Plant Physiology and Biochemistry 123 (2018) 406–413

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Ginsenosides and ginsenosidases in the pathobiology of ginseng-*Cylindrocarpon destructans* (Zinss) Scholten

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ARTICLE INFO

Keywords:
Antifungal activity
Araliaceae
Cylindrocarpon destructans (Zinss) Scholten
Ginsenosides
Glycosidase
Panax ginseng C.A. Meyer
Pathogenicity

ABSTRACT

To investigate the role that ginsenosides (and some of their metabolites) play in interactions between plants and phytopathogenic fungi (e.g. *Cylindrocarpon destructans* (Zinss) Scholten), we systematically determined the anti-fungal activities of six major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1), along with the metabolites of ginsenoside Rb_1 (Gypenoside XVII (G-XVII) and F_2), against the ginseng root pathogen *C. destructans* (Zinss) Scholten and non-ginseng pathogens *Fusarium graminearum* Schw., *Exserohilum turcicum* (Pass.) Leonard et Suggs, *Phytophthora megasperma* Drech. and *Pyricularia oryzae* Cav. Our results showed that the growth of both ginseng pathogens and non-pathogens could be inhibited by using the proto-panaxatriol (PPT) ginsenosides Re and Rg_1. In addition, the growth of the non-pathogens could also be inhibited by using proto-panaxadiol (PPD) ginsenosides Rb_1, Rb_2, Rc and Rd, whereas the growth of ginseng pathogen *C. destructans* (Zinss) Scholten was enhanced by ginsenosides Rb_1 and Rb_2. In contrast, ginsenoside G-XVII and F_2 strongly inhibited the hyphal growth of both *C. destructans* (Zinss) Scholten and the non-pathogens tested. Furthermore, addition of sucrose to the media increased the growth of *C. destructans* (Zinss) Scholten, whereas glucose did not affect the growth. Moreover, *C. destructans* (Zinss) Scholten and all four non-pathogens were able to deglycosylate PPD ginsenosides using a similar transformation pathway, albeit with different sensitivities. We also discussed the anti-fungal structure-activity relationships of the ginsenosides. Our results suggest that the pathogenicity of *C. destructans* (Zinss) Scholten against ginseng root is independent of its ability to deglycosylate ginsenosides.

1. Introduction

Panax ginseng C. A. Meyer, an important traditional Chinese herb, is highly valued both medically and economically. Because ginseng is usually cultivated on farms, the plant is susceptible to fungal diseases and rust rot caused primarily by the fungus *Cylindrocarpon destructans* (Zinss) Scholten (Farh et al., 2015; Lee, 2004; Rahman and Punja, 2005). Over thousands of years, plant-pathogen co-evolution has made the interaction between plants and fungi very complicated (Funnell et al., 2010). Pathogens infect the plant to obtain nutrients and produce harmful substances, and the plants defend themselves by producing large amounts of low-molecular-mass natural products (i.e. secondary metabolites) (Bednarek and Osbourn, 2009). Ginsenosides are secondary metabolites produced by *Panax* plants, including *P. ginseng* C. A. Meyer, and provide an effective defense against phytopathogenic fungi (Nicol et al., 2002, 2003; Zhao et al., 2012).

Ginsenosides are comprised of aglycone and sugar moieties (Fig. 1). Based on the carbon skeleton of aglycones, ginsenosides can be divided into dammarane and oleanane types (Jayakodi et al., 2015; Shin et al., 2015). According to the hydroxylation pattern of the parent aglycone and the position of sugar moieties, dammarane type ginsenosides are further classified into two types: proto-panaxadiol (PPD) and proto-panaxatriol (PPT) (Shin et al., 2015). Thirty-seven ginsenosides have been identified in the root of *P. ginseng* C.A. Meyer, and these account for 3%–6% of the total dry matter of the plant. The major PPD ginsenosides are Rb_1, Rb_2, Rc, Rd and PPT, along with Re and Rg_1 that account for more than 90% of the total ginsenosides (Güçlü-Üstündağ and Mazza, 2007; Ma et al., 2005; Qi et al., 2011). Ginsenoside Rb_1, the major component in the root, can reach up to 1.62% of the content in dry ginseng root (Ahmed et al., 2016; Shi et al., 2007).

