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Effects of exogenous methyl jasmonate and 2-isopentenyladenine on artemisinin production and gene expression in *Artemisia annua*

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Abstract: Artemisinin, produced in very low amounts in *Artemisia annua* L. plants, is one of the most effective drugs in treating malaria. In this study, the effect of exogenous applications of methyl jasmonate (MeJA) and 2-isopentenyladenine (2-iP) in artemisinin production and the main genes of its biosynthesis pathway within a 1-week time period were investigated. Both MeJA and 2-iP increased artemisinin content, but no correlation was found between gene expression and its content. In plants treated with 2-iP, despite increased gene expression following elicitor application, downregulation of gene expression was also observed, which might be attributed to the negative effects of rising artemisinin levels in gene expression. In plants treated with MeJA, the steady increase in artemisinin content was not explained by the few changes observed in gene expression, suggesting that some other mechanism may increase artemisinin. Our results indicate that the negative feedback mechanism of artemisinin is likely an obstacle for the selection of biotechnological strategies to increase artemisinin content. This may be overcome by studying the role of trichome in artemisinin production as a step toward achieving high yields of this valuable component.

Key words: *Artemisia*, artemisinin, gene expression, MeJA, 2-iP

1. Introduction

There are more than 225 million cases of malaria, which cause approximately 781,000 deaths each year (WHO Global Malaria Programme, 2010). Cinchona alkaloids and artemisinins are 2 plant medicines that have antimalarial activity and are used to treat severe malaria (Dobson, 1998). Artemisinin, one of the secondary metabolites, is a sesquiterpene lactone with an endoperoxide bridge in its structure that is essential for its activity against malaria (Cui & Su, 2009). Artemisinin production takes place in the secretory cells of *Artemisia annua* L. glandular trichomes and trichome density is highly associated with artemisinin yield in different plant tissues (Olsson et al., 2009; Olofsson et al., 2011). Artemisinin is currently considered the best therapeutic strategy against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum* Welch (Liu et al., 2006; Weathers et al., 2006). In 2004, the World Health Organization (WHO) recommended that artemisinin-based combination therapy (ACT) should be used to treat *P. falciparum*, and it has been adopted in most malaria-endemic countries (Hommel, 2008).

Because its chemical synthesis is complicated and economically unfeasible, *A. annua* is currently the only source of artemisinin (Abdin et al., 2003). Serious limitations for commercialisation of drugs based on artemisinin include a relatively low yield (0.01% to 0.8%) of artemisinin in *A. annua*. It also varies with environmental conditions, such as light and disease, and variety (Liu et al., 2006). Enhancing the production of artemisinin is highly desirable to increase drug availability at a suitable cost for the benefit of people in developing countries, who most often need treatment for malaria. Consequently, investigation of the artemisinin biosynthetic pathway is useful, particularly because it can provide an understanding of artemisinin production that can be applied to its synthesis by transgenic plants or genetically modified microbes. There have been many studies done to identify genes and enzymes involved in artemisinin biosynthesis (Figure 1). Studying the effects of exogenous factors such as phytohormones on secondary metabolite production and expression of main genes in its pathway can improve understanding of the biosynthetic pathway and its regulation.

Jasmonic acid (JA) and its methyl ester, MeJA, are well-known signalling molecules that play an important role in regulating the reprogramming of gene expression and in eliciting the biosynthesis of secondary metabolites in

