Rosmarinic acid accumulation in *Melissa officinalis* shoot cultures is mediated by ABA

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Abstract

Plant responses to elicitors are the result of a series of highly modulated consecutive changes in hormones or reactive oxygen species (ROS). Abscisic acid (ABA) is a stress hormone that coordinates the complex networks of stress responses and its content is rapidly changed in response to stresses. This study evaluated the effects of application of ABA (0, 5, 25, 50, and 100 µM) to shoot cultures of lemon balm (*Melissa officinalis* L.) in Murashige and Skoog (MS) liquid medium on growth, H₂O₂ production, rosmarinic acid (RA) content, total phenolic compound accumulation, phenylalanine-ammonia lyase (PAL) gene expression, and PAL activity. Our results showed that all the applied concentrations of ABA decreased the growth rate of shoots. Moreover, the expression of PAL, tyrosine aminotransferase (TAT), and rosmarinic acid synthase (RAS) genes, PAL activity, and the accumulation of total phenolic compound as well as RA were increased in the ABA-treated shoots. The highest content of RA was detected in the shoots treated with 100 µM ABA. The results suggested that both the PAL- and TAT-derived pathways were induced by ABA to increase RA accumulation in the shoot cultures of lemon balm. The results revealed that application of ABA led to up-regulation of respiratory burst oxidase homolog (RBOH) expression, which was correlated with the production of H₂O₂ in the shoots cultures. In addition, the cis epoxy carotenoid dioxygenase (NCED) gene, which encodes key enzyme involved in ABA biosynthesis was up-regulated. These results demonstrated that ABA treatment enhanced endogenous ABA content and rosmarinic acid synthesis in the shoot cultures of lemon balm.

Additional key words: 9-cis epoxy carotenoid dioxygenase, lemon balm, phenylalanine-ammonia lyase, rosmarinic acid synthase, tyrosine aminotransferase.

Introduction

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid. As a main active phenolic compound, it is commonly found in species of the subfamily Nepetoideae of the Lamiaceae, including perilla, rosemary, sage, mint, basil, and lemon balm (*Melissa officinalis* L.). The RA shows antiviral, antibacterial, anti-inflammatory, hepatoprotective, antiangiogenic, antidepressant, antineurodegenerative, antiallergic, and antioxidant activities (Petersen and Simmonds 2003). The biosynthesis of rosmarinic acid has been vastly investigated for two reasons: 1) rosmarinic acid has been shown to be a useful compound in the field of medicine, and as a food additive, 2) accumulated RA contributes to the defense against microbes. Moreover, the challenge to elucidate the compelling biosynthesis of RA which consists of two parallel biosynthetic pathways that should be regulated in a coordinated way is one of the other reasons for extensive studying of rosmarinic acid (Matsuno et al. 2002). Additionally, rosmarinic acid can be accumulated in plants as a defensive compound and protection against herbivores.

The initial report on the biosynthetic route of rosmarinic acid has demonstrated the involvement of two aromatic amino acids, L-tyrosine (tyrosine-derived pathway) and L-phenylalanine (phenylpropanoid pathway) (Ellis and Towers 1970). A coumaroyl-CoA is derived from phenylalanine in three well characterized enzymatic steps catalyzed by phenylalanine-ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumaric acid-CoA ligase (4CL). The second pathway

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Abbreviations: ABA - abscisic acid; MS - Murashige and Skoog; NCED - 9-cis epoxy carotenoid dioxygenase; PAL - phenylalanine-ammonia lyase; RA - rosmarinic acid; RAS - rosmarinic acid synthase; RBOH - respiratory burst oxidase homolog; ROS - reactive oxygen species; TAT - tyrosine aminotransferase.

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originates from tyrosine which results in the formation of 4-hydroxyphenyllactate (4HPL). Tyrosine aminotransferase (TAT) is the first enzyme in the tyrosine-derived branch of RA biosynthesis. The two ingredients of the parallel pathways, i.e., caffeoyl-CoA and 3,4-dihydroxyphenyllactic acid (DHPPL) are connected by rosmarinic acid synthase (RAS) catalyzing a transsterification reaction (Petersen 1991). Kim et al. (2013) have pointed out that addition of methyl jasmonate (MJ) into cell suspension culture of *Agastache rugosa* rapidly induced PAL but not TAT activity during RA accumulation. Similar results have been reported by Mizukami et al. (1992) who elicited the *Lithospernum erythrorhizon* cell suspension cultures by yeast extract. Conversely, some other reports have demonstrated that elicitors induced biosynthesis of RA and phenolic compounds in *Salvia miltiorrhiza* hairy roots, which was correlated with tyrosine-derived pathway (Yan et al. 2006).

