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Jasmonic acid serves as a signal role in smoke-isolated butenolide-induced tanshinones biosynthesis in *Salvia miltiorrhiza* hairy root

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ABSTRACT

Although smoke-water (SW) and butenolide (KAR_1) isolated from smoke show stimulating effects on the production of secondary metabolites in medicinal plants, the underlying mechanisms are not well understood. This study was initiated to investigate the signal molecule in the KAR_1-induced tanshinones biosynthesis in *Salvia miltiorrhiza* hairy root. KAR_1 treatment resulted in substantial improvement in the accumulation of jasmonic acid (JA), followed by an increase in lipophilic tanshinones production in *S. miltiorrhiza* hairy root. Treatment of external JA stimulated the biosynthesis of tanshinones without KAR_1 treatment. KAR_1-stimulated tanshinones biosynthesis was inhibited with the treatment of SHAM and PrGall, which are JA synthesis inhibitors. This study indicated that JA serves as a role in the signal transduction involved in KAR_1-stimulated tanshinones biosynthesis in the *S. miltiorrhiza*.

1. Introduction

The roots and rhizomes of *Salvia miltiorrhiza*, considered as one of the most renowned ones in traditional Chinese medicine (TCM) (Zhang et al., 2014), have been widely used for removing blood stasis and eliminating carbuncle throughout Chinese history (China Pharmacopoeia Committee, 2015). The lipophilic tanshinones including tanshinone I (T-I) and tanshinone IIA (T-IIA), isolated and identified from *S. miltiorrhiza*, have been considered as the most bioactive components (Ma et al., 2012; Wang et al., 2015). *Salvia miltiorrhiza* market supply is mostly relies on field planting, which is a time- and labor-consuming procedure (Gao et al., 2014; Yu et al., 2016). Hairy root is derived after infecting plantlets using the natural occurring *Agrobacterium rhizogenes*, which has been considered as a convenient platform for the mass production of bioactive components and supplement to traditional planting (Figlan and Makunga, 2017). Biotic elicitors (yeast extracts), abiotic elicitors (silver ion) and plant signal material (methyl jasmonate) have been widely used in enhancing tanshinones production in *S. miltiorrhiza* hairy root.

Plant-derived smoke-water (SW) and smoke-isolated butenolide (KAR_1) show stimulatory effects on seed germination as well as plant growth (Light et al., 2009; Mojzes et al., 2015; Martínez-Baniela et al., 2016; Morffy et al., 2016; Salomon et al., 2017). KAR_1 is an active ingredient in SW and has been considered as a new plant growth regulator. In previous report, the treatments of SW and KAR_1 could enhance lipophilic tanshinones production in *S. miltiorrhiza* (Zhou et al., 2014). However, the underlying signal transduction mechanism of KAR_1-induced tanshinones biosynthesis has not been well understood.

It is generally accepted that Jasmonic acid (JA) acts as a signaling compound which elicits the production of secondary metabolite biosynthesis. It has been investigated that external JA treatment could stimulate the biosynthesis of T-I and T-IIA in *S. miltiorrhiza* hairy roots (Wang et al., 2007). Treatment of KAR_1 resulted in an increase in tanshinones production in *S. miltiorrhiza* (Zhou et al., 2011). While relatively little is confirmed that JA regulates KAR_1-induced tanshinones in *S. miltiorrhiza*. We hypothesized that JA serves as a signal molecule in KAR_1-induced tanshinones biosynthesis in *S. miltiorrhiza*. Based on this hypothesis, pharmacological strategies were used in the research to study (1) the effects of KAR_1 on the level of endogenous JA and tanshinones in *S. miltiorrhiza* and (2) the effects of JA and the biosynthesis inhibitors treatments on tanshinones synthesis to further comprehend the signal transduction through which KAR_1 stimulated tanshinones production in *S. miltiorrhiza*.

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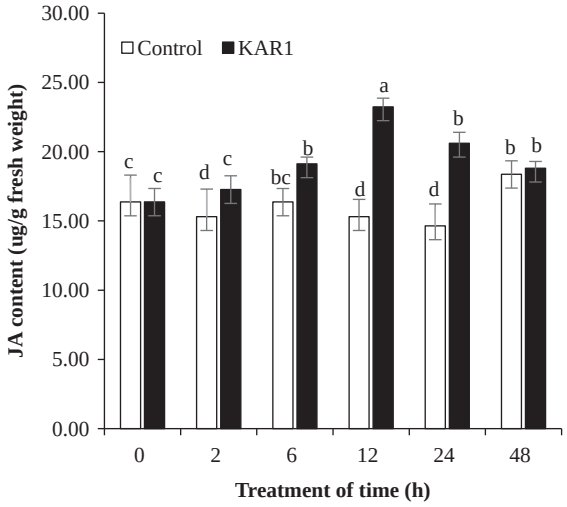

Fig. 1. Effects of KAR_1 on JA level in *S. miltiorrhiza* hairy root. Experiments were carried out in triplicates and expressed as mean ± SD. Bars with different letters differ significantly at $P < .05$ considering Fisher's LSD test.

