

Original Article

Expression Pattern of *pmt*, *erf1* and *jap1* Genes in *Nicotiana benthamiana* and *Atropa belladonna* Plants under UV Radiation, Wounding and Methyl Jasmonate Treatments

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Abstract

The Solanaceae plants produce a variety of interesting biologically active products including the steroid alkaloids solanidine, nicotine and tropane alkaloids. Putrescine N-methyltransferase (PMT) is an enzyme that catalyses S-adenosylmethionine-dependent methylation of putrescine in one of the primary steps of nicotine and tropane alkaloids biosynthesis pathway. Two tobacco members of the AP2/ERF-domain transcription factors family called NtORC1 and NtJAP1 were shown to up regulate the activity of the NtPMT promoter in *Nicotiana tabacum* L. under environmental stresses. In this study, semiquantitative RT-PCR analysis showed that expression patterns of *PMT*, *ERF1* and *JAP1* genes in shoots and roots of *Nicotiana benthamiana* Domínguez and *Atropa belladonna* L. were examined under methyl jasmonate, UV radiation and wounding treatments. Plants were harvested half an hour after each treatment. The expression pattern of examined genes showed differences between plant tissues under different treatments.

Key words: *Nicotiana benthamiana*, *Atropa belladonna*, Alkaloid biosynthesis, PMT, NtORC1, NtJAP1, NbERF1

Abbreviations: PMT, Putrescine N-methyltransferase, AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR, UV, ultraviolet, MJ, Methyl Jasmonate, JA, Jasmonic Acid

Introduction

Plants of Solanaceae family produce some of interesting biologically active steroid alkaloids including solanidine, nicotine and tropane alkaloids [1-2]. Alkaloids are particularly important in medicine due to their high biological activities[3-4]. The use of plants as medicines has a long history in the treatment of various diseases [5-6]. Tropane alkaloids, which have been used for a long time by ancient people, have several synthetic analogs. Plant roots produce a remarkable variety of secondary metabolites, such as alkaloids, flavonoids and terpenoids. Formation of pyrrolidine ring is one of the early steps leading to biosynthesis of both nicotine and tropane alkaloids.

In this phenomenon, pyrrolidine derives from putrescine by the action of an S-adenosyl-L-methionine-dependent putrescine-N-methyltransferase (PMT) [2,7]. The activity of PMT enzyme has primarily been detected in roots of some tropane alkaloid-producing plants such as *Atropa belladonna* L., *Hyoscyamus niger* L. and *Datura stramonium* L., and this has been confirmed by RNA gel blot analyses for *A. belladonna* [2]. Secondary metabolites that are produced by plants function for defending plants against pathogens and herbivores, and have diverse physiological roles, such as attracting pollinators and screening UV light [8-10]. The biosynthesis of these products because of their defensive activities is controlled at transcription stage in response to biotic and abiotic

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stresses in a cell-specific manner. In tobacco plants, two transcriptional factors including NtORC1 and NtJAP1 (AP2/ ERF-like TFs), have been shown to activate the *PMT* promoter. The regulation of gene expression by jasmonates has been recently reviewed and 82% amino acid identity was shown between the AP2/ERF-like protein NbERF1 and tobacco NtORC1 [9].

In this study, the expression patterns of *PMT*, *ERF1* and *JAP1* genes in shoots and roots of *Nicotiana benthamiana* L. and *A. belladonna* were examined under methyl jasmonate, UV radiation and wounding treatments.

Material and Methods

Plant materials and treatments

A. belladonna seeds were collected from Noushahr of Mazandaran province in the North of Iran (N:51°,30'; E:36°,39') and *N. benthamiana* seeds were bought from Biotechnology Research Institute of Isfahan. Seeds were germinated in pots containing Tref Peat Moss at 23 °C under 16 h photoperiod. After three mount of germination treatments were performed. Plant leaves were scratched by scalpel for wound treatment. In UV radiation treatment, plants were exposed to UV radiation for half an hour and methyl jasmonate (100mM) was sprayed on leaves. Gene expression patterns were measured in shoots and roots of treated plantlets.