Previous studies have shown that ginsenosides are secreted into the soil by the ginseng root, and all ginsenosides can inhibit the growth of non-ginseng pathogens. However, ginsenosides are not effective at dealing with root pathogens, and they can even beneficial for them. PPT

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https://doi.org/10.1016/j.plaphy.2017.12.038
Received 14 September 2017; Received in revised form 15 December 2017; Accepted 26 December 2017
Available online 29 December 2017

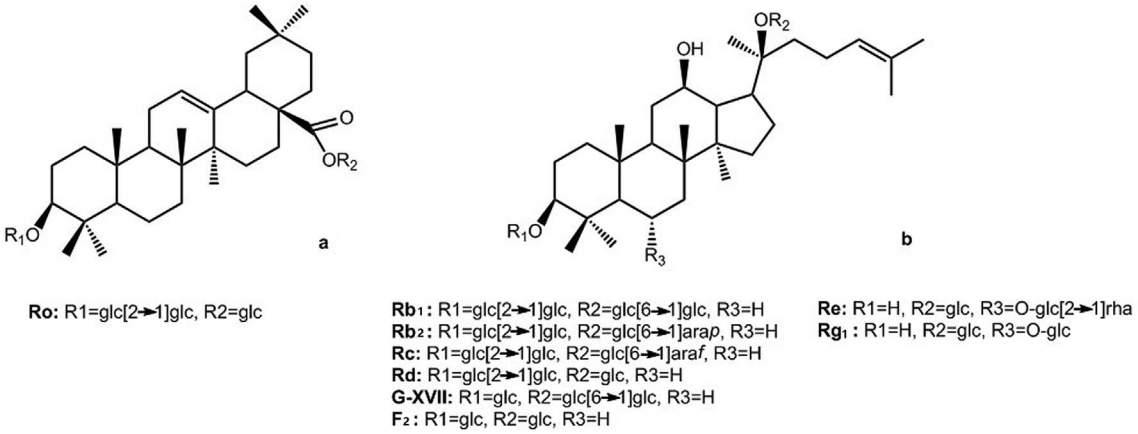

Fig. 1. Structures of common ginsenosides of *Panax ginseng* C. A. Meyer. Two classes of ginsenosides are shown: the oleanane type (a) and the dammarane type (b). Glc stands for glucose, ara*p* for arabinose (pyranose form), ara*f* for arabinose (furanose form) and rha for rhamnose.

and PPD both inhibit the growth of non-ginseng pathogens, whereas they exhibit different activities on the pathogen *C. destructans* (Zinss) Scholten. In this regard, PPT can inhibit its growth, whereas PPD can significantly enhance its growth (Nicol et al., 2002, 2003; Zhao et al., 2012). Recent studies have found that the ginsenoside Rb_1 can stimulate the growth of the ginseng pathogen *Pythium irregulare* Buisman, while its metabolite F_2 inhibits its growth (Ivanov et al., 2016). The stimulating effect from ginsenosides on the growth of pathogens is presumed to arise from the utilization of ginsenosides as a nutritional source, in which ginsenosides are metabolized to less polar products (structurally similar to sterols) that can be transported into the hyphae of the pathogen and can act as a growth hormone (Bernards et al., 2011; Neculai et al., 2009; Yousef and Bernards, 2006).

To investigate the role of individual ginsenosides on ginseng pathogens and the significance of their metabolism on the pathogenic behavior of *C. destructans* (Zinss) Scholten, we determined the antifungal activity of six major ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1), as well as the metabolites of ginsenoside Rb_1 (Gypenoside XVII (G-XVII) and F_2) against the ginseng root pathogen *C. destructans* (Zinss) Scholten and non-ginseng pathogens *Fusarium graminearum* Schw., *Exserohilum turcicum* (Pass.) Leonard et Suggs, *Phytophthora megasperma* Drech. and *Pyricularia oryzae* Cav. We also tested the effect of some sugars on fungal growth, and screened for the ability of fungi to deglycosylate ginsenosides by using extracellular glycosidases.

