Journal of Biotechnology 239 (2016) 106–114

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Fungal elicitors enhance ginsenosides biosynthesis, expression of functional genes as well as signal molecules accumulation in adventitious roots of *Panax ginseng* C. A. Mey

Jinxin Li[a], Shujie Liu[b], Juan Wang[a,*], Jing Li[a], Dahui Liu[c], Jianli Li[a,b], Wenyuan Gao[a,*]

[a] *Tianjin Key Laboratory for Modern Drug Delivery and High Efficiency, School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China*
[b] *Key Laboratory of Systems Bioengineering, Ministry of Education, Tianjin University, Tianjin 300072, China*
[c] *Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, 65023, China*

ARTICLE INFO

Article history:
Received 1 July 2016
Received in revised form 6 October 2016
Accepted 12 October 2016
Available online 13 October 2016

Keywords:
Adventitious root
Fungal elicitor
HPLC-ESI–MS^n
Biosynthetic genes
Signal molecules

ABSTRACT

In this work, we selected three fungi strains (*Aspergillus niger, Aspergillus flavus* and *Aspergillus oryzae*) as elicitors prepared from mycelium or fermentation broth to improve ginsenosides production in adventitious roots culture. The results showed that ginsenosides production (29.90 ± 4.67 mg g^{-1}) was significantly enhanced upon elicitation with 200 mg L^{-1} *A. niger* elicitor prepared from mycelium, which was 3.52-fold of untreated group. HPLC-ESI–MS^n analysis was performed, showing that ginsenoside Rb_3 was present after treatment with the *A. niger*. Furthermore, we found that *A. niger* significantly enhanced accumulation of Nitric oxide (NO), salicylic acid (SA) and jasmonic acid (JA) involved in plant defense response, and significantly up-regulated the expression of the geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPS), squalene synthase (SS), squalene epoxidase (SE), dammarenediol synthase (DS), Two cytochrome P450 (CYP) genes (CYP716A47 and CYP716A53v2) and three UDP-glycosyltransferases (UGT) genes (UGTAE2, UGT94Q2 and UGTpg100).

1. Introduction

Panax ginseng C. A. Mey. (*P. ginseng*), which belongs to the Aralilaceae family, is one of the traditional Chinese medicines. Ginsenosides are the major bioactive constituents in ginseng and have a wide range of pharmacological activities, such as anti-aging and anticancer (Chen et al., 2008; Wang et al., 2013). In the proposed biosynthesis pathway (Fig. 2), ginsenosides are synthesized from the key precursor, 2, 3-oxidosqualene, which is transformed into dammarenediol, β-amyrin, or cycloartenol by dammarenediol synthase (DS), β-amyrin synthase and cycloartenol synthase (CAS), respectively. Two cytochrome P450 (CYP) genes, CYP716A47 and CYP716A53v2, catalyzing the generation of protopanaxadiol and protopanaxatriol separately, were identified in *P. ginseng* (Han et al., 2012, 2011). In addition, the three UDP-glycosyltransferases (UGT) genes UGTAE2, UGT94Q2 and UGTpg100, transforming a glucose moiety from UDP-glucose to dammarenediol, ginsenoside Rh_2 and protopanaxatriol to generate ginsenosides Rh_2, Rg_3 and Rh_1 respectively, were also discovered in *P. ginseng* (Jung et al., 2014; Wei et al., 2015). In recent years, *P. ginseng* has been used in medicine, food and cosmetic, which has increased the demand of ginseng. Furthermore, the supply of ginseng mainly depends on the field cultivation which is an extremely time-consuming and labor-intensive process (Wu et al., 2009). Admittedly, it is also possible to enhance the ginsenoside concentration significantly in cultivated ginseng roots by simple selection followed by vegetative propagation (Murthy et al., 2014). Therefore biotechnology is certainly not the only alternative to increase the production of ginsenosides. This is also true for artemisinin in *Artemisia annua* as well as for other secondary metabolites in other plant species (Xiang et al., 2015). However, tissue and organ cultures have been exploited as a biotechnological alternative for more efficient and controllable production of active metabolites.

