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RESEARCH ARTICLE

Effect of methyl jasmonate on expression of some genes related to shikonin biosynthetic pathway in *Lithospermum officinale*

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ABSTRACT: Shikonin is a naphthoquinone with some important medicinal properties and is found in gromwell plant. There are so many biotechnological approaches proposed for the productivity enhancement, and elicitation is recognized as one of the most effective strategies for increasing the production of secondary metabolites in plant *in vitro* cultures. Moreover, a deeper understanding of the mechanisms and factors affecting shikonin biosynthesis can led to the design of more intelligent and efficient biological production systems. To this aim, in the present study, the expression of some genes related to the shikonin biosynthetic pathway including *PAL*, *4CL*, *HMGR*, *GPPS* and *PGT* in *in vitro* cultures of *Lithospermum officinale* in response to methyl jasmonate (MJ) at different times, were investigated by real-time PCR. The results showed that MJ had a significant effect on increasing gene expression levels in elicited samples compared to control samples. Additionally, we found that the studied genes respond to MJ with different pattern, in which the highest increase in gene expression level was observed for *PGT* while the lowest increase was observed for *GPPS*. Maximum and minimum transcript levels were obtained in most genes at 4 and 96 h post-elicitation, respectively. It was also found that phenylpropanoid pathway genes respond better to MJ than terpenoid pathway genes. The results of the present study would increase our knowledge about elicitor signal transduction pathways, and may be particularly useful for enhancement of shikonin production in plant cell cultures of *L. officinale.*

KEYWORDS: Gromwell, Gene Expression, Shikonin, Methyl Jasmonate, Real-time PCR.

ABBREVIATIONS: methyl jasmonate (MJ), phenylalanine ammonia-lyase (*PAL*), cinnamic acid 4-hydroxylase (*C4H*), 4-coumaroyl CoA ligase (*4CL*), 3-hydroxy-3-methylglutaryl-CoA reductase (*HMGR*), geranyl diphosphate synthase (*GPPS*), p- hydroxybenzoic acid geranyl transferase (*PGT*), m-geranyl- p- hydroxybenzoic acid (GBA)

INTRODUCTION

The genus *Lithospermum* (Gromwell) is composed of perennial plants belonging to the family Boraginaceae, is a rich source of phenylpropanoid compounds including naphthoquinones. Shikonin and its stereoisomer, alkanin, are the most important naphthoquinones in *Lithospermum* and their acetyl, deoxy, isobutyryl, and isovaleryl derivatives are commonly found in *Lithospermum* [7, 27, 44]. Shikonin and related compounds are used as natural colorants in the food, textile, and cosmetic industries. Moreover, shikonin is an active pharmaceutical ingredient with antimicrobial, anti-cancer, anti-oxidant, and burns and wounds healing properties [6, 24, 44].
Although shikonin is naturally found in the root of the gromwell plant, however, poor germination, sensitivity to

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viral infection, and time-consuming production procedures are the major drawbacks in the production of shikonin from natural resources. Therefore, using alternative methods is inevitable. Biotechnological methods have some important advantages such as independence from natural resources, higher production rate, homogeneity, higher concentrations, and the possibility of production on a large scale. These approaches successfully were applied for the over-production of several plant-origin pharmaceutical compounds [10, 24]. Generally, in biotechnological methods, plant cell, tissue or organs are used as production systems and there are so many inducing strategies are integrated with these systems to overproduce of target metabolites [25].

The application of elicitors is one of the most effective approaches to the over-production of plant secondary metabolites. An elicitor can be defined as a chemical or physical agent that induces the plant defense systems. Elicitors may be of external origin, such as cellular components of pathogenic bacteria or fungi, or of internal origin, such as compounds released from plants by plant-microbe interactions [30, 33]. Elicitors trigger plant defense responses, which results in improved resistance to a wide range of biotic and abiotic stresses. Increasing secondary metabolites production such as shikonin is one of the consequences of elicitation. Jasmonates, especially MJ, are one of the most well-known elicitors used in *in vitro* culture-based production systems, and their increasing effects on the production of many plant secondary metabolites have been proven [11, 38].