Plant harvesting

Plants were harvested half an hour after each treatment. The harvested shoots and roots of were separately frozen in liquid nitrogen and then transferred to -80 °C refrigerator.

Total cellular RNA extraction

Plant tissues (80mg) were excised and pulverized in liquid nitrogen with mortar and pestle. Total cellular RNA was extracted using BIOZOL Total RNA Extraction Kit.

cDNA library synthesis and RT-PCR

In order to investigate the expression patterns of *PMT*, *ERF1* and *JAP1* in shoots and roots of *N. benthamiana* and *A. belladonna* plants, semi-quantitative RT-PCR was carried out. First-strand cDNA was synthesized from five µg of total RNA using an oligo-d (T) primer. The oligonucleotide primers used for amplification of the *PMT* gene were F: 5'-CGTTGAATTCACAGCTACTGGGAAGCTCCA

-3' (position 333–363) and R: 5'-AAATGGATCCGAGGACGAGGGTTACACGA-3' (position 734–763) according to the sequence of the *PMT* gene from *N. benthamiana* (GenBank ID: EU165356.1). The oligonucleotide primers used for amplification of the *ERF1* gene were F: 5'-CGGAATTCTGCCATGAGTTCAAGTAACG-3' (position 174–195) and R: 5'-CGGGATCCGAAAATTGAGCCGAGCTCTCG-3' (position 534–555) according to the sequence of the *ERF1* gene from *N. benthamiana* (GenBank ID: CQ808982.1). The oligonucleotide primers used for amplification of the *JAP1* gene were F: 5'-CGGAATTCTGGAATTACCGGAGTTGTGG-3' (position 273–294) and R: 5'-TTCTCGAGGACGATGAAGAAGAAGAACATAC TCC-3' (position 596–619) according to the sequence of the *JAP1* gene from *N. tabacum* (GenBank ID: CQ808845.1). Meanwhile the RT-PCR reaction for the house-keeping gene (tubulin gene) using specific primers (F:5'-GGGGCGTAGGAGGAAAGCA-3' and R:5'-GCTTCACAACAACTTCTTCAG -3') was designed according to the conserved regions of tubulin genes. The housekeeping gene was used as an internal control to estimate whether equal amounts of RNA were used among samples. The products from PCR were analyzed on 1% agarose/TBE gel where the expected size of the amplified *PMT*, *ERF1*, *JAP1* and tubulin fragments were 400, 380, 350 and 700 bp respectively.

Statistical analyzes

One-way ANOVA method and Duncan tests were used. Semi-quantitative Measurement of gene expression were examined by Total lab software.

Results

Expression of *PMT* gene

Expression of *PMT* gene in *N. benthamiana*

Results showed that the expression level of *PMT* gene in roots of *N. benthamiana* control plants was about 5 times higher than that of the leaves. Under methyl jasmonate, UV radiation and wounding treatments, the expression level of *PMT* gene in leaves was not altered and in roots, only under methyl jasmonate treatment, its expression level reduced significantly. The expression level of the *PMT* gene was not significantly modified under wounding and UV radiation treatments (Fig. 1).

Expression of *PMT* gene in *A. belladonna*

The results showed that the expression level of *PMT* gene in leaves was very low and methyl jasmonate, UV radiation and wounding treatments had no significant effects on its expression level. In roots, although the expression levels under the methyl jasmonate and UV radiation decreased. No significant differences were observed in the expression level of *PMT* gene between wounding treatment and control group (Fig. 2).

Expression of *ERF1* gene

Expression of *ERF1* in *N. benthamiana*

The expression level of *ERF1* gene in leaves of control plants was about 3 times higher than that of the roots. The results showed that UV radiation and wounding treatments had no significant effect on *ERF1* expression levels in leaves of *N. benthamiana* although its expression decreases under methyl jasmonate treatment. In roots, however the expression level of *ERF1* gene had no significant difference with control under UV radiation, wounding and methyl jasmonate treatments (Fig. 3).