The production of phenolic secondary metabolites can be maximized by elicitors, such as MJ, salicylic acid (SA), ABA, polyamines, metal ions, H$_2$O$_2$, UV-B, and yeast extracts (Ma et al. 2013). Plants respond to elicitor due to a series of highly modulated consecutive changes in their hormones or ROS (reactive oxygen species) (Jabs 1999, Ton et al. 2009). The ABA has been recognized as a stress hormone coordinating the complex networks of stress responses (Zhang et al. 2006). In some plant species, changes in the amount of endogenous ABA may play an important role in the induction of biosynthesis of RA and related phenolic compounds (Vagner et al. 1998, Gagne et al. 2011, Hao et al. 2012, Villalobos-Gonzalez et al. 2016). Further evidence supports the idea that ABA content is highly modulated by regulation of the different steps of its biosynthesis (Thompson et al. 2007, Rodriguez-Gacio et al. 2009). Several recent studies have investigated the capability of ABA to induce the accumulation of phenolic compound in cell and tissue cultures as well as in field experiments (Gagne et al. 2011, Ibrahim and Jaafar 2013, Liang et al. 2013, Villalobos-Gonzalez et al. 2016, Murcia et al. 2017).

The aim of this study was to investigate the effect of ABA on RA accumulation in the shoot cultures of *Melissa officinalis* in order to reveal the contribution of two metabolic pathways to RA biosynthesis. Further, the correlations between enzyme activities and the expression of corresponding genes were determined.

**Materials and methods**

**Plants, culture conditions, and ABA treatment:** Seeds of *Melissa officinalis* L. were purchased from Pakanbazr seed company (Isfahan Province, Isfahan, Iran). Seeds were surface sterilized with 70 % (v/v) ethanol for 20 s and 20 % (m/v) sodium hypochlorite solution for 8 min. Then, they were rinsed five times in sterile distilled water. For germination, seeds were placed in autoclaved hormone free of 1/2 Murashige and Skoog (1962; MS) medium supplemented with various concentrations of (±)-abscisic acid (ABA), polyamines, metal ions, H$_2$O$_2$, UV-B, and yeast extracts (Ma et al. 2013). Plants respond to elicitor due to a series of highly modulated consecutive changes in their hormones or ROS (reactive oxygen species) (Jabs 1999, Ton et al. 2009). The ABA has been recognized as a stress hormone coordinating the complex networks of stress responses (Zhang et al. 2006). In some plant species, changes in the amount of endogenous ABA may play an important role in the induction of biosynthesis of RA and related phenolic compounds (Vagner et al. 1998, Gagne et al. 2011, Hao et al. 2012, Villalobos-Gonzalez et al. 2016). Further evidence supports the idea that ABA content is highly modulated by regulation of the different steps of its biosynthesis (Thompson et al. 2007, Rodriguez-Gacio et al. 2009). Several recent studies have investigated the capability of ABA to induce the accumulation of phenolic compound in cell and tissue cultures as well as in field experiments (Gagne et al. 2011, Ibrahim and Jaafar 2013, Liang et al. 2013, Villalobos-Gonzalez et al. 2016, Murcia et al. 2017).

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**H$_2$O$_2$ assay:** The content of H$_2$O$_2$ was measured according to Alexieva et al. (2001). Fresh shoot tissue (0.1 g) was macerated in 1.5 cm$^3$ of 0.1 % (m/v) trichloatoacetic acid (TCA). After centrifugation (12 000 g, 4 °C, 15 min), 0.5 cm$^3$ of the supernatant was then mixed with 0.5 cm$^3$ of 10 mM potassium phosphate buffer (pH 7) and 1 cm$^3$ of 1 M KI. The absorbance of mixture was measured at 390 nm using a U/VVis spectrophotometer (*Ultrospec™ 3100 pro*, GE Healthcare Life Sciences, Pittsburgh, USA). Results were calculated with help of a H$_2$O$_2$ standard curve.