Understanding of secondary metabolites biosynthetic pathways and identification of the underlying genes, enzymes, and metabolic pathways intermediates, and investigation of impacts of different inducers and inhibitors lead to enhancing the production efficiency in biological systems [34, 39]. It has been known that shikonin biosynthetic pathway composed of phenylpropanoid and terpenoid pathways. In the phenylpropanoid pathway, elimination of ammonia from phenylalanine by *PAL* gives cinnamic acid. Other steps of this pathway followed by *C4H* and *4CL* led to the formation of 4-hydroxybenzoic acid. In the terpenoid pathway, the activity of *HMGR* and *GPPS* led to the formation of geranyl diphosphate. Linking the phenylpropanoid and terpenoid pathways is performed by *PGT*, which leads to the formation of GBA. Following several metabolic steps, GBA is converted into shikonin [5, 36, 40, 44].

Studying the gene expression variations in response to different treatments, provide valuable information about the regulation of plant secondary metabolic pathways [2, 26]. This information can be useful for enhancing the production of metabolites [13, 34, 47]. Due to the importance of the topic and because there is little molecular knowledge about the *Lithospermum* secondary metabolites pathways, the current study was performed to investigate the expression of several shikonin-related genes in response to MJ treatment in *in vitro* cultures of *Lithospermum officinale*.

MATERIALS AND METHODS

Plant culture

Seeds of *L. officinale* were collected from Arasbaran area in East Azarbaijan province, Iran and stored at -20°C. Alternating temperature treatment (including one week at 4°C and one week at 25°C for 6 weeks) was used for breaking seed dormancy. After the appearance of the first signs of germination, seeds were planted in pots containing peat moss and maintained in greenhouse at 25±5°C and 40 to 60% relative humidity. Seedlings were periodically irrigated (once every two weeks) with Hoagland's solution [16].

Callus culture

Leaf and stem segments of the plant, were used as explants for callus induction. The explants were mainly obtained from plants grown in the greenhouse, however, some micro-propagated plants were also used for callus induction. Explants were prepared and sterilized as follows: First, young shoots (about 5 cm) were separated from the source plants. Then, the explants were washed under running tap water for 5 minutes to remove surface contaminants. In the next step, explants were placed in 70% ethanol for 20 seconds and 1.5 % sodium hypochlorite containing 2-3 drops of Tween-20 for 2 minutes. Then, plant materials were transferred to laminar airflow cabinet and rinsed 5 times with sterile distilled water. Explants were divided into roughly equal-sized pieces (1 cm) and then soaked in sterile distilled water for 10 minutes. The excess water was removed from the explant surfaces using sterile wathman filter paper. Linsmaier and Skoog basal medium [22] containing NAA (2 mg/l) and BAP (0.2 mg/l) were used for callus induction. To avoid browning of tissues, ascorbic acid (200 mg/l) was applied. Callus cultures were maintained in a dark growth room at 24±2°C.

Cell culture and elicitation

To establish cell suspension cultures, two-month-old calli with best growth characteristics, were transferred to a liquid medium. The cultures were agitated on an orbital shaker at 140 rpm. Cell suspension culture medium was similar to the callus induction except the vitamin concentration in the suspension culture medium was 2 fold higher than in the callus medium. Two weeks after the transferring of calli to the suspension medium, cell cultures were treated with 100 µM MJ, and sampling was carried out at 4, 8, 24, 48, and 96 hours after elicitation. Samples were immediately frozen in liquid nitrogen and stored at -70°C until further analyses.

RNA extraction

The method of Majidi and Bahmani [23] was used for RNA extraction. Briefly, this method involves the use of CTAB buffer, sodium citrate and helper buffers. Precipitation is carried out using lithium chloride and isopropanol. For removing DNA contamination, samples were treated with *DNase* I according to the manufacturer's instructions (Sinacolon, Iran). The extracted RNA samples were stored at -70°C. Quality and quantity assessments of the extracted RNAs were performed using 1% agarose gel electrophoresis and spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, USA).

cDNA synthesis

cDNAs were synthesized using the Easy cDNA Synthesis Kit (Pars tous, Iran) which contains the MMLV reverse transcriptase and random hexamers and oligo dT primers. For each sample, 2 µg RNA was used as template and cDNA synthesis was done at 47°C for 60 min. cDNAs were diluted (1:5) with nuclease-free water and stored at -20°C.