Expression of *ERF1* in *A. belladonna*

Results showed a similar expression pattern changes in leaves and roots of *A. belladonna* plants under different employed treatments. The expression level of *ERF1* gene in both tissues under the wound and UV radiation treatments did not significantly changed compared to control, but under the methyl jasmonate treatment increased up to three times (Fig. 4)

Expression of *JAP1* gene

Expression of *JAP1* gene in *N. benthamiana*

The results showed that the expression level of *JAP1* gene is very low in shoots and roots of *N. benthamiana* control plants. In shoots, all employed treatments increased the expression level of *JAP1* gene compared to the control. In roots, however the wounding treatment had no significant effect on *JAP1* gene expression but the two other treatments increased its expression compared to the control (Fig. 5).

Expression of *JAP1* gene in *A. belladonna*

Based on our results, no expression of *JAP1* gene in leaves and roots of *A. belladonna* plants were observed in control group and under different employed treatments.

Discussion

Pyrrolinium alkaloids, including nicotine, are synthesized in tobacco root from arginine and/or ornithine via putrescine. Putrescine N-methyltransferase (*PMT*; EC 2.1.1.53) catalyzes the S-adenosyl methionine-dependent N-methylation of putrescine and is the first committed step in the biosynthetic pathways that lead to both nicotine and tropane alkaloids [11].

Two AP2/ERF-like transcription factors in tobacco including NtORC1 and NtJAP1, have been shown that activate the *pmt* promoter. The AP2/ERF-like protein NbERF1 shows 82% amino acid identity with tobacco NtORC1 [9]. Tobacco *pmt* contains functional GCC-box and G-box sequences in the proximal promoter region, which are trans-activated by ectopic over-expression of NIC2-locus ERFs (ERF189 and ERF221/ORC1) or MYC2-clade bHLHs (NtMYC2b and NbbHLH1) in cultured tobacco cells or protoplasts [12-14]. Shoji and Hashimoto (2011) indicated that NtMYC2 controls nicotine biosynthetic genes in two combinatorial ways including directly binding the G-box in the target promoters or up-regulating the NIC2-locus ERF genes.

Our results showed that the *pmt* expression levels in roots of both *N. benthamiana* and *A. belladonna* are higher than the leaves. Higher levels of *pmt* expression in roots is consisted in previous studies [15-16].

In this study, expression level of *erf1* gene in leaves of *N. benthamiana* was about 3 times higher than the roots but *erf1* expression in roots of *A. belladonna* was about 2 times higher than the leaves. Kathleen De Boer *et al* (2011) showed that in mock conditions, *ORC1* transcript levels in the leaves of tobacco plants were lower than the roots, which is in agreement with our observation in *A. belladonna*. Elicitation is considered as an important strategy to improve *in vitro* production of secondary metabolites. In cell cultures, biotic and abiotic elicitors have effectively stimulated the production of plant secondary metabolites [17]. It is known that elicitors bind to their receptor proteins that generate signals, which are transmitted to the sites of gene expression via different components, such as Ca^{2+} /ion fluxes, medium alkalinization and cytoplasmic acidification, oxidative burst, jasmonate and nitric oxide *etc.* [18].