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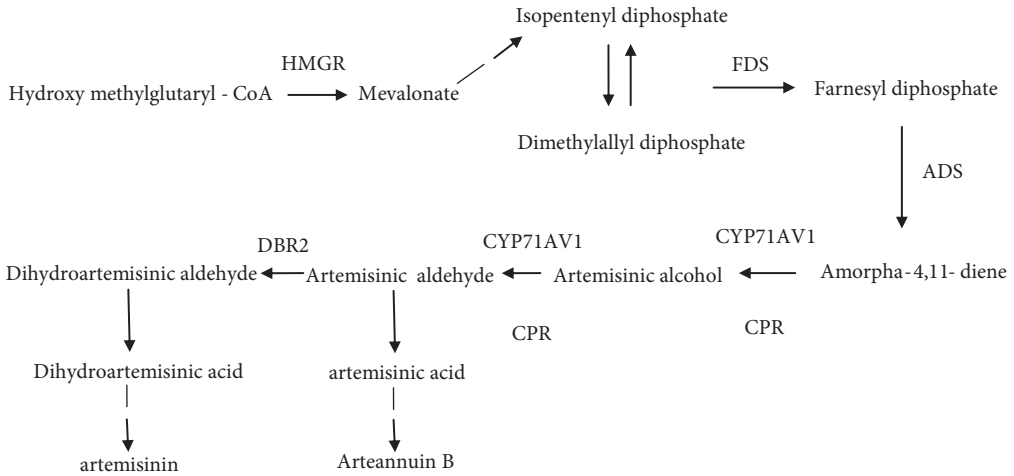

Figure 1. Biosynthesis pathway of artemisinin in *Artemisia annua*. HMGR, HMG-CoA reductase; FDS, farnesyl pyrophosphate synthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; DBR2, artemisinic aldehyde Delta11(13) reductase.

plant cells (Pauwels et al., 2008). There have been many successful efforts to enhance artemisinin production by the use of MeJA or JA, both in cell suspension and in the whole plant (Baldi and Dixit, 2008; Caretto et al., 2010; Wang et al., 2010; Maes et al., 2011; Wu et al., 2011). Some of these studies also investigated the influence of the treatments on the expression of biosynthetic genes. JA seems to increase the density and size of glandular trichomes on *A. annua* leaves and enhance artemisinin production (Liu et al., 2009; Maes et al., 2011).

Cytokinins are another group of plant hormones that play an important role in many physiological and developmental processes in the plant, such as the regulation of shoot and root growth, leaf senescence, chloroplast development, stress response, and pathogen resistance (Mok & Mok, 2001). A few studies have described the relationship between cytokinins and artemisinin, which found that an increase in endogenous and exogenous cytokinins increased artemisinin levels in *A. annua* transgenic plants and hairy roots, respectively (Geng et al., 2001; Weathers et al., 2005). Recent work, however, has reported that BAP merely stimulated the glandular trichome formation and did not induce artemisinin biosynthesis (Maes et al., 2011).

For a better understanding of how MeJA and 2-iP affect artemisinin production, we measured the effects of their exogenous applications to the time-course production of artemisinin and related gene transcripts in plants, with the aim of obtaining new insight for metabolic engineering.

2. Materials and methods

2.1. Plant growth and phytohormones treatment

Seeds of high artemisinin-yielding *A. annua* from Gonbad, Golestan Province, Iran, were received from the Iranian Biological Resource Centre and used in the experiments. The seeds were surface sterilised with 70% (v/v) ethanol for 5 min and washed 3 times with sterile distilled water. Seeds were then placed on wet Whatman paper in a petri dish in a growth chamber with a photoperiod of 16 h light/8 h dark and light of 3000 lx (fluorescent source) at 25 °C and 70% relative humidity, and were grown for 2 weeks. After germination, the plantlets were transplanted into individual plastic pots containing peat moss and sand at the ratio of 2:1 and grown under the same conditions. They were watered every day and fertilised with half-strength Hoagland solution once a week. Three-month-old uniform plants were selected for the experiments.

MeJA and 2-iP were purchased from Duchefa. In order to treat the plants, 300 µM MeJA in 0.8% ethanol (according to Wang et al., 2010) and 5 µM 2-iP in 0.5% NaOH (according to Weathers et al., 2005) were individually sprayed on the surface of the experimental set of plants, while the control plants were sprayed with only 0.8% ethanol and 0.5% NaOH, respectively. Sampling was carried out at 12, 24, 48, 72, and 168 h after treatment. For each sampling, 5 leaves under the third visible leaf from the apex were harvested and mixed, frozen in liquid nitrogen, and stored at –80 °C immediately for RNA analysis. For the quantification of artemisinin, plants were cut 3 cm above the soil level and dried in a forced-air oven at 45 °C for 48 h, according to the method of Pu et al. (2009).