2. Materials and methods

2.1. Hairy root culture and treatment

Salvia miltiorrhiza hairy root has been established after infecting plantlets using *A. rhizogenes* bacterium (ACCC10060). It was maintained in 6, 7-V medium containing sucrose of 30 g/l. For this experiments, the hairy root was incubated in flasks of 250 ml on an orbital shaker (120 rpm) at 25 °C in the dark (Zhang et al., 2014). *Salvia miltiorrhiza* hairy root of 18 days old was used in this experiment (Ge and Wu, 2005). They were treated with a solution of either (1) KAR_1 (10^{-9} mol/l), (2) JAMe (10^{-6} mol/l), (3) KAR_1 (10^{-9} mol/l) and salicylhydroxamic acid (SHAM, JA synthesis inhibitor, 100 μmol/l), which was dissolved in DMSO (0.1 mmol/l SHAM in 20 μl DMSO), (4) KAR_1 (10^{-9} mol/l) and n-propyl gallate (PrGall, JA synthesis inhibitor, 100 μmol/l), which was dissolved in ethanol and (5) JAMe and SHAM. The same amount of DMSO and ethanol were used in the control. Treatments of SHAM and PrGall were performed 30 min before treatments of KAR_1 or JAMe. The concentrations of liquid used in this experiment were determined in earlier experiments. The hairy root was harvested, frozen with liquid nitrogen and stored at −80 °C for further experiment. All experiments were performed in triplicate.

2.2. Preparation of KAR_1 solution

Compound of KAR_1 was isolated from smoke water. SW was prepared following the method of Light et al. (2009) with smoldering plant materials of *Crataegus pinnatifida* and *Magnolia denudata* through 500 ml water for 45 min at a ratio of 6 kg of raw plant materials for 10 l of distilled water. Before burning, the plant material was cut into small pieces and subjected to UV-B radiation with the dosages of 10 kJ/m^2 for 4 h at a distance of 30 cm above it and dried at 105 °C for 24–48 h. KAR_1 was isolated from smoke-water following the method of Light et al. (2009) and 10^{-9} M was chosen for this experiment.

2.3. HPLC analysis of tanshinones

The extraction of tanshinones in *S. miltiorrhiza* hairy root was conducted following the methods described by Liang et al. (2017) with slight modification. Briefly, the sample (about 50 mg), being ground into powder with a mortar and pestle, was extracted with 20 ml 70% ethanol for 12 h. Then the extract was sonicated for 15 min (300 W, 25 kHz) and centrifuged at 10,000×*g* for 15 min. The supernatant was filtered with 0.45 μm microporous membrane and a sample injection volume of 20 μl used for further analysis. The contents of tanshinones was analyzed on an HPLC system (Agilent-1260) coupled with a C18 (250 mm × 4.6 mm, 5 μm). The detection wavelength was set at 280 nm and temperature of the column component was maintained at 30 °C. A gradient elution of A (0.2%-methanoic acid-ammonium) and B (acetonitrile) was used as followed: t = 0 min, 5% B; t = 10 min, 20% B; t = 15 min, 25% B; t = 20 min, 25% B; t = 25 min, 20% B; t = 28 min, 30% B; t = 36 min, 30% B.

2.4. HPLC–ESI–MS/MS analysis of Jasmonic acid (JA)

JA levels were assayed and calculated following the method of Pan et al. (2010). The hair roots was obtained per time point (0, 2, 6, 12, 24, 48 h, after treatment) and immediately frozen in liquid nitrogen, and each replicate was ground to a fine powder at liquid nitrogen temperatures using a Retsch model MM 301 ball mill (Retsch Inc., Newtown, PA, USA). The 100 mg powder was accurately weighed into a 5 ml centrifuge tube with a spatula; and 1 ml extraction solvent (n-propanol/water/concentrated hydrochloric acid: 200/100/0.2, v) was added to each tube, which was placed on a shaker at a speed of 100 rpm for

