METHYLJASMONATE-INDUCED TROPANE ALKALOID PRODUCTION, PUTRESCINE N-METHYLTRANSFERASE (PMT) AND HYOSCYAMINE 6β-HYDROXYLASE (H6H) GENES EXPRESSION IN ATROPA BELLADONNA L. (SOLANACEAE) IN VITRO-PROPAGATED PLANTLETS AND HAIRY ROOTS.

Azar Moradi a, Mozafar Sharifi a, Amir Mousavi b

aDepartment of plant science, Faculty of biological science, Tarbiat Modares University, Tehran, Iran
bNational Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

Email: msharifi@modares.ac.ir

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Abstract

Atropa belladonna as a medicinally important herbaceous plant has a large amount of hyoscyamine and scopolamine tropane alkaloids in its organs. These alkaloids are used for the production of some medicines. In this study, the effect of methyl jasmonate (MJ) as a signaling molecule on the tropane alkaloid production and expression of two important genes involved in their biosynthetic pathway were investigated by using in vitro-propagated plantlet and hairy root systems. MJ treatment decreased the growth parameters in both the plantlets and hairy roots. Scopolamine rose in plantlet root and shoot but declined in hairy roots by MJ. Hyoscyamine increased in shoot but fell in root and hairy root after MJ application. Transcriptional analysis revealed that MJ treatment up-regulates the expression of the genes encoding the key enzymes putrescine N-methyltransferase and hyoscyamine 6β-hydroxylase. The results of this survey suggest that the expression of h6h and pmt genes and accumulation of tropane alkaloids are regulated differently in A. belladonna tissues.

Keywords: Agrobacterium rhizogenes, Atropa belladonna, Hyoscyamine 6β-hydroxylase, Methyl jasmonate, Putrescine N-methyltransferase, Tropane alkaloids.

INTRODUCTION

Tropane alkaloids (TA) hyoscyamine and scopolamine are anticholinergic agents acting on the parasympathetic nervous system [1]. Solanaceae genera such as Atropa, Datura, Duboisia, Hyoscyamus and Scopolia usually have a high amount of these alkaloids. In most cases, hyoscyamine is the major alkaloid. However, there is a higher industrial demand for scopolamine which is the more valued of these two alkaloids. Accordingly, there is increasing interest in obtaining cultures with enhanced content of scopolamine [2].

The first step taken in the biosynthesis of these alkaloids is that the N-methylation of putrescine has been catalyzed by putrescine N-methyl transferase (PMT) [3]. Scopolamine, which is the 6, 7-β-epoxide of hyoscyamine, is formed from hyoscyamine by means of 6β-hydroxyhyoscyamine (Fig. 1). Hyoscyamine 6β-hydroxylase (H6H), encoded by the gene h6h, catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine as well as the epoxidation of 6β-hydroxyhyoscyamine to scopolamine [4]. It has been demonstrated that when a sufficient amount of the H6H enzyme is present, scopolamine is accumulated, but when the supply of hyoscyamine is enhanced by feeding, the amount of H6H is limiting [5].

There has been a long-standing interest in raising the tropane alkaloids production. On the other hand, inducibility of the tropane alkaloids biosynthetic pathway is not yet understood fully. To overcome the present limitations, one approach is elicitation. An elicitor is a compound that activates specific genes through an unknown mechanism [6], resulting in the synthesis of almost all chemical classes of secondary plant metabolites [7]. Methyl jasmonate (MJ) as a signaling molecule is considerable for inducing the expression of key enzymes and biosynthetic pathway of tropane alkaloids in Scopolia parviflora [1]. Therefore, MJ induces particular enzymes to catalyze biochemical reactions to form defense compounds of low molecular weight in plants [8, 9]. Three genes for Fig(1) Biosynthetic pathway of nicotine and tropane alkaloids [28]. ArgDC, arginine decarboxylase; OrnDC, ornithine decarboxylase; PMT, putrescine N-methyltransferase; DAO, diamine oxidase; TR, tropinone reductase; H6H, hyoscyamine 6β-hydroxylase.
PMT (pmt1, pmt2 and pmt3) were isolated from Nicotiana sylvestris which could confer jasmonate-responsive expression [10]. Like many genes of plant secondary metabolism, pmt genes are expressed in an organ-specific pattern [11]. Two identified isozymes PMT1 and PMT2 with accession numbers Q8S7W6 and Q8XJ42 have been reported for A. belladonna [12]. Hyoscyamine and scopolamine in A. belladonna are found in roots and aerial parts. It has been reported that these two tropane alkaloids mostly synthesize in young root cells and translocate to aerial parts of the plant.