2.2. Determination of artemisinin concentration

Artemisinin was measured by a procedure described by Ferreira and Gonzalez (2009), with some modifications. Approximately 50 mg of dry leaf powder were extracted with 20 mL of petroleum ether (50–70 °C) in an ultrasonic bath for 15 min. The supernatant was transferred to a 50-mL round bottom flask and dried using a rotary evaporator.

The residue was dissolved in 3 mL of acetonitrile, filtered through a nylon filter (0.20 μm), and then transferred to HPLC sampling vials. Artemisinin was separated using a Nucleosil 100 C18 column (125 mm × 4.0 mm, 5 mm), and detection was conducted at 210 nm. Acetonitrile and 0.1% ice acetate (40:60, v/v) were used as the mobile phase with a flow rate of 0.7 mL/min and column temperature of 25 °C. Artemisinin (purity > 97%) was provided by the Institute of Medicinal Plants, Iran. The artemisinin concentration was calculated from a 5-point standard curve varying from 0.2 to 1 mg/mL artemisinin with 50 μL of injection volume.

2.3. Real-time fluorescent quantitative amplification

For expression analysis of artemisinin biosynthetic genes, 100 mg of leaves was collected from treated *A. annua* and ground to a powder in liquid nitrogen, and total RNAs were isolated with the RNeasy Plant Mini Kit (QIAGEN). Their concentrations were determined spectrophotometrically, and the qualities were checked by agarose gel electrophoresis. Reverse transcription was performed using a First Strand cDNA Synthesis Kit (Fermentas) and 18S rRNA was the internal reference with primers generated for it according to the method of Zeng et al. (2008). The qRT-PCR primers for artemisinin biosynthetic genes, HMGR, FDS, ADS, CYP71AV1, CPR, and DBR2, were designed with primer3 and perlprimer software using the following criteria: melting temperature of 60 °C, PCR amplicon length from 150 to 200 bp, and primer sequence length from 19 to 20 nucleotides with guanine-cytosine contents from 50% to 60%. The primers that were designed for amplification of target genes from *A. annua* are listed in the Table. The quantitative assay based on SYBR Green was conducted by using an iQ5 (BIO-RAD) machine under the following conditions: 95 °C for 30 s followed by 35 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 30 s. Relative fold changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ comparative method (Livak & Schmittgen, 2001).

2.4. Statistical analysis

Experiments were performed at least in triplicate and fold change values were expressed as mean ± standard deviation (SD). Data were analysed statistically with a t-test. The statistically significant difference values ($P < 0.05$) are labelled with a single asterisk (*), and the statistically very significant difference values ($P < 0.01$) are labelled with double asterisks (**).

3. Results

3.1. Artemisinin production

Artemisinin was analysed at various intervals in plants treated with MeJA and 2-iP and, compared to untreated controls, artemisinin production significantly increased. MeJA increased artemisinin levels until 72 h after application, with increases of 63%, 68%, 165%, and 169% at 12, 24, 48, and 72 h posttreatment, respectively, compared to the controls. At 168 h, MeJA showed no difference in artemisinin production compared to the controls. Plants treated with 2-iP showed increased artemisinin levels only during the first 24 h after application, reaching an increase of 83% compared to the control, after which levels were no different from those of the controls (Figure 2).

3.2. Expression of main genes in artemisinin pathway

To determine if MeJA and 2-iP affect transcription of the main genes in artemisinin biosynthesis, we used qPCR to study the responses of the genes HMG-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FDS), amorpha-4,11-diene synthase (ADS), cytochrome P450 monooxygenase (CYP71AV1), cytochrome P450 reductase (CPR), and artemisinic aldehyde Delta11(13) reductase (DBR2). Plants treated with MeJA showed only slight changes in the transcription levels of these genes compared to the control. HMGR gene expression

Table. Primer sequences designed for real-time amplification of artemisinin biosynthetic genes of *Artemisia annua*.