**Total phenolic and rosmarinic acid content:** Total phenolic content was quantified using Folin-Ciocalteu assay (Singleton and Rossi 1965). Plant tissue (0.1 g) was homogenized in 3 cm$^3$ of extraction buffer containing 80 % (v/v) methanol in a pre-chilled mortar. The homogenate was centrifuged at 15 000 g for 15 min. In reaction test tube, 0.02 cm$^3$ of the sample extract was mixed with 0.12 cm$^3$ of 20 % (m/v) Na$_2$CO$_3$ and 0.15 cm$^3$ of Folin-Ciocalteu reagent. After 30 min at dark and room temperature, absorbance was measured in 765 nm. Total phenolic content was expressed as mg of gallic acid per g of fresh mass.

Rosmarinic acid extraction and analysis followed the methods described by Öztürk et al. (2010). Methanol extract of shoots (0.2 cm$^3$) was added to 0.2 cm$^3$ of 0.5 M ZrOCl$_2$. 8 H$_2$O and 4.6 cm$^3$ of ethanol. After 5 min, the absorbance of reaction mixture was measured at 362 nm.
Rosmarinic acid (Sigma) at the range of 0 - 0.004 mM was used as a standard.

**PAL activity analysis:** PAL activity was assayed according to Abell and Shen (1987). The reaction mixture contained 2.5 cm$^3$ of 0.1 M Tris-HCl (pH 8.5) with 12 mM phenylalanine and 0.5 cm$^3$ of enzyme extract. The reaction mixture without phenylalanine was added as controls. At the onset of reaction and an hour later, increase in absorbance due to PAL activity was recorded at 290 nm. One unit was defined as the amount that caused an increase of 0.01 in the absorbance per h.

**HPLC analysis of ABA content:** Quantification of ABA was performed using an HPLC system (Waters, Massachusetts, USA) based on the methods of Kelen et al. (2004) with slight modifications. For extraction of ABA, 1 g of fresh shoots was homogenized with 10 cm$^3$ of 80 % (v/v) methanol containing butylated hydroxytoluen and ascobic acid and stirred overnight at 4 °C. The extract was filtered through a Whatman filter paper and the methanol was evaporated under vacuum at -35 °C. The residue was dissolved in 0.5 M potassium phosphate buffer, and adjusted to pH 8.5 with 0.2 M KOH. The ethyl acetate was added in volume equal to volume of mixture. The solution was vortexed and the ethyl acetate phase was discarded and the remaining ethyl acetate was freeze dried by evaporating at -35 °C. The pH of the aqueous phase was adjusted to 2.5 with 0.2 M HCl. The residue was dissolved in ethyl acetate, and the extract was evaporated under vacuum to remove ethyl acetate. The dry residue was dissolved in 0.5 cm$^3$ of methanol and filtered through a 0.22 µm membrane and stored at -80 °C until analysis. The samples (0.02 cm$^3$) was injected into a reverse-phase column (C18, 250 × 4.6 mm). The mobile phase consisted of acetic acid (0.2 %) and methanol (100 %) (50:50, v/v), and the flow rate was 0.7 cm$^3$ min$^{-1}$. Apparatus wavelength was adjusted to 265 nm. Identification of unknown peaks was based on retention time and UV absorption spectral comparison with known standards of (±)-ABA.

**RNA extraction and cDNA synthesis:** RNAs were extracted by DENAzist kit (DENAzist Asia Co., Mashhad, Iran) from fresh leaves according to manufacturer’s instructions. The recombinant DNase I treatment was used to remove contaminating DNA from RNA preparations and RNA (500 ng) was reverse transcribed to first strand cDNA using PrimeScript RT Enzyme Mix I (TaKaRa Bio, Otsu, Japan), which were later used as templates for real-time PCR.