Real-time PCR

The sequences of target genes were obtained from the NCBI GenBank sequence database. Then the primers were designed (Table 1) by the Primer Premier 5.0 (Premier Biosoft International, USA). Primers with melting temperatures between 60-65°C without any secondary structure or unspecific pairing were selected. Moreover, the specificity of primers was confirmed using Primer- BLAST (NCBI) and the primers were synthesized by Bioneer (Korea).

Table 1. List of primers used in real-time PCR.

Gene	Primer sequences 5' → 3'	Size of amplicon (bp)
GAPDH	ctttggattcaggaacccagagg gcaccacccttcaagtgagcag	120
PAL	aggggacggatagttatggtgtaac ccttgttgctgagtggggtaatg	164
4CL	gcaaaacttggtcagggttatgg ggttacgaggcaaagaggcac	172
HMGR	acagaggatgtaaggcaatttatgc agcggctcttttcgcagtt	110
GPPS	actagacctttacaggcgcattg cttccaacttctcaccagccat	101
PGT	ttgttccaagcatcgcctacc tttataccagcttttgcgtcgtc	112

Real-time PCR was done on Rotor-Gene Q (Qiagen, Germany) using Sina Green HS-qPCR Mix kit (Sinacolon, Iran). PCR was performed with following program: an initial 10 min denaturation at 95°C followed by 40 cycles of 15 s denaturation at 95°C, 20 s annealing at 57°C, and 25 s extension at 72°C. Finally, the melting curves were acquired by a stepwise increase of the temperature from 60 to 95°C to confirm the specificity of the amplified products. Gene expression analysis was performed using the Pfaffl relative quantification method [28]. *GAPDH* was used as the reference gene to normalize gene expression levels amongst the samples. Each experiment was performed in three biological replicates and two technical replicates and data were analyzed using REST 2009 software.

RESULTS

We found that the germination rate of *Lithospermum* seeds is very low. This low germination rate may result from physiological seed dormancy [9]. Among different treatments, alternating temperature treatment increased the germination rate up to 90%, whereas other treatments (sulfuric acid, gibberellic acid and cold treatment) were not effective. Moreover, we found that peat moss is the best medium for *Lithospermum* seedling growth and a mixture of peat moss and perlite (1:1 v/v) is in the next position. Seedlings were emerged after two weeks, and subsequently successful flowering and seed production under greenhouse conditions were observed (Fig. 1).

Callus culture results (Fig. 2) showed that the leaf samples had a higher rate of callus induction in compared to stems.

Figure 1. Cultivation of gromwell in greenhouse. a: comparison of seedling growth at peat moss (above) and the mixture of peat moss and perlite (below). b: flowering stage (70 days after planting), c: seed production stage (100 days after planting).

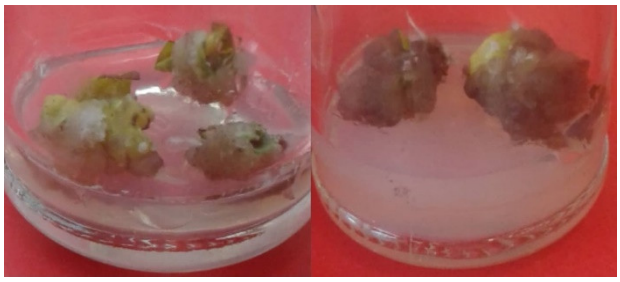

Figure 2. Callus cultures of *Lithospermum officinale*.

The initiation of callus started 12–14 days after culture from the cut ends of the explants. We also found that the explants of *Lithospermum* are more sensitive to disinfection and wound, in which increasing in disinfection time and physical stresses led to cell death. In the callus stage, we observed browning of samples, however, application of ascorbic acid (200 mg/l) successfully diminished the browning of the cultures. In suspension cell cultures, one week after transferring the calli to the liquid medium, we observed the generation of fresh cells, indicate that the selected medium is appropriate.