Target gene	GenBank accession number	Primer sequences	Amplicon lengths (bp)
HMGR	AF142473.1	F 5′-TGCTGGTTCTCTTGGTGGAT-3′ R 5′-CTCCAACTGTGCCAACCTCT-3′	189
FDS	AF112881.1	F 5′-GAACTCGCCAATGAGGAACA-3′ R 5′-TTTCAGCACCGCTTGGACT-3′	200
ADS	DQ241826.1	F 5′-TGTCAATGAGGAGTATGCCC-3′ R 5′-GTCTCCCATACGTGTGAAGT-3′	183
CYP71AV1	DQ872632.1	F 5′-CTCCACTACCCTTGGTTCTG-3′ R 5′-TCGTATTCTGCACCCATGAC-3′	195
CPR	DQ984181.1	F 5′-GCTCGGAACAGCCATCTTAT-3′ R 5′-CCGAAGCCTTCTGAGTCATC-3′	175
DBR2	EU704257	F 5′-CCAATGGAAGTGAGGAGGAA-3′ R 5′-CAAGGTCAGGATTCGAGACA-3′	173

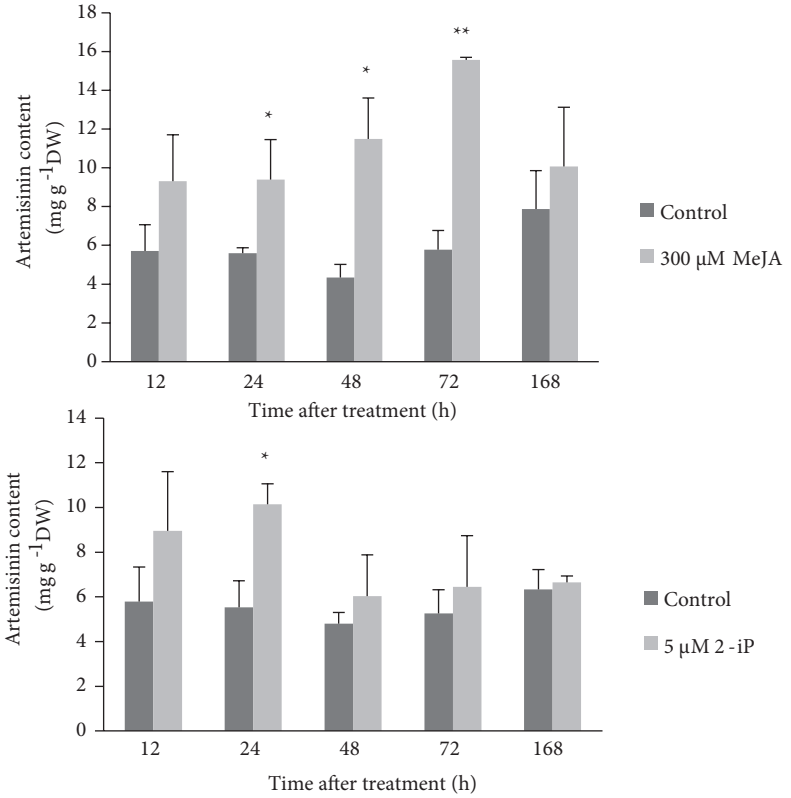

Figure 2. Time-course effects of MeJA and 2-iP on artemisinin production in *A. annua* plants. Values are mean ± SD. The results were analysed using t-test on the artemisinin contents in treated plants compared to the control. A single asterisk represents a significant difference at $0.01 < P < 0.05$ and double asterisks represent a highly significant difference at $P < 0.01$ between the control and treated plants.

decreased 1.5-fold at 24 h, and then increased 3-fold by the 48-h mark. It then decreased again 1.3-fold by 72 h after treatment. FDS gene expression increased 3-fold at 24 h after application, although by 48 h this dropped to an increase of only 1.5-fold over the controls. ADS transcript levels also decreased approximately 4.2-, 3.5-, and 1.7-fold, respectively, 12, 24, and 72 h after MeJA application. CYP71AV1 showed a decrease at 12 h, and CPR showed a slight increase at 24 and 72 h posttreatment. DBR2 had 2 decreases, 3- and 2-fold, at 12 and 72 h, respectively, and 2 increases of 2-fold at 48 and 168 h after treatment.

