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Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^+ and yeast elicitor

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Abstract

This work examined the accumulation of diterpenoid tanshinones and related secondary metabolism pathways in hairy root cultures of *Salvia miltiorrhiza* Bunge (Lamiacae) induced by a biotic elicitor, the carbohydrate fraction of yeast extract (YE, 100 μg mL^{-1}), and an abiotic elicitor, Ag^+ (30 μM). The activity of 3-hydroxy-3-methylglutaryl CoA redutase (HMGR) was only stimulated by Ag^+, and 1-deoxy-D-xylulose 5-phosphate synthase (DXS) was stimulated by both, but more strongly by YE. While the non-MVA pathway inhibitor fosmidomycin inhibited the tanshinone accumulation induced by both elicitors, the MVA-pathway inhibitor mevinolin only suppressed the Ag-induced tanshinone accumulation. The results suggest that the tanshinone accumulation induced by the two elicitors was mainly synthesized via the non-MVA pathway (DXS activity), but could depend on crosstalk between the MVA and non-MVA pathways. Furthermore, the pretreatment of hairy roots with Ag^+ for 24–48 h potentiated the YE-induced tanshinone production and DXS activity.
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Keywords: Tanshinones; Isoprenoid pathways; Elicitors; *Salvia miltiorrhiza*; Hairy root culture

1. Introduction

Plant hairy roots are transformed roots formed after the infection of wounded plant tissue by the soil bacterium, *Agrobaterium rhizogenes*. Hairy root culture represents a convenient experimental system for plant science research and a promising bioprocess for the production of medicinal plants and their active constituents, which are mostly plant secondary metabolites [1]. *Salvia miltiorrhiza* Bunge (Lamiaceae) is a well-known Chinese herbal plant, and its roots, known as Danshen in Chinese, are widely used for the treatment of menstrual disorders and blood circulation diseases, and for the prevention of inflammation. The chief bioactive ingredients of *S. miltiorrhiza* roots are the diterpenoid pigments, particularly the phenanthrofurane quinone derivatives generally known as tanshinones [2]. Hairy root culture of *S. miltiorrhiza* has been established as a potential means for tanshinone production [3].

In higher plants, terpenes or isoprenoids are synthesized via at least two different pathways, the mevalonate (MVA) pathway occurring in the cytosol and the non-MVA, 1-deoxy-D-xylulose 5-phosphate (DXP) pathway in the plastids of cell [4,5] (Fig. 1). HMGR (3-hydroxy-3-methylglutaryl CoA reductase) catalyzes the formation of mevalonate from 3-hydroxy-3-methylglutaryl CoA, an initial and important step in the MVA pathway, and 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) are the first two enzymes in the DXP pathway. Although diterpenes are generally believed to be synthesized via the non-MVA pathway, there has been growing evidence for the involvement of the cytosolic MVA pathway in diterpene biosynthesis and the existence of crosstalk between the two pathways for isoprenoid biosynthesis in some plants [5]. The specific biosynthetic pathways for tanshinone production in the *S. miltiorrhiza* roots are still not clear.

This work aims to further investigate the separate and combined effects of biotic and abiotic elicitors on the secondary metabolism activities of *S. miltiorrhiza* hairy

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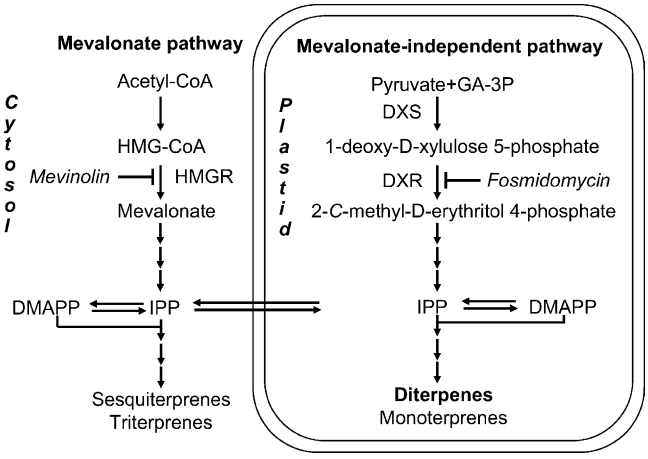

Fig. 1. An overview of the cytosolic mevalonate pathway and plastidial mevalonate-independent pathway for the biosynthesis of terpenoids (isoprenoids) in plant cells. (DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; GA-3P, glyceraldehyde-3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; IPP, isopentenyl diphosphate. Mevinolin and fosmidomycin are inhibitors of HMGR and DXR, respectively.)

roots in suspension cultures, and to identify the specific metabolic pathways responsible for the biosynthesis of tanshinones. The carbohydrate (polysaccharide) fraction of yeast extract was used as a biotic elicitor and Ag^+ as an abiotic elicitor.