The efficiency of real-time PCR primers was from 98.2 to 103.8 percent and these are the optimum values for quantification assays. Expression analysis of *GAPDH* gene showed that the amount of expression of this gene is constant in different conditions, therefore, it can be used as a suitable reference gene for relative quantification of gene expression assays.

The results of gene expression of shikonin related genes, showed that MJ has a significant effect on all studied genes (Fig. 3). We found that the up-regulation of studied genes at the early time treatment (including 4, 8, and 24 hours after elicitation) was higher than the longer time point treatment (including 48 and 96 hours after elicitation). All studied genes other than *PGT* showed the highest increase in expression at 4 hours after elicitation, while the lowest level of transcripts was observed in most genes at 96 hours after elicitation. At long period of elicitation (96 h), *PAL* gene still maintained high levels of its transcripts, while other genes showed a significant decrease in expression at this time point.

The highest increase in gene expression level was observed for the *PGT* gene (392.5 fold) at 24 hours after elicitation, while, the *GPPS* gene showed the lowest increase in expression (8.2 fold). *GPPS* transcripts levels were slightly increased at 4 and 8 hours after elicitation, then dramatically decreased at 24, 48, and 96 hours after treatment. The highest down-regulation rate among all genes (-6.0 fold) was observed in *GPPS* gene at 96 hours. Interestingly, results indicated that the terpenoid pathway-related genes (*HMGR* and *GPPS*) and phenylpropanoid pathway-related genes (*PAL* and *4CL*) respond to the elicitation with different patterns. It was shown that the expression level of *PAL* and *4CL* genes were significantly higher than the *HMGR* and *GPPS* genes, under elicitation.

DISCUSSION

Jasmonates, as elicitors, trigger the production of secondary metabolites and protect plants against a wide range of stresses. Jasmonates initiate the cell reprogramming process and gene expression changes which lead to the activation of different secondary metabolic pathways such as phenylpropanoids, terpenoids, and alkaloids [3, 37, 38]. Moreover, it has been reported that MJ increased the shikonin production in cell and hairy root cultures of Boraginaceae [32, 35, 43].

In line with previous findings, the results of the present study also showed that the expression of genes related to