The sequences of the **ras** and **pal** genes were obtained from the NCBI database (GenBank accession Nos. FR670523.1 and FN665700.1, respectively). The primers of **nced** (9-cis epoxy carotenoid dioxygenase) corresponding to conserved regions of **nced** were determined using a nucleotide alignment that included **nced**s from a range of plant species: *Sesamum indicum* (XM-011084544.2), *S. indicum* (XM-011099822.2), *Scutellaria baicalensis* (KC760149), *Fragaria × ananassa* (JX013945.1), *Fragaria vesca* (XM-004300619.2), *Erythranthe guttata* (XM-012981017.1), and *Daucus carota* (NM-001329172.1). The primers for **actin** and **tat** genes were taken from Doring et al. (2014). RNA-seq data (GSE100970) was used for design primers of **rboh** gene (NADPH oxidase B).

**Real-time quantitative PCR analysis:** Relative gene expression was determined by real-time qPCR using a Rotor-Gene Q instrument (Qiagen, Hilden, Germany). The PCR mixture (0.01 cm$^3$) contained 10 µM of each primer, 50 ng of the cDNA template and 0.005 cm$^3$ of SYBR Premix Ex Taq (TaKaRa Bio). The thermal cycle conditions were as follows: pre-incubation at 95 °C for 10 min, 40 cycles of 95 °C for 5 s, 58 - 62 °C for 10 s, and 72 °C for 20 s. The purity of the amplified products was confirmed by melting curve analysis and agarose gel electrophoresis. All reactions were performed twice.

**Data analysis:** The data were analyzed by ANOVA in the computer software SAS v. 8. Mean comparisons were performed with the least significant difference (LSD) test.

**Results**

The shoots fresh mass (SFM) was significantly affected by ABA at all applied concentrations with the maximum reduction in the shoots treated with 100 µM of ABA (Fig. 1). Evaluation of H$_2$O$_2$ production (Fig. 2A) indicated...
a significant enhancement of \( \text{H}_2\text{O}_2 \) content in all ABA-treated shoots with maximum at the highest ABA concentration. Also, the activity of PAL was increased with increasing ABA concentration (Fig. 2B). The content of phenolic compounds was 1.09-, 3.24-, 1.5-, and 1.7-fold higher in the shoots exposed to 5, 25, 50, and 100 mM of ABA, respectively, compared to the control (Fig. 3A). Similarly, rosmarinic acid content was a significantly enhanced in all the ABA-treated shoots compared to the control. The highest content of rosmarinic acid was detected in the shoots treated with 100 \( \mu\text{M} \) ABA two weeks after elicitation, which was 14 times higher than that of the control (Fig. 3B). One week after elicitation with 50 \( \mu\text{M} \) of ABA, a 55-fold increase was observed in the endogenous ABA content, which was the maximum amount of ABA detected in the elicited shoots (Fig. 4).

The effects of various concentrations of ABA on expressions of \( \text{PAL}, \text{TAT}, \text{RAS}, \text{RBOH}, \) and \( \text{NCED} \) genes were investigated using real-time PCR analysis (Fig. 5). Evaluation of gene expression profile indicated that \( \text{PAL} \) was significantly up-regulated in all the ABA-treated shoots. The expressions of \( \text{TAT} \) gene were significantly up-regulated at the ABA concentrations of 25, 50, and 100 \( \mu\text{M} \) compared to the control. Moreover, gene expressions of both \( \text{RAS} \) and \( \text{RBOH} \) genes were up-regulated at all concentrations of ABA compared to the control, but the highest expression of \( \text{RBOH} \) gene was detected in the shoots treated with 25 \( \mu\text{M} \) ABA. The expression of \( \text{NCED} \) gene was significantly up-regulated in the shoots treated with 25 and 50 \( \mu\text{M} \) of ABA.
Discussion

The present study investigated whether ABA-application alters expressions of the genes involved in the biosynthesis of RA and its accumulation in the shoot cultures of lemon balm. ABA is often described as a growth inhibitor since the reduced growth under stress condition is correlated with the increased ABA content; moreover, applied ABA prevents germination and seedling growth (Hedden and Thomas 2006). In this study, substantial differences in growth rate were observed between the ABA-treated and control shoots.

Fig. 4. Effect of different concentrations of ABA on endogenous ABA in Melissa officinalis shoots one week after application. Means ± SDs, n = 3. Values followed by different letters are significantly different at P ≤ 0.05 according to LSD test.