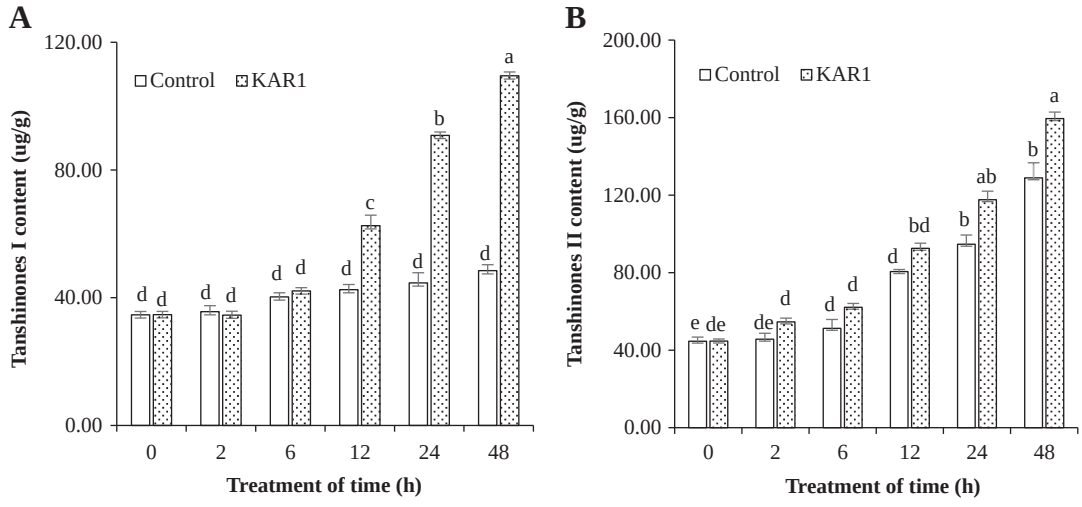

Fig. 2. Effects of KAR_1 on the contents of tanshinone I (A), tanshinone IIA (B) in *S. miltiorrhiza* hairy root. Experiments were carried out in triplicates and expressed as mean ± SD. Bars with different letters differ significantly at $P < .05$ considering Fisher's LSD test.

30 min at 4 °C. Then, the samples were placed into 2 ml of dichloromethane shake gently for 30 min, centrifuged for 15 min (4 °C, 3500 rpm), the supernatant was transferred to 5 ml tube, and added 0.5 ml dichloromethane to residue with vortex instrument for 30 s, the supernatant was combined and the solvent mixture was concentrated (not completely dry) using a nitrogen evaporator with nitrogen flow. The sample was dissolved in 0.5 ml methanol and 50 μl sample solution was injected into reversed C18 Gemini HPLC column for HPLC-ESI-MS/MS analysis.

2.5. Statistical analysis

One-wayANOVA using SPSS 20.0 was conducted at each time point to identify the accumulation of JA and secondary metabolites and difference was considered significant when $P < .05$.

3. Results and discussion

3.1. Effects of KAR_1 on JA production in S. miltiorrhiza hairy root

A rapid increase in endogenous JA level in *S. miltiorrhiza* hairy root was observed with the application of KAR_1 as shown in Fig. 1. The level of JA in the treated hairy root displayed a time-dependent increase within 12 h post-treatment of KAR_1, reaching 23.24 mg/g at 12 h and then showed a slight decrease, which was 18.81 mg/g at 24 h. By 48 h post-treatment the content of JA declined to 18.37 mg/g, which was not significantly different compared to the control. Treated hairy root had 40.73% significantly more JA than the control at 24 h ($P < .05$) and 11.32% less than that ($P < .05$) at 12 h after KAR_1 application. Overall, a promotion in the level of endogenous JA was observed after 12 h treatment of KAR_1.

3.2. Effects of KAR_1 on tanshinones production in S. miltiorrhiza hairy root

As displayed in Fig. 2, the content of T-I and T-II in the control and treated hairy root displayed a time-dependent increase during the treatment process, reaching 109.47 mg/g and 159.47 mg/g at 48 h during the treatment process, which was 215.50% ($P < .05$) and 256.78% ($P < .05$) significantly higher than that at 0 h respectively. Comparing to the control, treatment of KAR_1 caused a more increase in the content of T-I, and the highest levels were observed to be 77.67 mg/g at 24 h and 90.03 mg/g at 48 h post-treatment. It could be also observed that KAR_1-stimulated improvement in T-I level took place following the JA burst, peaking at 2.59-fold of that in control at 48 h after treatment.