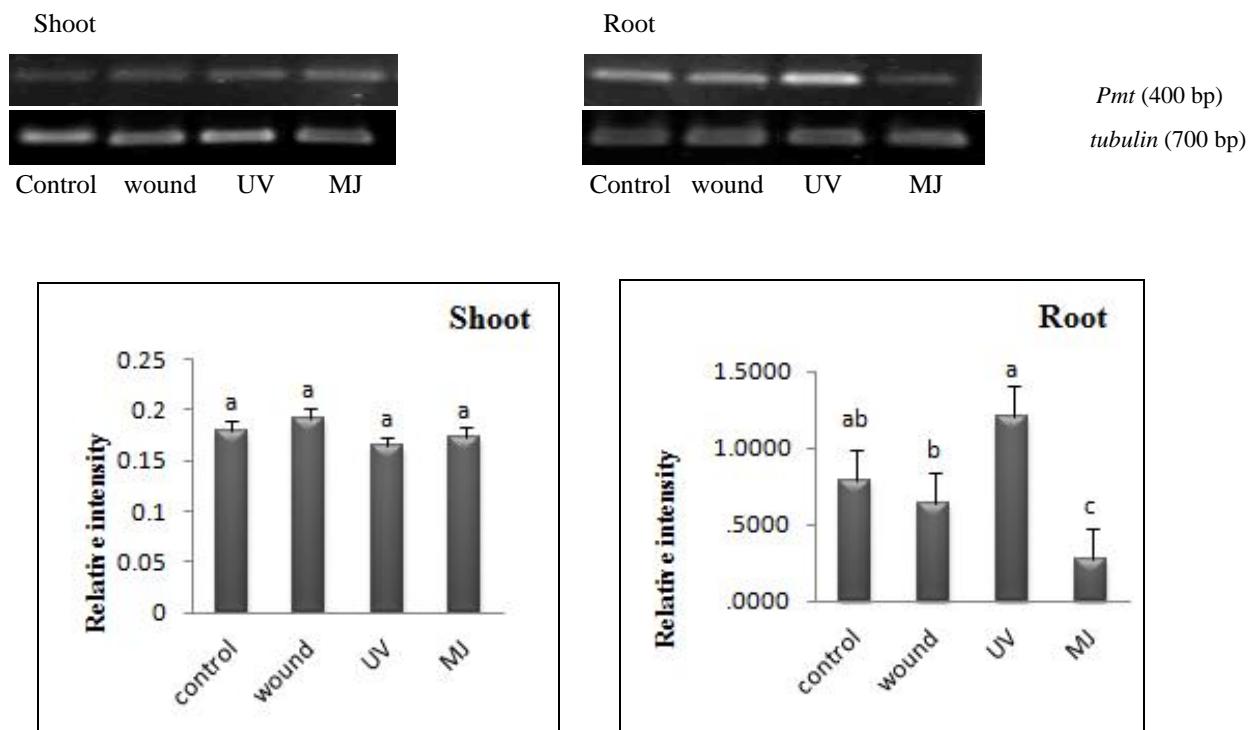


Fig. 1 Expression levels of *pmt* gene under different employed treatments in shoots and roots of *N. benthamiana*. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P < 0.05$). Tubulin: internal housekeeping gene