The transcript levels of genes in plants exposed to 2-iP were more variable. At 12 h, expression levels of HMGR, FDS, and DBR2 increased approximately 8-, 6.5-, and 5.5-fold relative to the control, but CYP71AV1 decreased 4-fold. At 24 h, transcript levels of ADS, CYP71AV1, and DBR2 decreased 4.5-, 6-, and 7-fold, respectively, and CPR increased 2-fold. At 48 h, HMGR, ADS, and CYP71AV1 decreased 1.5-, 7.5-, and 5-fold, respectively. In contrast, gene expression levels for CPR and DBR2 increased 1.7- and 4.5-fold, respectively. At 72 h all of the studied genes increased between 2- and 17-fold. At the final measurement at 168 h, ADS and DBR2 were downregulated 4- and 2.5-fold, respectively, and FDS was upregulated 3.5-fold (Figure 3).

4. Discussion

Enhancement of secondary metabolite production by biotic and abiotic elicitors has been reported in numerous plants (e.g., Bota and Deliu, 2012; Ch et al., 2012; Hao et al., 2012; Yamaner et al., 2013). JA and its analogue MeJA are small signalling molecules induced in response to wounding or pathogen attack in plants (Wasternack, 2007). They elicit secondary metabolites, including terpenoids, flavanoids, alkaloids, and phenylpropanoids (Tamogami et al., 1997; Brader et al., 2001; Farmer et al., 2003; Martin et al., 2003; Wang et al., 2008; Zayed & Wink, 2009). The current study showed that MeJA treatment increased artemisinin production in intact plants with few changes in the expression of the studied genes and no significant correlation was found between gene expression and artemisinin content. This suggested that MeJA increased in artemisinin production through another mechanism, in which artemisinin content continuously increases until 72