Generally, treatment by various types of elicitors induces a rapid increase in the endogenous ABA content (Gagne et al. 2011, Hao et al. 2012, Villalobos-Gonzalez et al. 2016, Kuyyogsuy et al. 2018). Therefore, both the content of endogenous ABA and the expression of pivotal gene associated with the ABA biosynthetic pathway (NCED) increased in the shoot cultures of lemon balm treated with ABA. The ABA content in shoots was increased within 7 d after ABA application and its maximum was detected in the shoots treated with 50 µM ABA. Increase in the expression of NCED leads to an increase in the ABA content (Castellarin et al. 2016) also in response to environmental stresses (Agusti et al. 2007, Kuyyogsuy et al. 2018). In our study, the expression of NCED was up-regulated in the plants treated with 50 µM ABA compared to the control. Results showed that although expression of NCED was not increased in the shoots treated with 5 µM ABA, the endogenous ABA was increased in the shoots, suggesting that the increase could be due to the decreased catabolism of this hormone. Our finding revealed that the ABA application could induce an increase in the endogenous ABA together with via up-regulation of the NCED gene expression. Thus, it could be suggested that the exogenous ABA stimulated ABA biosynthesis through increasing expression of NCED.

The applied ABA can also trigger the generation of H2O2 in the plant cells or tissues (Hu et al. 2010, Ye et al. 2011). ROS, especially H2O2 and O2•−, are involved in the ABA signaling pathway, thus, ABA increases ROS accumulation which leads to changes in gene expression and in metabolism. After foliar applications of ABA antioxidative properties of cells might be up-regulated by the increase in the content of phenolics and flavonoids (Ibrahim and Jaafar 2013). Accumulation of ROS in plants is generally attributed to several possible sources, including plasma membrane-localized NAD(P)H oxidase, oxalate oxidase, apoplastic peroxidase, and amino oxidase (Bolwell 1999). Previous studies have found that the H2O2 generated by NADPH oxidase encoded by RBOH plays an important role in plant responses to biotic and abiotic stresses. The results of the present study indicated that the expression of RBOH, which is a key player in controlling H2O2 accumulation, was significantly up-regulated by ABA application.

Phenylalanine ammonia lyase (PAL) is known as a key enzyme in RA biosynthetic pathway (Dong et al. 2010). Several studies have revealed that exogenous ABA could induce the activation of PAL (Hao et al. 2012). The results of the present study revealed that exogenous ABA induced the activation of PAL and synthesis of RA in the shoots of lemon balm. Here, we reported that ABA induced the accumulation of RA and phenolic compounds. Our findings are consistent with those of the previous studies on other plant species which reported that the content of RA and phenolic compounds was increased by application of biotic and abiotic elicitor (Yan et al. 2006, Bauer et al. 2009, Kim et al. 2013, Ejtahed et al. 2015). In this study, ABA was used as an elicitor to examine the relationship between the expression of rate-limiting enzymes in the two RA biosynthesis pathways and accumulation of RA in the shoot cultures. The expressions of PAL, TAT, and RAS genes were significantly up-regulated as the result of ABA treatment. Expressions of these genes are correlated with the RA content in several plants (Weitzel and Petersen 2010, Doring et al. 2014). These results suggested that both the PAL- and TAT-derived pathways were induced by ABA to increase RA accumulation in the shoot cultures of lemon balm.

In summary, ABA application could effectively induce RA accumulation by the induction of the PAL activity, as well as by the up-regulation of PAL, TAT, and RAS genes. In addition, the up-regulated expression of NCED gene by ABA treatment is correlated with the enhancement of ABA content. In this study, ABA not only mediates the accumulation of endogenous ABA, but also controls the production of H2O2 by changing the expression pattern of RBOH gene.
ABA-INDUCED ROSMARINIC ACID ACCUMULATION

Fig. 5. Relative transcriptions of PAL (A), TAT (B), RAS (C), NCED (D), and RBOH (E) genes in Melissa officinalis shoots treated with different concentrations of ABA in relation to the housekeeping gene actin. Means ± SDs, n = 3. Values followed by different letters are significantly different at P ≤ 0.05 according to LSD test.

References


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