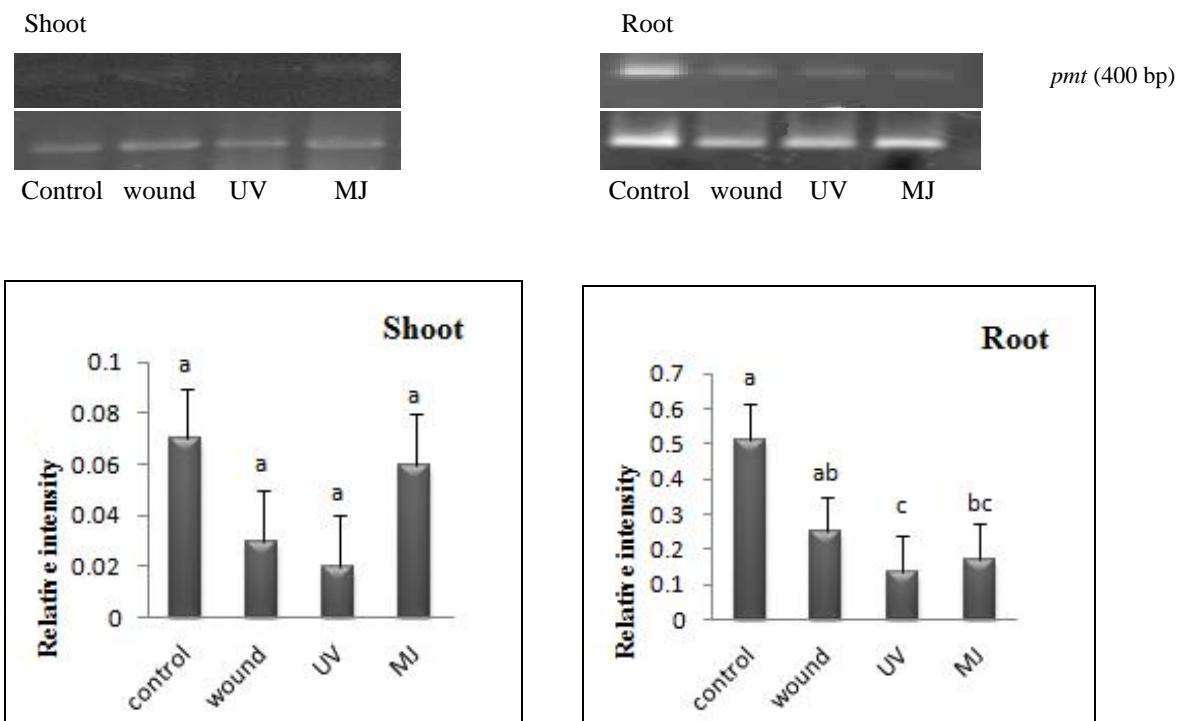


Fig. 2 Expression levels of *pmt* gene under different employed treatments in shoots and roots of *A. belladonna*. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P < 0.05$). Tubulin: internal housekeeping gene

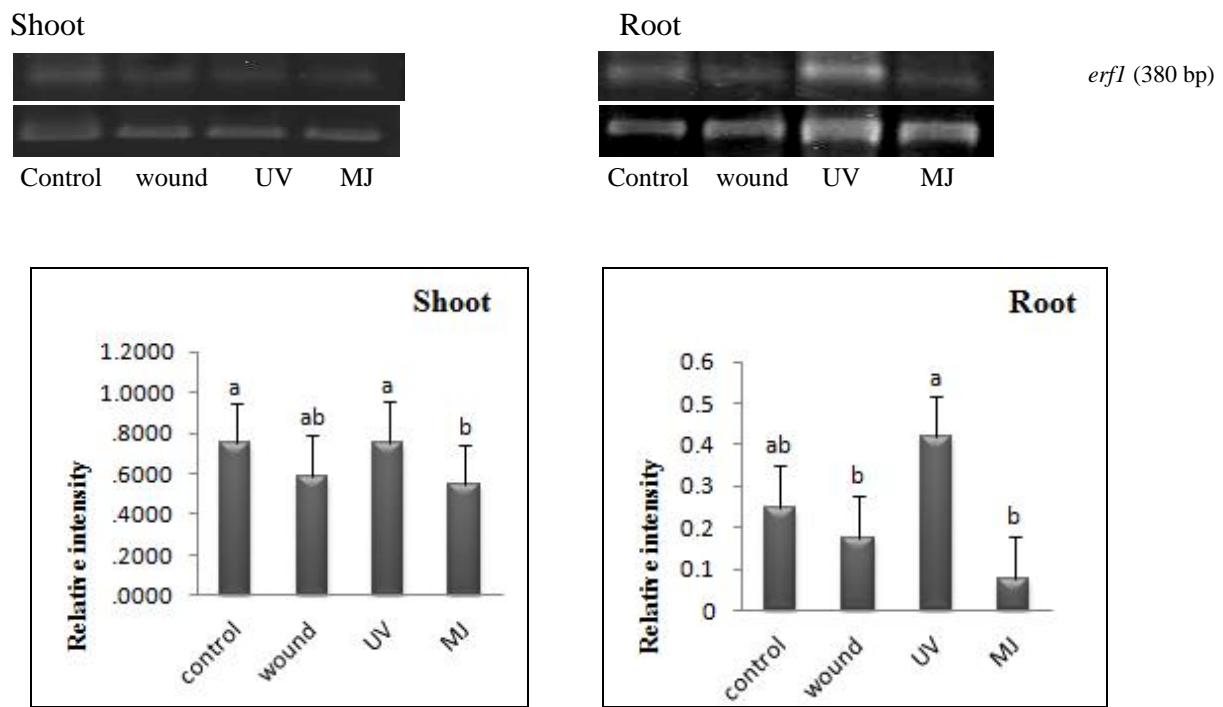


Fig. 3 Expression levels of *erf1* gene under different employed treatments in shoots and roots of *N. benthamiana*. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P < 0.05$). Tubulin; internal housekeeping gene