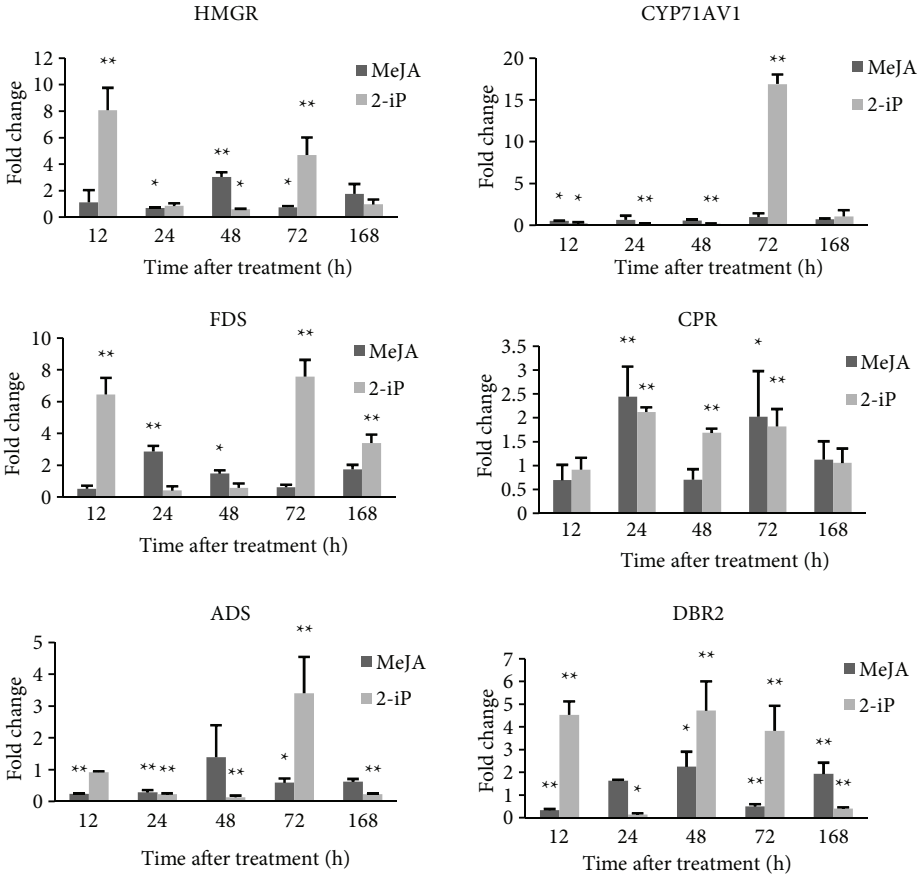

Figure 3. Changes in HMGR, FDS, ADS, CYP71AV1, CPR, and DBR2 genes in *A. annua* plants treated by MeJA and 2-iP. Data represent the means of $2^{-\Delta\Delta CT}$ ± SD. Statistical analyses using a t-test were carried out for significant differences between treated plants and the control. A single asterisk represents a significant difference at $0.01 < P < 0.05$ and double asterisks represent a highly significant difference at $P < 0.01$ between the control and treated plants.

h after application. Reactive oxygen species (ROS), which are known to be induced by the stress hormone MeJA (Zhang & Xing, 2008), is one way for such an increase to occur without substantial changes in gene expression. The final step of artemisinin biosynthesis, the transformation of dihydroartemisinic acid (DHAA) to artemisinin, involves a nonenzymatic photooxidative step to add the final 3 oxygen atoms to the molecule (Brown & Sy, 2004). This suggests that spontaneous transformation of DHAA to artemisinin may have occurred in a plant treated with MeJA. This is supported by new findings that $^1O_2$ might be involved in SA/MeJA-mediated signalling for artemisinin biogenesis in *A. annua* (Guo et al., 2010), and that ROS may play a role in artemisinin production in *A. annua* (Mannan et al., 2010). However, because the observed increases in artemisinin did not yield a corresponding increase in transcript levels, the MeJA effect may instead be on the glandular trichomes. Recent breeding work has shown a positive relationship between trichomes and artemisinin levels (Graham et al., 2010) and other studies suggest that the main effect of JA is on trichomes. This caused significant increases in nonglandular trichome production of leaves in *Arabidopsis* (Traw & Bergelson, 2003; Maes & Goossens, 2010) and glandular trichome density in *Lycopersicon esculentum* (Boughton et al., 2005). Recent studies have reported the same effect of JA on the stimulation of trichome formation and increases in its density and size on *A. annua* leaves, therefore increasing artemisinin production (Liu et al., 2009; Maes et al., 2011). It was suggested that enzymes of artemisinin biosynthesis are located in apical cells of glandular secretary trichomes of *A. annua* (Olsson et al., 2009), and that artemisinin accumulates in its trichome (Covello et al., 2007). Although Wang et al., (2010) observed no significant changes in glandular trichomes on the *Artemisia* leaves treated with MeJA, there is a need for further investigation.

The current study also showed that 2-iP, a cytokinin phytohormone, increased artemisinin production and its

biosynthetic genes were upregulated, yet no correlation was found between gene expression and artemisinin content. This lack of correlation along with downregulation of most of the genes that was observed at 24 or 48 h after treatment suggests the presence of another factor that may be affecting gene expression.

Artemisinin is phytotoxic even to *A. annua* (Duke et al., 1987), and evidence exists for a feedback mechanism. *A. annua* seedlings grown in increasing levels of artemisinin showed that there was end production feedback inhibition on ADS and CYP transcript levels (Arsenault et al., 2010). It was interesting that in the current study in plants treated by 2-iP, when artemisinin levels decreased to nearly the same level as the control at 72 h, no negative feedback mechanism was observed and the expression level of all genes increased.

Our results suggest that both 2-iP and MeJA increased artemisinin content, but the MeJA response was greater and more stable than the response to 2-iP. Due to the self-regulating mechanism that seems to limit excessive accumulation of artemisinin in *A. annua* (Arsenault et al., 2010), achieving high yields of artemisinin by metabolic engineering through overexpression of the main pathway genes should be viewed with caution. MeJA's effect on artemisinin production and few changes in gene expression suggest that this elicitor may increase artemisinin levels, not by affecting transcription, but by some other mechanism, possibly by affecting trichomes. Therefore, studying the various effects of MeJA on trichomes and expression of genes involved in their formation and development, as well as artemisinin production, could be the next step for understanding production controls, eventually leading to increased yields of this valuable therapeutic.

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