2. Methods

2.1. Microbial and ginsenoside materials

Fungal *Cylindrocarpon destructans* (Zinss) Scholten, *Fusarium graminearum* Schw., *Exserohilum turcicum* (Pass.) Leonard et Suggs, *Phytophthora megasperma* Drech. and *Pyricularia oryzae* Cav. were isolated from local plants and were a gift from the Jilin Academy of Agricultural Sciences. All isolates were maintained on V8 juice medium (the centrifugal supernatant of V8 juice 20%, Calcium carbonate 0.2% and agar 2%, autoclaving at 121 °C for 20 min) in the dark at 25 °C. The total ginsenosides from *Panax ginseng* C.A. Meyer were purchased from Hongjiu Biotechnology Co., Ltd., China. Ginsenoside standards were purchased from Chengdu Mansite Biotechnology Co., Ltd., China.

2.2. Preparation and analysis of individual ginsenosides

The pure ginsenosides (Rb_1, Rb_2, Rc, Rd, Re and Rg_1) were prepared from total ginsenosides by using chromatographic methods, including macroporous resin and silica gel as follows. The dry total ginsenosides powder was dissolved in distilled water and then adsorbed onto a macroporous resin column (D-101, Tianjin Chemical, China). The column was sequentially eluted with distilled water, ethanol/water (3/2, v/v) and ethanol/water (9/1, v/v). Part of the ethanol/water (3/2, v/v) fraction was concentrated and dried by rotary evaporation, and separated further by silica gel column chromatography eluted with n-butanol/acetidin/water (4/4/1, v/v/v). Five fractions were collected with R*f* values. According to the orders of R*f* values from large to small, these fractions were named as F-I, F-II, F-III, F-IV and F-V respectively. F-II, F-III and F-IV were defined as ginsenoside monomer Re, Rd and Rc. F-I was further separated and purified by silica gel column chromatography eluted with methanol/ethanol/acetidin (3/7/55, v/v/v) and finally yielded Rg_1 monomer. F-V was further eluted from the silica gel column with chloroform/methanol/water (65/35/10, v/v/v, lower phase) to obtain single Rb_1 and Rb_2.

Ginsenoside monomers G-XVII and F_2 were obtained by transforming ginsenoside Rb_1 using crude enzyme preparation from *C. destructans* (Zinss) Scholten as described by Zhao et al., (2012).

After freeze-drying, the ginsenoside monomers were dissolved in methanol to a concentration of 1 mg mL^{-1} for HPLC (High Performance Liquid Chromatography) analysis using a Shimadzu system equipped with a unitary C-18 column (4.6 mm × 250 mm, 5 μm). The mobile phase was distilled water (A) and acetonitrile (B), with the following gradient: 22.5% B (0–10 min), 22.5–70% B (10–40 min), 100% B (40–50 min). The column was eluted at 0.8 mL min^{-1} at room temperature. An elution profile was obtained using a UV/Vis detector at 203 nm (Supplement Fig. S1).

The ^{13}C-NMR (Nuclear Magnetic Resonance) spectrum of ginsenoside monomers was acquired on a Bruker Av 600 NMR spectrometer operating at a carbon frequency of 150 MHz with deuterated methanol as the solvent and tetramethylsilane (TMS) as the internal standard (detail in Supplement Table S1).

2.3. Bioassay for the sensitivity of fungi to ginsenosides

Fungi were tested for their sensitivity to ginsenosides by measuring the diameter of mycelial colonies grown on V8 medium supplemented with these compounds. The medium containing different concentrations of ginsenosides (0.03%, 0.06%, 0.125%, 0.5% and 2%, w/v) was prepared by mixing an appropriate volume of ginsenoside stock solution that had been filtered through a 0.2 μm filter. The ginsenoside stock solution was prepared using methanol at a concentration of 1% in the final volume of medium for both the treatment and control. A bioassay plate (60 mm diameter) contained approximately 7 mL of solid medium. A block (5 mm diameter) was taken from the edge of an actively growing colony on V8 medium and placed in the center of an experimental plate. Test cultures were incubated in the dark at 25 °C for