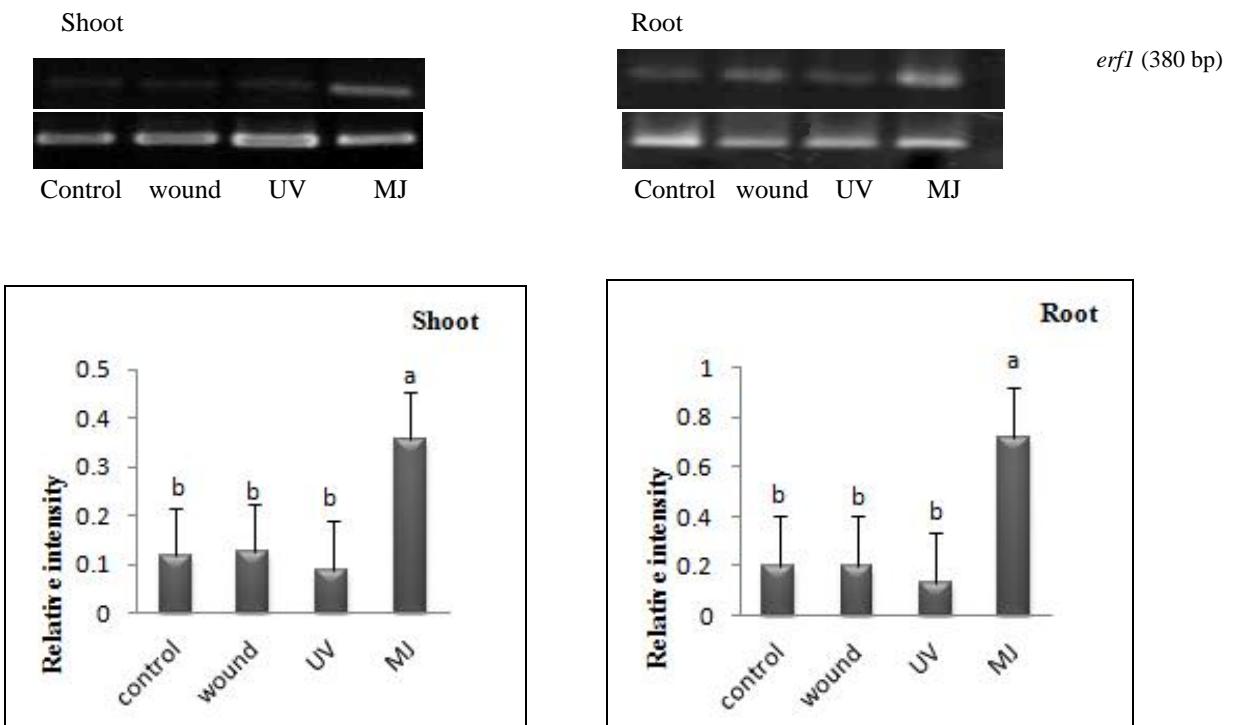


Fig. 4 Expression levels of *erf1* gene under different employed treatments in shoots and roots of *A. belladonna*. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P < 0.05$). Tubulin; internal housekeeping gene