To our knowledge, there has not been any report so far on the methyl jasmonate elicitation of gene expression and tropane alkaloid production in different organs of Atropa belladonna. For this reason, in the present work, we investigated the effect of methyl jasmonate on the tropane alkaloids production in root and shoot of micropropagated plantlets and in Agrobacterium rhizogenes transformed hairy roots of A. belladonna while comparing the alkaloids content with the expression levels of pmt1, pmt2 and h6h.

RESULTS AND DISCUSSIONS

MJ effects on growth and tropane alkaloids production in hairy roots
The growth index of hairy root cultures treated by 0.02 and 0.04 mM of MJ significantly declined compared to the control (Fig. 2). However, the 0.01mM of MJ treatment did not have a negative effect on this index. MJ as a signalling molecule has some inhibitory effects on the growth and a variety of other activities in plants [13]. MJ concentration is important for its inhibitory effects in some species. Kang et al. [1] reported that the MJ treatment of 0.01 and 0.1 mM did not have an inhibitory effect on root growth index in Scopolia parvifolia while it has been reported that MJ concentration above 0.01 mM inhibited root growth in some species [14]. These observations suggest MJ specific signalling effect on hairy root growth.

![Fig(2) The growth index of hairy root cultures treated for two weeks by various concentrations of MJ (0, 0.01, 0.02 and 0.04 mM).](image)

When the plantlets were treated by 0.01 mM of MJ, the shoot and root DW decreased to 53% and 60% respectively compared to the controls that were in agreement with those previously reported by Kang et al. [1], who found that Scopolia parviflora shoot and root biomass decrease in the presence of MJ. Although hyoscyamine content in plantlet roots remained unchanged after being treated by 0.01 mM of MJ, it increased meaningfully in shoots (3.32 mg g⁻¹ DW) compared to the control (0.01 mg g⁻¹ DW). MJ treatment of 0.01 mM enhanced scopolamine content to 0.43 mg g⁻¹ DW and 0.5 mg g⁻¹ DW in roots and shoots respectively compared to the controls that were 0.01 and 0.02 mg g⁻¹ DW in that order (Fig. 6).

![Fig(4) Roots and shoots growth of in vitro-propagated A. belladonna plantlet after four weeks of treatment with 0 (control), 0.01, 0.04, 0.1 and 1 mM of MJ.](image)

Fig(3) The effects of various concentrations of MJ on hyoscyamine and scopolamine production in hairy root cultures. All the values are the means of three biological replicates. Common letter in each group indicates no significant differences (P < 0.05).

Effects on growth and production of tropane alkaloids in plantlets
Fig. 4 shows A. belladonna plantlets being treated with 0, 0.01, 0.1 and 1 mM of MJ for four weeks. The plantlets did not grow in 0.1 and 1mM of MJ possibly due to its toxicity. Thus, MJ effects on the growth of plantlets and the production of TA were examined at 0 and 0.01mM concentrations. The length of shoots and roots significantly declined at 0.01 mM of MJ compared to the control (P < 0.05) (Fig. 5).

![Fig(3) The effects of various concentrations of MJ on hyoscyamine and scopolamine production in hairy root cultures.](image)
In this study, MJ induced a significant increase in the hyoscyamine accumulation at 0.01 mM in shoots but not in the corresponding roots. The result was consistent with the observations of Zabetakis et al. [15]. Based on a study in *Hyoscyamus muticus* MJ induced the accumulation of methylputrescine in root cultures and yielded only a modest increase in tropane alkaloid levels [16].