3.3. Effects of exogenous JA on tanshinones production in S. miltiorrhiza hairy root without KAR_1

JAMe was selected in the experiment to study the effects of exogenous JA on tanshinones level without treatment of KAR_1. As shown in Fig. 3, the profile of T-I in *S. miltiorrhiza* hairy root showed a slight increase during the treatment both in the control and in the treated ones. The content of T-I in the treated samples showed a significant increase in the later phase from 12 to 48 h after treatment, which was recorded to be 148.60% (24 h) and 173.99% (48 h) higher than that of control. Treatment of JA significantly promoted the concentration of T-IIA compared to the control. JAMe treatment slightly stimulated T-I level, overstepping as much as 20.29% of the KAR_1 response at 48 h post-treatment.

3.4. Inhibition of KAR_1-induced tanshinones production with JA biosynthesis inhibitors

The effects of KAR_1, JAMe and JA biosynthesis inhibitors on the level of T-I and T-IIA in *S. miltiorrhiza* hairy root is shown in Fig. 4A. The increase in T-I accumulation was reduced significantly ($P < .05$) with the inhibitors of JA synthesis, SHAM and PrGall, which were 39.95% (SHAM, $P < .05$) and 27.87% (PrGall, $P < .05$). While they were significantly higher than the control recording 44.82% (SHAM, $P < .05$) and 73.95% (PrGall, $P < .05$). As displayed in Fig. 4B, the KAR_1-stimulated T-IIA biosynthesis was blocked by SHAM ($P < .05$). The production of T-IIA was significantly higher comparing to the control in spite of JA biosynthesis was inhibited by SHAM ($P < .05$). PrGall, JA biosynthesis inhibitor, showed inhibition in KAR_1-induced improvement of T-IIA.

4. Discussion

The treatments of smoke-water and smoke-isolated KAR_1 could enhance the biosynthesis of tanshinone I and tanshinone IIA in *S. miltiorrhiza* (Zhou et al., 2014). While relatively little is known about the signal transduction mechanism involved in KAR_1-regulated tanshinones biosynthesis. This study showed the relation between KAR_1, JA and tanshinones production in *S. miltiorrhiza* hairy root. An improvement in the level of JA was observed in the treatment of KAR_1, occurring earlier than the stimulation of biosynthesis of T-I and T-IIA,

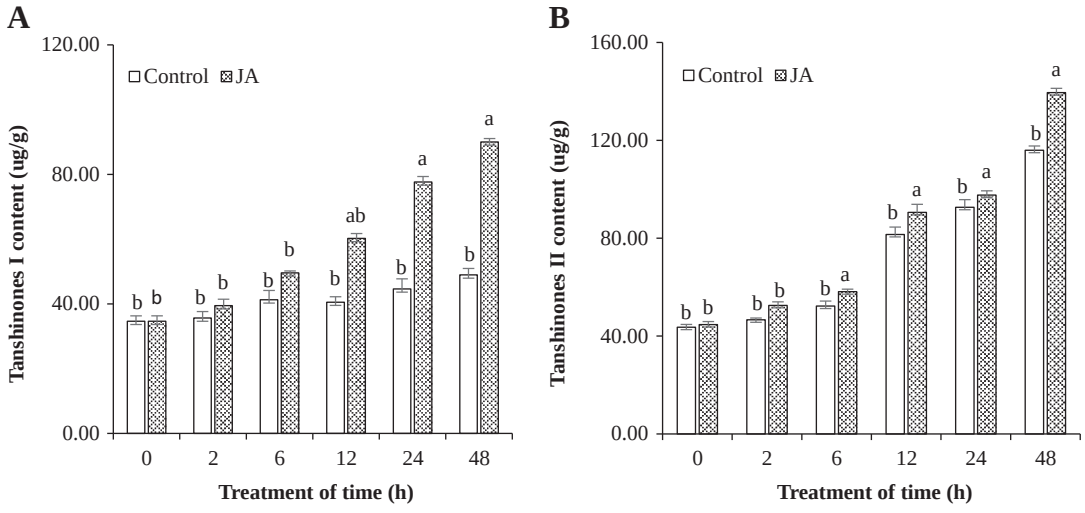

Fig. 3. Effects of exogenous JA on the contents of tanshinone I (A), tanshinone IIA (B) in *S. miltiorrhiza* hairy root. Experiments were carried out in triplicates and expressed as mean ± SD. Bars with different letters differ significantly at $P < .05$ considering Fisher's LSD test.

