). The expression of *PAL* gene significantly increased 24 h after MeJa treatment and reached its maximum value (10.25 times) 48 h after spraying, then significantly declined (Fig. 2).

Table 2. Analysis of variance for the effect of times after methyl jasmonate application on the expression of eugenol-o-methyl transferase (*EOMT*) and phenylalanine ammonia lyase (*PAL*) genes in basil.

Source of Variance	*EOMT*	*PAL*
Sampling time	62.77**	82.52**
Error	1.01	0.61
CV (%)	14.97	10.99

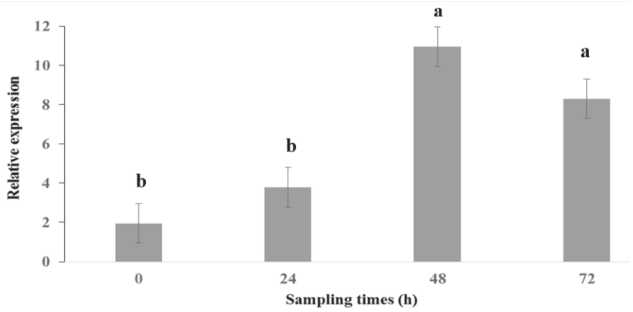

Fig. 1. The effect of times after 0.5 mM methyl jasmonate treatment on the expression of eugenol-o-methyl transferase (*EOMT*) gene in basil.

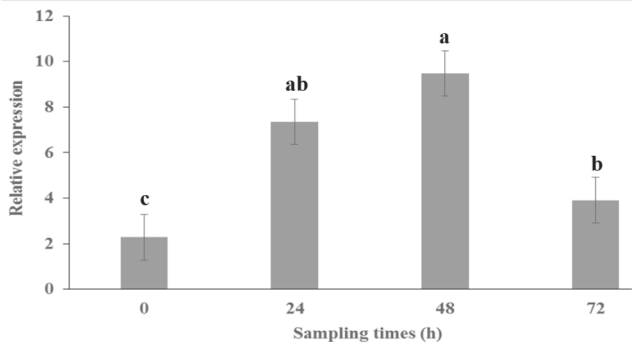

Fig. 2. The effect of times after 0.5 mM methyl jasmonate treatment on the expression of phenylalanine ammonia lyase (*PAL*) gene in basil.

In this study, the expression of two key genes, *PAL* and *EOMT*, involved in the biosynthesis of phenylpropanoids, was investigated in basil under MeJa treatment. It has been demonstrated that jasmonate (JA) or its derivative, MeJa, could induce the expression of genes involved in the accumulation of secondary metabolites in plants (Kim et al. 2005). Methyl eugenol, as an important phenylpropanoid

compound in basil, is produced through the phenylpropanoid pathway initiated by the *PAL* enzyme (Gang et al. 2001). *PAL* catalyzes the first step of the phenylpropanoids pathway in which L-phenylalanine is deaminated to trans-cinnamic acid and is considered as an important regulation point between primary and secondary metabolism (Dixon and Paiva 1995). Many phenolic compounds produced via this pathway can be induced by stresses and elicitors such as JA and MeJa (Lewinsohn et al. 2001). Therefore, it has been suggested that elicitors such as MeJa may increase the expression of the genes involved in the biosynthesis of these compounds.

In our study, MeJa with a concentration of 0.5 mM increased the expression of *EOMT* and *PAL* genes. The expression of both genes reached the maximum amount 48 h after treatment, but the expression of *PAL* gene significantly increased earlier than that of the *EOMT* gene. To our knowledge, the effect of the MeJa on the expression of *PAL* and *EOMT* genes has not been investigated, but basil treatment with 2 mM salicylic acid has increased the expression of the chavicol o-methyl transferase gene 72 h after treatment (Zarei et al. 2015).

Chitosan treatment in basil has also increased the expression of this gene (Naderi et al. 2014). The expression of the *PAL* gene and its enzyme activity was induced 1 d after chitosan application in basil and the changes in *PAL* gene expression at different harvest stages were consistent with the amount of phenylpropanoid compounds (Naderi et al. 2014). Any external stress or inducer applied to the plant is initially perceived by membrane receptors, and then a signal is transmitted to the downstream, which leads to the production of secondary messenger molecules. These secondary messengers promote calcium-dependent protein interactions which lead to the transcription of the stress-responsive genes through phosphorylation. In the meantime, genes activated up to 24 h after stress perception are called early responsive genes while those activated 48 h and more after stress treatment are called late responsive genes (Heidarvand and Malli Amiri 2010).

In our investigation, MeJa, as an inducer, activated significantly the expression of the *PAL* gene 24 h after treatment. This gene catalyzed the first step of the phenylpropanoid pathway in which L-phenylalanine is deaminated to trans-cinnamic acid, hence, its expression was induced earlier. The *EOMT* gene reached its maximum expression 48 h after MeJa treatment. This gene is located in the last steps of the phenylpropanoid biosynthesis pathway. In addition, not all stresses lead to the expression of the *EOMT* gene, as many products can be produced at the end of the phenylpropanoid biosynthesis pathway and the type of product depends on its frequency in the plant (Lewinsohn et al. 2001).

In general, the expression of the *PAL* gene was more than that of the *EOMT* gene. This result may be due to the low amount of eugenol in basil as well as the low amount of that in leaves compared with that in the stem (Grayer et al. 2004). Hence, the low expression of the *EOMT* gene in leaves in our investigation can be expected since the expression of both genes was studied in leaves.

In conclusion, results of the current study indicated that external application of MeJa can increase the expression of key genes involved in phenylpropanoid biosynthesis in basil. Thus, more research is needed to study the expression of other important genes involved in this pathway as well as their relationship with phenylpropanoid accumulation under MeJa and other treatments with chemical elicitors. Future studies could help provide the possibilities for genetic manipulation of this pathway to enhance production of valuable compounds in basil.

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