**Expression pattern of pmt and h6h in MJ-treated hairy roots**

The results show that h6h expression levels in *A. belladonna* hairy roots declined by increasing the concentration of MJ and this lower expression level corresponds to the lower accumulation of scopolamine under MJ treatment (Fig. 7). Transcript levels of pmt1 had no significant difference from the control under various concentrations of MJ (Fig. 7) whereas the expression of pmt2 rose by increasing MJ concentration. The pmt2 expression profiles were similar to the pattern of hyoscyamine production. Based on the results, the stimulatory effects of MJ on hyoscyamine production are at least partly due to the activation of the PMT2 enzyme by the induction of its gene expression.

It has been reported that PMT enzyme is very sensitive to the environment where the roots are growing [19]. Moreover, Biondi et al. [20] have documented a considerable increase of PMT activity as well as an increase in polyamine contents and tropane alkaloids after methyl jasmonate treatment in transformed roots of *Hyoscyamus muticus*. However, it has been shown that overexpression of pmt in *A. belladonna* does not affect tropane alkaloid levels either in transgenic plants or in hairy roots [21]. Both hyoscyamine and scopolamine production improved in hairy root cultures of *Datura metel*, whereas in *H. muticus* only hyoscyamine contents increased by pmt gene overexpression [22].

In *A. belladonna* MJ induced pmt gene expression and lead to hyoscyamine accumulation, indicated that the same biosynthetic pathway in the related plant species can be differently regulated.
h6h, pmt1 and pmt2 genes in roots and shoots of the plantlets. As indicated, MJ increased h6h expression in roots but not in the shoots. The expression profiles of h6h were identical to the pattern of root scopolamine production. Recently h6h expression has been reported in aerial parts of some Solanaceae plants [23, 24]. In our study, the h6h transcript was clearly presented in both roots and shoots. This result is similar to that of Kai et al. [25] who displayed h6h activity in roots, stems and leaves from Anisodus acutangulus. However, the result is not similar to earlier findings that emphasize root-specific expression of h6h gene [11].

Suzuki et al. [11] reported that Abpmt1 mRNA was much more abundant than Abpmt2 in the roots of A. belladonna. However, Shoji et al. [10] reported that none of the pmt genes was expressed in shoots of Nicotiana sylvestris. Our results also showed no pmt1 and pmt2 transcripts in shoots of A. belladonna plantlets whereas pmt2, but not pmt1, transcripts were present in the roots, and these increased markedly in the presence of 0.01 mM MJ. The fact that MJ increased the induction of pmt2 in the roots of A. belladonna indicated that the hyoscymine production occurred in roots. On the other hand, this result indicates that higher accumulation of hyoscymine in the shoots of MJ treated plantlets is due to enhanced production in root and translocation from there to the aerial parts of the plantlets. However, our observation considered pmt2 more responsible than pmt1 for hyoscymine production in A. belladonna plantlets.

**MATERIALS AND METHODS**

**Plantlet preparation and elicitation**

The seeds of A. belladonna were collected from Sari-Garmestan of Mazandaran province in the North of Iran (N: 53°, 9'; E: 36°, 140'). The seeds were dried at room temperature, surface sterilized and germinated on MS [26] medium with a 16 h photoperiod. The obtained plantlets were used for explant (a node with a bud) preparation. The explants were cultured in MS media containing 0.2 mg/L IAA supplemented by the sterilized methyl jasmonate in final concentrations 0, 0.01, 0.1 and 1 mM with 16 h photoperiod. The buds were grown and the produced shoots were rooted. Four plantlets were used in three replications for each treatment. After 4 weeks shoots and roots of the plantlets were separated at 4 °C and their fresh weights and lengths were determined. Next, they were frozen in the liquid nitrogen and then kept at −80 °C.

**Hairy root preparation and elicitation**

The A. belladonna hairy root culture used in this study was derived after inoculation of leaf explants with a Ri T-DNA bearing Agrobacterium rhizogenes (R15834), as described previously [27]. The roots were maintained on hormone-free MS medium in 100 ml flasks and subcultured in the medium every 15-20 days and incubated at 24 ± 2 °C, in shakers at 100 rpm. Hairy roots of approximately 1.5 g fresh weight (FW) were inoculated in 20 ml of free hormone MS medium, adjusted to pH 5.8 before autoclaving (at 121 °C for 15 min) and were placed in 100 ml flasks. Sterilized MJ with filtration were added at concentrations of 0.01, 0.02, and 0.04 mM into the medium. The cultures were incubated as described above for 2 weeks. The control is referred to as non-treated. Finally, the hairy roots were separated from the medium and then blotted and weighed for FW. The samples of the root were placed at 60 °C for 48 hours and then waited for dry weight (DW). The growth was represented by a growth index (GI) which was calculated using the following formula: GI=the harvested fresh weight (g)/the inoculated fresh weight (g).