However, the contents of bioactive metabolites in adventitious roots are often lower than those of the whole plant. It was reported that the contents of ginsenosides in *P. ginseng* and *P. quinquefolium*

Abbreviations: NO, nitric oxide; SA, salicylic acid; JA, jasmonic acid; GPS, geranyl diphosphate synthase; FPS, farnesyl diphosphate synthase; SS, squalene synthase; SE, squalene epoxidase; DS, dammarenediol synthase; CYP, cytochrome P450; UGT, UDP-glycosyltransferases.

* Corresponding authors.
E-mail addresses: drwangjuan@163.com (J. Wang), biochemgao@163.com (W. Gao).

http://dx.doi.org/10.1016/j.jbiotec.2016.10.011
0168-1656/

adventitious roots were lower than those cultivated plants (Huang et al., 2010; Liu et al., 2014). The contents of flavonoids and glycyrrhizic acid in adventitious root of *Glycyrrhiza uralensis* Fisch were lower than those of whole plants (Wang et al., 2013). Fungal elicitors applied in plant cultures have been regarded as effective methods for improving the production of bioactive compounds. Liu et al. (1997) showed that artemisinin production was increased from 390 mg L^{-1} to 550 mg L^{-1} when the cultures of *Artemisia annua* L. hairy root were elicited with a homogenate of *Aspergillus oryzae* (Liu et al., 1997). Boue et al. (2000) reported that elicitors derived from mycelia extracts of *Aspergillus flavus* enhanced isoflavonoid production in soybean cotyledon tissue. It had been reported that *A. niger* as elicitor could enhance the production of bioactive compounds including gymnemic acid (triterpenoid saponin) (Chodisetti et al., 2013), artemisinin (Hasanloo et al., 2013), Ajmalicine (indole alkaloids) (Namdeo et al., 2002) and other compounds. Furthermore, the report showed that fungal elicitors enhanced effectively the production of secondary metabolites by regulating biosynthetic genes expressions which were involved in secondary metabolites biosynthesis pathway In *S. miltiorrhiza* hairy root cultures, *T. atroviride* D16 can stimulate tanshinone production by influencing the expression of related genes involved in the tanshinone biosynthesis pathway (Ming et al., 2013).

Moreover, fungal elicitor can induce generation of signal molecules and secondary metabolites biosynthesis (Xu, 2005). Nitric oxide (NO), salicylic acid (SA) and jasmonic acid (JA) are important signal molecules in plants. In addition, they are integral parts of signal transduction pathway involved in plant defense response (Yendo et al., 2014). NO, SA and JA production in plant cells increases after treatment with fungal elicitors. Furthermore, the signal moleculars that elicitors induced led to accumulation of bioactive compounds (Yendo et al., 2014). Fungal elicitors inducing the accumulation of SA in plant cell and JA has been investigated in signaling pathway for the accumulation of bioactive compounds in the plant (Xu et al., 2006). In particular, JA induces a wide variety of plant secondary products including terpenoids, flavonoids, alkaloids, and other compounds (Zhao et al., 2005).

Until now, few reports have described the effects of fungal elicitors isolated from ginseng rhizosphere soil on the production of signal molecules (NO, SA and JA), biosynthetic genes expression and bioactive compounds accumulation in adventitious roots of *P. ginseng*. What's more, the effect of fungal elicitor on the expression of three UDP-glycosyltransferases (UGT) genes UGTAE2, UGT94Q2 and UGTpg100, transforming a glucose moiety from UDP-glucose to dammarenediol, ginsenoside Rh_2 and protopanaxatriol to generate ginsenosides Rh_2, Rg_3 and Rh_1 respectively, has not been reported. Therefore, in this study, we investigated the impacts of concentration and contact time of fungal elicitors prepared from mycelium or fermentation broth on production of signal molecules, expression of biosynthetic genes and accumulation of bioactive compounds in adventitious root culture of *P. ginseng*. We also identified the ginsenoside composition based on HPLC-ESI–MS^n after fungal elicitor treatment.