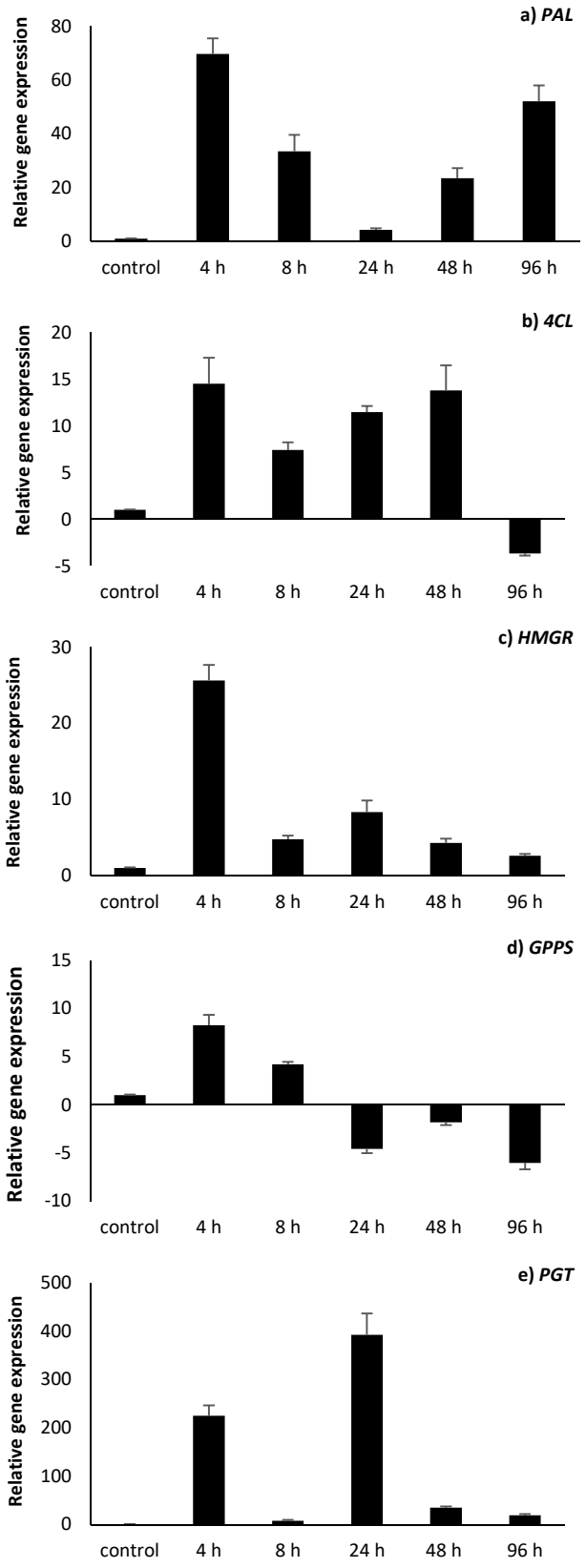

Figure 3. Shikonin biosynthesis-related gene expression changes in response to methyl jasmonate. a: *PAL*, b: *4CL*, c: *HMGR*, d: *GPPS*, e: *PGT*. Columns indicate the relative expression levels of genes at different time points (error bars, SD; n = 3).

the shikonin pathway in *L. officinale* cell cultures is sensitive to MJ. However, related genes to shikonin pathway respond to MJ with different patterns especially in fold change, up-regulation time point, and transcript stability in cell cultures. It has been shown that the increased lzevel of intrinsic and extrinsic MJ can lead to widespread modulation at transcription, mRNA stability, translation, and post-translational modifications, leading to the overproduction of plant secondary metabolites. It has been known that, MJ causes massive changes in plant metabolism by acting on transcription factors including WRKY, MYB, AP2, NAP, G/HBF-1 and ERF [1, 15].

High up-regulation at early stage and maintaining the increased level of transcripts of *PAL* gene at a long time after elicitation, indicate that this enzyme has a key role in response to stresses. As phenylpropanoids are major components of plant response to a wide range of stresses including biotic and abiotic stresses, an early and stable response of *PAL* to MJ was expected [32]. In agreement with our findings, other researchers also, have reported a high correlation between increased expression level of *PAL* gene and the production of phenolic compounds [18, 19].

Relatively lower up-regulation of *GPPS* in comparison with other genes may be due to deficiency of enzyme precursor i.e. dimethylallyl pyrophosphate (DMAPP). DMAPP deficiency has also been reported as a key limiting factor to the production of other terpenoid-based plant secondary metabolites. One of the proposed solutions to solve this problem is the overexpression of isopentenyl diphosphate isomerase (*IPPI*), which leads to an increase in the amount of DMAPP [17, 31]. Overexpression of *GPPS* is another strategy to increase the terpenoid content. Given that the product of *GPPS*, i.e. geranyl diphosphate, is an important metabolic intermediate for the biosynthesis of shikonin, gibberellic acids, abscisic acid, and carotenoids, therefore the overexpression of *GPPS* can lead to extensive growth and developmental changes in plants. Transgenic studies in tobacco and camelina showed that the overexpression of *GPPS* can lead to accumulation of terpenoid content, bigger leaves, improvement in the overall growth, bigger internodes, and earlier flowering [20, 41, 45].

In line with our findings, it has been shown that the secondary metabolism-related gene expression decreased after prolonged elicitation by MJ, which referred to the accumulation of intracellular secondary metabolites. This is a protective feedback strategy to avoid the over-accumulation of intracellular secondary compounds and

consequent cell toxicity and death. However, since there is a correlation between the transcript levels of shikonin related genes and shikonin production, the prolonged maintenance of the transcript levels of plant secondary metabolites related genes can improve the production efficiency in biological systems. The application of treatments such as ultrasound, DMSO, and β-cyclodextrin that lead to secretion of secondary metabolites to the extracellular environment, without intracellular accumulation, is an efficient approach for overproduction of plant secondary metabolites over a long period of time [8, 21].