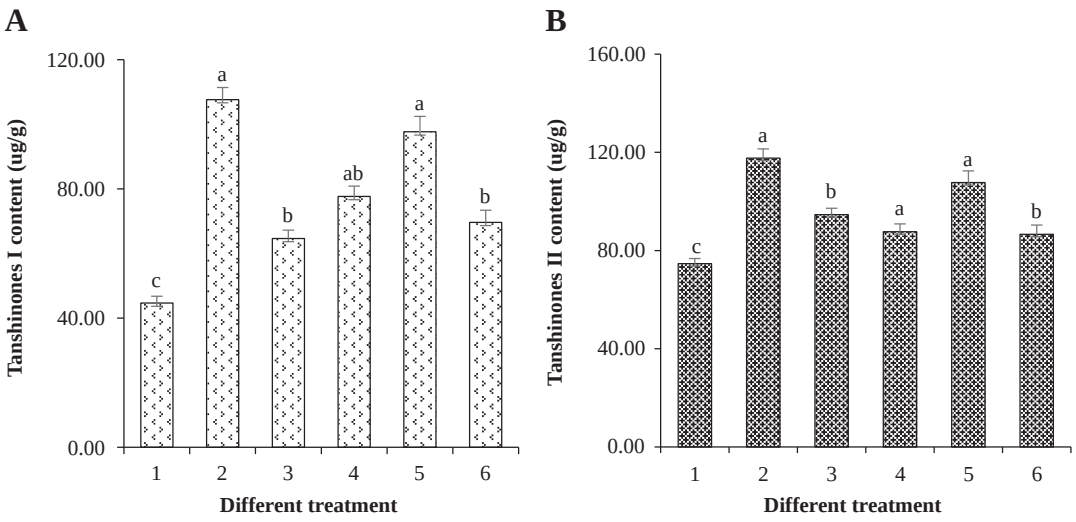

Fig. 4. Effects of KAR_1 on the contents of tanshinone I (A), tanshinone IIA (B) in *S. miltiorrhiza* hairy root with different treatment. 1. Control, 2. KAR_1, 3. KAR_1 + SHAM, 4. KAR_1 + PrGAll, 5. JA, 6. JA + SHAM. *S. miltiorrhiza* hairy root were treated with KAR_1 (10^{-9} mol/l) and JAMe (10^{-6} mol/l). SHAM (100 umol/l) and PrGall (100 umol/l) were added 30 min before the treatment with KAR_1 or JAMe. Experiments were carried out in triplicates and expressed as mean ± SD. Bars with different letters differ significantly at $P < .05$ considering Fisher's LSD test.

which indicated that JA might play a role of a signal molecule in the KAR_1-induced tanshinones biosynthesis. Exogenous JA treatment could improve the accumulation of tanshinones, implying that JA could independently promote the tanshinones synthesis in *S. miltiorrhiza* hairy root, which further suggests that JA acts as a signal molecule in inducing tanshinones biosynthesis. The KAR_1-stimulated tanshinones biosynthesis was inhibited by synthesis inhibitors of JA, which strongly implied that JA was involved in the biosynthetic pathway of KAR_1-induced tanshinones synthesis. It could also be said that tanshinones biosynthesis was induced at least partially by JA pathway. In spite of JA synthesis inhibitor suppressing the biosynthesis of endogenous JA in the treatment of KAR_1, the *S. miltiorrhiza* hairy root contained significantly more tanshinones than that in control, suggesting that JA was not the only signal to induce the accumulation of tanshinones. It is possible that KAR_1 may induce tanshinones biosynthesis through other signal pathway in case JA signal pathway was obstructed in *S. miltiorrhiza*. PrGall also showed inhibition in KAR_1-induced improvement of T-IIA, indicating presence of another signal pathway via which KAR_1-stimulated tanshinones biosynthesis.

In recent years, KAR_1 as a novel exogenous elicitor to stimulate secondary metabolites production has attracted more and more researchers. Therefore understanding the molecular mechanism of plant to KAR_1 is an important step in the improvement of secondary metabolites. JA signaling pathway has been considered to be involved in elicitor-induced secondary metabolism (Nham et al., 2017). Actually different signal molecule, for example salicylic acid and nitric oxide are involved in the biosynthetic pathways for secondary metabolite. This study showed that JA serves as a signaling involved in KAR_1-stimulated tanshinones biosynthesis, while it was not the unique signaling for tanshinones synthesis.

Conflict of interest

Authors declare that there is no conflict of interests regarding the publication of this work.

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