2. Materials and methods

2.1. Adventitious root culture

The adventitious roots of *P. ginseng* were cultured in the liquid 3/4 strength Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 5.0 mg L^{-1} IBA, 0.1 mg L^{-1} KT and 4% sucrose. 1 g fresh weight of adventitious roots were cultured in Erlenmeyer flask containing 100 mL of the same medium (pH was adjusted 5.8 ± 0.2 before autoclaving at 121 °C for 25 min) on a rotary shaker (120rm) at 23 ± 2 °C and subcultured every 4 weeks.

2.2. Elicitor preparation

Aspergillus niger, *Aspergillus flavus* and *Aspergillus oryzae* were isolated from ginseng rhizosphere soil and maintained in our laboratory. Elicitors were prepared from *A. niger*, *A. flavus* and *A. oryzae* cultured in 200 mL Potato Dextrose Agar (PDA) medium at 28 °C in 500 mL flasks with shaking at 150 rpm for 7 days. Then, the mycelium with spores was filtered through 4 layers of cheesecloth and dried under vacuum at 50 °C for 24 h, before being ground to a powder using a mortar. The dry cell powder was dissolved in water, and a solution of 10 g L^{-1} concentration was obtained. The filtrate was centrifuged at 10,000*g* for 20 min and filtered through a 0.45 μm filter. In the end, the filtrate was evaporated to about a third of its original volume under vacuum at 60 °C. The solution of cell powder and concentrated filtrate was sterilized at 121 °C for 15 min before use. The concentration of the solutions was determined on its total carbohydrate. The total carbohydrate was analyzed by Anthrone-sulfuric acid method using glucose as the standard.

2.3. Elicitor treatment

Adventitious roots (10 g L^{-1}) were inoculated into ten 250 mL conical flasks containing 100 mL 3/4 MS liquid medium supplemented with 5.0 mg L^{-1} IBA, 0.1 mg L^{-1} KT and 4% sucrose at 23 ± 2 °C, and sub-cultured at intervals of four weeks. For elicitation, six elicitors prepared from mycelium (FM) and fermentation broth (FFB) were added to the 28-day-old adventitious root culture at five different concentrations 0, 50, 200, 400, 800 mg L^{-1} respectively. After elicitor treatment for one week, the adventitious roots were harvested. Then the dry weight and the content of bioactive compounds were analyzed. Furthermore, we also investigated effect of the best elicitor treatment time (0 h, 12 h, 24 h and 48 h) on accumulation of signal molecule (SA, JA and NO), ginsenosides content and expression level of functional genes involved in ginsenosides biosynthesis pathway.

2.4. Quantitation of SA, JA and NO of roots after treatment with A. niger in different time

0.25 g of adventitious roots (control group and elicitor group) was ground into powder using a mortar and pestle chilled with liquid nitrogen. Extraction and analyses of SA and JA were performed as described previously (Segarra et al., 2006).

The NO extracts were prepared by homogenizing 0.2 g of adventitious roots (control group and elicitor group) in a mortar on ice, using 1.0 mL distilled water. The content of NO was measured based on the principle of color reaction using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from 0.2 g sample from both the elicitor treated and control groups using the Plant RNA Kit (OMEGA, USA). 2.4 μg of total RNA was used for first strand cDNA synthesis with the HiFiScript 1Strand cDNA Synthesis Kit (CWBIO, China) according to the manufacturer's instructions. First-strand cDNAs were used as a template for RT-PCR reactions, which were performed as follows: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 57 °C for 1 min, and 72 °C for 50 s; with a final 2 min extension at 72 °C. The PCR products