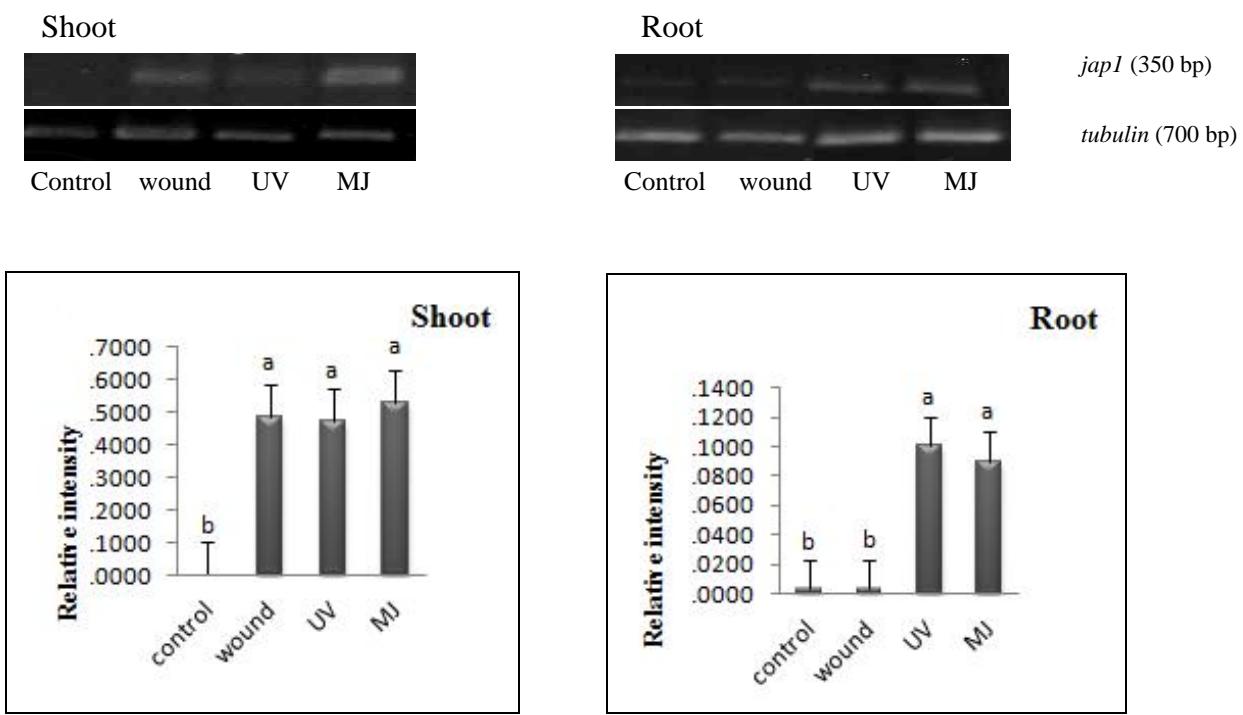


Fig. 5 Expression levels of *jap1* gene under different employed treatments in shoots and roots of *N. benthamiana*. All the values are the means of three biological replicates. Values without a common letter are statistically different ($P < 0.05$). Tubulin; internal housekeeping gene

Expression of *pmt*, *erf1* and *jap1* genes under wounding treatment

Only the *jap1* was more expressed under wounding treatment in *N. benthamiana* leaves. In the other cases, wounding treatment had no effect on gene expression. Sachan & Falcone (2002) indicated the low *pmt* expression levels in leaves of the *N. tabacum*, which was abruptly upregulated by wounding. They also indicated that maximum expression of *pmt* under wounding treatment was seen at the 30 min and 1 h time points, which then sharply decreased 2 h after wounding. Different responses obtained for the *pmt* expression levels in *Arabidopsis* and tobacco by Sachan & Falcone (2002), had lead them to suggest that the *pmt*-transcriptional response to wounding may be a specific phenomenon in tobacco leaves [11]. In this study, the treated plants harvested 30 min after wounding and no differences were seen in expression levels of *pmt* and *erf1* genes.

We found difference between behavior of *jap1* with the two other genes against wounding treatment and it could be perhaps because of existence of different pathways in response to wounding stress.

Expression of *pmt*, *erf1* and *jap1* genes under by UV treatment

Jansen *et al* (2008) indicated that Production of nicotine increased in tobacco callus exposed to UV-B [19]. Receptor proteins that bind to elicitors generate signals that are transmitted to the sites of gene expression via different components, such as Ca^{2+} /ion fluxes, medium alkalinization and cytoplasmic acidification, oxidative burst, jasmonate, nitric oxide etc. [18].