In addition to the similarities, the expression patterns of shikonin pathway genes in several studies also show differences. These differences are might be due to several factors such as plant species or genotypes within species, epigenetic changes and other unknown factors [14, 35, 46]. In general, the study of secondary metabolic pathways, due to their high diversity is currently facing many obstacles, and understanding the details of pathways and regulatory mechanisms affecting of the pathways are still unknown. The increasing use of high-throughput technologies, especially genomics, transcriptomics, proteomics and metabolomics can improve our knowledge of secondary metabolites biosynthesis pathways to accelerate and optimize the biological production systems [4, 12, 26, 29, 42].

CONCLUSION

Considering the appropriate response of *in vitro* cultures of gromwell plant to elicitor and up-regulation of secondary metabolites-related genes, it can be concluded that *in vitro* cultures of *L. officinale* is an effective approach for shikonin production. However, it seems that to maximize the production of shikonin in *in vitro* cultures, in addition to the use of MJ, it necessary to employ other effective strategies. High and stable expression of terpenoid pathway genes, especially *GPPS*, can lead to an overall improvement in the shikonin biosynthesis. Using the treatments that increase the exudation of secondary metabolites into the extracellular medium is also helpful in this regard. With the progress of the multi-omic technologies, secondary metabolisms underlying pathways will be uncovered and lead to increase the production efficiency of secondary metabolites in biotechnological systems.

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بررسی اثر متیل جاسمونات بر بیان برخی ژن‌های مرتبط با مسیر بیوسنتزی شیکونین در *Lithospermum officinale*

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چکیده

شیکونین یک نفتوکینون حاوی برخی ویژگی‌های دارویی مهم است که در گیاه سنگدانه یافت می‌شود. روش‌های بیوتکنولوژیک فراوانی برای افزایش بهره‌وری تولید متابولیت‌های ثانویه از طریق کشت‌های درون شیشه‌ای گیاهی پیشنهاد شده‌اند که الیسیتاسیون یکی از موثرترین استراتژی‌های شناخته شده می‌باشد. به علاوه، شناخت عمیق‌تر مکانیسم‌ها و عوامل موثر بر بیوسنتز شیکونین می‌تواند به طراحی سیستم‌های تولید زیستی هوشمندانه‌تر و کارامدتر منجر گردد. بدین منظور، در مطالعه حاضر، بیان برخی از ژن‌های مرتبط با مسیر بیوسنتز شیکونین از جمله *PAL*، *4CL*، *HMGR*، *GPPS* و *PGT* در کشت‌های درون شیشه‌ای *Lithospermum officinale* در پاسخ به متیل جاسمونات در زمان‌های مختلف، توسط تکنیک real-time PCR مورد بررسی قرار گرفت. نتایج نشان داد که متیل جاسمونات اثر معنی‌داری بر افزایش بیان ژن‌ها در نمونه‌های تحریک شده در مقایسه با نمونه‌های شاهد داشت. به علاوه مشخص شد که ژن‌های مورد مطالعه به طرز متفاوتی به متیل جاسمونات پاسخ می‌دهند، به طوری که بیشترین میزان افزایش در ژن *PGT* و کمترین مقدار در ژن *GPPS* مشاهده شد. حداکثر و حداقل سطوح رونوشت‌ها در اغلب ژن‌ها به ترتیب در زمان‌های ۴ و ۹۶ ساعت پس از الیسیتاسیون به دست آمد. همچنین مشخص شد که ژن‌های مسیر فنیل‌پروپانوئیدی واکنش بهتری را در پاسخ به متیل جاسمونات در مقایسه با ژن‌های مسیر ترپنوئیدی از خود نشان می‌دهند. نتایج مطالعه حاضر، دانش ما را در مورد مسیرهای پیامرسانی الیسیتورها افزایش می‌دهد و ممکن است خصوصا برای افزایش تولید شیکونین در کشت‌های سلولی گیاهی *L. officinale* مفید واقع شود.

کلمات کلیدی: سنگدانه، بیان ژن، شیکونین، متیل جاسمونات، Real-time PCR.