**Extraction and analysis of tropane alkaloids (TA)**

The samples of roots, shoots and hairy roots were prepared by extracting 1.5 g (FW) of them with 30 ml of 95% ethanol for 14 h in a reflux system. The extracts were centrifuged, and then dried using a rotary vacuum evaporator. The residues were dissolved in 5% (v/v) sulphuric acid and washed with diethylether (1:1 v/v). The produced aqueous phases were collected and the pH values were adjusted to 10 with NaOH. The alkaloids were extracted by three volumes (30, 40 and 40 ml) of chloroform and again evaporated. The residues were dissolved in methanol (HPLC grade) and analysed by HPLC for determination of hyoscyamine and scopolamine contents. A filtered sample was transferred to the HPLC employing a HPLC operating system (Gilson, France) equipped with an ODS-3 (4.6 mm i.d. × 25 cm) column filled with perfectsil target (5µm) and a UV detector (Knauer, UV 3000) operating at a wavelength of 260 nm. The isocratic mobile phase was a mixture of distilled water 83%, methanol 15%, acetic acid 1.5% and triethylamine 0.5% and the flow rate was 1.1 ml min⁻¹. The column temperature was 25 °C and 20 mL of the extracts was injected each time. For quantitative analysis, the calibration curve was drawn using scopolamine hydrobromide and hyoscymine sulfate. The alkaloids were quantified by comparing the retention times with data obtained from the standards and co-chromatograms of the standards and samples. Three replications were carried out for each treatment and the averages were expressed as mg g⁻¹ DW of plant tissues.

**Total cellular RNA extraction**

Total RNA was extracted from all samples using RNX-plus kit (Cinagen) according to manufacturers’ instruction. The RNA samples were quantified by measuring the absorbance at 260 nm and the purities were assessed by 260/280 nm ratio and electrophoresis on 1% agarose.
cDNA library synthesis and RT-PCR

Primer pairs were designed in order to detect the mRNAs encoding hyoscyamine 6β-hydroxylase (H6H) and Putrescine N-methyltransferase (PMT1, PMT2 isozymes) using Gene runner and Oligo 5 softwares. All the primers were specific for the gene sequence and were designed against the A. belladonna h6h sequence under the accession number ABO17154. The potato tubulin gene-specific primers (RAc1, accession number AB047313) were used as an internal control in semi-quantitative assays (Table. 1).

cDNA was generated by mixing 6 µl of total RNA extract in 6 µl DEPC water plus 1µl Oligo dt (0.5µg/µL) and 0.5 µl ribonuclease inhibitor (40 U µL⁻¹) in a ban marry at 72° C for 10 min. Then, 4 µl RT- PCR buffer (5x), 1µl dNTP (10 mM), 0.5 µl ribonuclease inhibitor (40U µL⁻¹) and 1 µl M-MuLV reverse transcriptase (200 U µL⁻¹) were added and incubated at 42°C for 2 hours. For semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), potato tubulin gene was used as internal control. PCR was carried out using PCR master kit (Cinnagen). The amplification reaction mixture consisted of 8 µl deionized water, 10 µl master mix, sense and antisense primers at 0.8 mmol L⁻¹ in a final volume of 20 µl. For all PCR programs, the reaction mixtures were initially denatured at 94 °C for 2 min, followed by 32 cycles (Table. 1) of 93 °C for 40s, 52–58 °C for 45s and 72 °C for 1min, and ended with 7 min of extension at 72 °C. PCR products were separated by electrophoresis on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide.

Statistical analysis

All the experiments were carried out in triplicate. The data were expressed as means±SD. The analysis of variance and the Duncan’s test (P<0.05) of mean comparison were performed using the MSTATC program ver. 1.4.

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References


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