The results obtained by Susheel Kumar Raina *et al* (2012) indicated that Methyl jasmonate, UV radiation and wounding treatments on *Catharanthus roseus* leaves resulted in transcript accumulation of CrMPK3, a mitogen activated protein kinase. These treatments also increased activation of MAPK in *C. roseus* leaves. They also showed that amino acid sequence of CrMPK3 showed 86% identity with WIPK from *N. benthamiana* [20]. Here we observed that the expression levels of *pmt* and *erf1* genes in *N. benthamiana* roots had no significant differences under UV treatment with control plants, but the level of *jap1* transcript was dramatically increased in shoots and roots of *N. benthamiana*. Our studies

have also shown that UV radiation treatment decreased *pmt* expression level in roots of *A. belladonna* and no significant changes observed in the level of *erf1* expression in shoots and roots of this plant. Based on the results, we suggest that probably there are different pathways for UV radiation signaling and these pathways are not identical in different plant species.

Expression of *pmt*, *erf1* and *jap1* genes under methyl jasmonate treatment

Based on results obtained by Tsubasa Shoji and Takashi Hashimoto (2011) it is proposed a model for jasmonate-inducible gene expression by two types of transcription factors in nicotine biosynthetic pathway. The G-box in the promoters of nicotine biosynthetic genes, such as *pmt*, in the ground state without the jasmonate signaling, is bound by the MYC2–JAZ complex, which recruits chromatin-remodeling factors to repress the MYC2-target genes actively. After the perceived the bioactive jasmonate, JA–Ile by the COI1–JAZ receptor complex, JAZ proteins are degraded. Releasing of an active NtMYC2 transcription factor increases the expression of *pmt* and other nicotine biosynthetic genes [14]. NtORC1 and NtJAP1 are two AP2/ ERF-like TFs in tobacco that activate the *pmt* promoter. [9]. The tobacco *pmt* promoter, contains the GCC- and G-boxes and a TA-rich region (TATTATAT) positioned between for jasmonate regulation [21]. This sequence also is reported in the *N. benthamiana* *pmt* promoter [14]. Based on the results obtained by Tsubasa Shoji and Takashi Hashimoto (2011) NtMYC2 controls nicotine biosynthetic genes by directly binding to the G-box in the target promoters such as *pmt* promoter, and by up regulating the NIC2-locus in *erf* genes.

We have not observed any changes in *pmt* expression levels in the leaves of *N. benthamiana* and *A. belladonna* under MJ treatment. Winz and Baldwin (2001) indicated that *pmt* expression in leaves of *N. tabacum* not affected by JA, which is in agreement with our results [22]. We observed that expression of *pmt* decreased in roots of both two plants whereas Suzuki *et al* (1999) indicated that unlike *N. tabacum*, the *pmt* expression in *A. belladonna* roots was not changed by methyl jasmonate treatment [11]. The mechanism of action of methyl jasmonate (100mM) in reducing *pmt* expression is unclear. It is suggested that in future studies, the effect of different concentrations of methyl jasmonate on expression of these genes be

examined to determine the concentration of methyl jasmonate that stimulates expression of these genes.

Here methyl jasmonate decreased *erf1* expression in shoots and roots of *N. benthamiana* and also *erf1* transcript accumulation dramatically increased in shoots and roots of *A. belladonna*. The expression of *jap1* in the shoots and roots of *N. benthamiana* was increased in response to MJ. This result is interesting because according to our results, no expression of *jap1* was observed in leaves and roots of *N. benthamiana* control groups and different treatments.

In addition, our results indicate that, compared to PMT and JAP1, closely relationship between PMT and ERF1. Previously De Sutter *et al* (2005) had shown that ORC1 and to a lesser extent JAP1, can promote induction of PMT in transfected tobacco BY-2 protoplasts [13]. In addition, a stimulatory effect was not detected between *jap1* and *jam* (The gene encoding the MAPKK protein) and suggesting a specificity of JAM1 for ORC1 and perhaps other NIC2 ERFs [13].

Finally, based on the results obtained in this study, while the performed treatments had different effects on two plant species, it seems that the UV radiation, methyl jasmonate and wounding treatments induce gene expression and production of secondary metabolites by different pathways.

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