2. Materials and methods

2.1. Hairy root culture

The *S. miltiorrhiza* hairy root culture was derived after the infection of plantlets with a Ri T-DNA bearing *Agrobacterium rhizogenes* bacterium (ATCC15834) in the laboratory of Dr. Steven F. Chen at The University of Hong Kong [6]. Stock cultures of the hairy roots were maintained on solid, hormone-free MS medium [7] with 8 g L^{-1} agar and 30 g L^{-1} sucrose but without ammonium nitrate, at 25 °C in the dark. Experiments in this study on the effects of elicitors were all carried out in shake-flask suspension cultures, with 125-mL Erlenmeyer flasks each containing 25 mL medium on an orbital shaker set at 110–120 rpm and 25 °C, as described elsewhere [8]. Hairy roots were harvested from the culture medium by filtration and blotted dry with paper towels (yielding the fresh weight, fw), and then dried at 45 °C in an oven until constant weight (dry weight, dw).

2.2. Preparation of elicitors and metabolic inhibitors

The yeast elicitor (YE) was the carbohydrate (polysaccharide) fraction of yeast extract prepared by ethanol precipitation, as described by Hahn and Albersheim [9]. Briefly, 25 g of yeast extract (Sigma, St. Louis, MO, Cat. Y4250) was dissolved in 125 mL of distilled water, followed by the addition of 100 mL of ethanol. The solution was allowed to precipitate for 4 days at 4 °C in a refrigerator, and the supernatant was decanted. The remaining gummy precipitate was redissolved in 125 mL of distilled water and subjected to another round of ethanol precipitation. The final precipitate was dissolved in 100 mL of distilled water, sterilized by autoclaving at 121 °C for 2 h and stored at 4 °C in a refrigerator before use. The elicitor dose was expressed by the total carbohydrate content, which was determined by the phenol–sulfuric acid method [10] using sucrose as a standard.

Silver ion Ag^+ was supplied to the culture with a concentrated silver thiosulfate ($Ag_2S_2O_3$) solution prepared by mixing $AgNO_3$ and $Na_2S_2O_3$ at 1:4 molar ratio as described previously [8]. Mevinolin (Sigma, Cat. M2147) was used as an inhibitor of the mevalonate pathway [11] and fosmidomycin (sodium salt FR-31564, Molecular Probes Inc., Eugene, OR) as an inhibitor of the non-MVA pathway [4]. Both have been proven specific and effective for the respective pathways, mevinolin for HMGR and fosmidomycin for DXR (Fig. 1). Stock solutions of mevinolin (Mev, 5 mM) and fosmidomycin (Fos, 100 mM) were prepared in distilled water and sterilized by filtration (0.22 μm membrane).

Elicitor and inhibitor treatments were applied to the hairy root culture on day 18 post inoculation, at concentrations (Ag^+ 30 μM, YE 100 μg mL^{-1}, Mev 5 μM and Fos 100 μM) chosen based on previous studies to produce the maximum responses. All treatments were performed in triplicate, and the results were represented by the mean ± standard error (S.E.).

2.3. Analysis of tanshinones

Tanshinones were extracted from the roots with methanol (MeOH) (ca. 100 mg/mL). Tanshinones in the culture medium were extracted with ethyl acetate (1:1, v/v), which was then evaporated to dryness and redissolved in methanol. Tanshinone content in the MeOH extract solution was determined by the HPLC method and instruments as described previously [8] with a 25 cm × 0.46 cm Alltech Econosphere 5 μ C-l8 column. Three tanshinone species, cryptotanshinone (CT), tanshinone I (T1) and tanshinone IIA (T2A) (Fig. 2), were detected and quantified with authentic standards obtained from the Institute for Identi-

Fig. 2. Molecular structures of three major tanshinones in *Salvia miltiorrhiza* roots.

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