Investigation of ginsenosides in different tissues after elicitor treatment in *Panax ginseng*

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**A B S T R A C T**

**Background:** The effect of methyl jasmonate (MJ) on ginsenoside production in different organs of ginseng (*Panax ginseng* Meyer) was evaluated after the whole plant was dipped in an MJ-containing solution. MJ can induce the production of antioxidant defense genes and secondary metabolites in plants. In ginseng, MJ treatment in adventitious root resulted in the increase of *dammarenediol synthase* expression but a decrease of *cycloartenol synthase* expression, thereby enhancing ginsenoside biosynthesis. Although a previous study focused on the application of MJ to affect ginsenoside production in adventitious roots, we conducted our research on entire plants by evaluating the effect of exogenous MJ on ginsenoside production with the aim of obtaining new approaches to study ginsenoside biosynthesis response to MJ *in vivo*.

**Methods:** Different parts of MJ-treated ginseng plants were analyzed for ginsenoside contents (fine root, root body, epidermis, rhizome, stem, and leaf) by high-performance liquid chromatography.

**Results:** The total ginsenoside content of the ginseng root significantly increased after 2 d of MJ treatment compared with the control not subjected to MJ. Our results revealed that MJ treatment enhances ginsenoside production not in the epidermis but in the stele of the ginseng root, implying transportation of ginsenosides from the root vasculature to the epidermis. Application of MJ enhanced protopanaxadiol (PPD)-type ginsenosides, whereas chilling treatment induced protopanaxatriol (PPT)-type ginsenosides.

**Conclusion:** These findings indicate that the production of PPD-type and PPT-type ginsenosides is differently affected by abiotic and biotic stresses in the ginseng plant, and they might play different defense mechanism roles.

1. **Introduction**

*Panax ginseng* Meyer, which is commonly known as Korean ginseng, is one of the most important traditional medicines in East Asia. Triterpene glycoside saponin, named ginsenoside, is the main bioactive ingredient in *P. ginseng* and is known to exhibit various pharmacological and physiological effects including anticancer [1–3], antidiabetic [4,5], immunomodulatory [16], neuroprotective [1], radioprotective [7], anti-amnestic [1], and antistress properties [8,9]. The natural role of saponins in plants has been suggested to play a defensive role against pathogen and pest attacks [10]. The most important physiological role of ginsenosides in the ginseng plant is part of the defense mechanisms from pathogen attacks [11]. Naturally occurring ginsenosides are present to protect the ginseng from microbial and fungal infection; the bitter taste of ginsenosides makes them antifeedants [12–16].

Ginsenoside is contained in ginseng root at >4% by dry weight [17]. Ginsenosides are classified into two groups by the skeleton of aglycones, namely dammarane type and oleanane type. Dammarane-type tetracyclic structure is unique in ginseng, although other oleanane-type triterpenes are also observed in other plants. Dammarane-type ginsenosides consist mainly of two types that are classified according to their aglycone moieties, protopanaxadiol (PPD) and protopanaxatriol (PPT) ginsenoside.

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Ginsenoside backbones are synthesized via the isoprenoid pathway by cyclization of 2,3-oxidosqualene mediated by dammarenediol synthase (DDS) or β-amyrin synthase (β-AS). Although many reports have been published regarding the pharmacological effects of ginsenosides, little is known about the ginsenoside biosynthesis pathway or its regulation. Complete cDNA clones for several enzymes from ginseng have been reported. The genes encoding squalene synthase (SS), squalene epoxidase (SE), β-AS, lanosterol synthase, cycloartenol synthase (CAS), and DDS have been identified. Metabolic engineering such as overexpression or gene silencing of those genes has altered ginsenoside levels. Upregulation of ginsenoside levels by elicitors is also an attractive strategy to achieve greater ginsenoside quantities [18].

The accumulation of secondary metabolites can be enhanced by exposing plant cell and tissue cultures to biotic and abiotic elicitors [19]. When plants perceive environmental changes, they generate biological responses through specific signal transduction. Methyl jasmonate (MJ) has been reported to play an important role in the production of antioxidant defense genes and secondary metabolites in plants [20–22]. It has been reported that MJ stimulates ginsenoside production in cultured ginseng cells, hairy root, and adventitious roots [23–26]. MJ also increases the production of soyasaponin in cultured Glycyrrhiza glabra cells [27] and saikosaponin in the adventitious roots of Bupleurum falcatum [28]. The stimulation of saponin production by MJ treatment may be mediated by the upregulation of the genes involved in the biosynthesis of these saponins.

Elicitation using MJ treatment has been conducted on ginseng hairy roots and adventitious roots. Treatment of in vitro cultures with MJ may increase the production of ginsenosides up to ninefold [29]. However, no elicitation studies with MJ have been done with the entire P. ginseng plant. Although ginseng root is usually used for medicinal purposes, ginsenosides are distributed in many parts of the ginseng plant, including the root, leaf, and berry. Different parts of the plant contain distinct ginsenoside profiles [2], which may exhibit different pharmacological activities. We conducted our research on whole 3-yr-old ginseng plants. The aim of the present study was to investigate which organs of the ginseng plant respond to elicitor treatment in vivo, thereby potentially enhancing ginsenoside production.

2. Materials and methods

2.1. Ginseng materials and treatment

Three-yr-old ginseng plants hydroponically cultured in perlite and peat moss at 23 ± 2°C under white fluorescent light (60–100 μmol/m2/s) in a controlled greenhouse (kindly provided by i-farm in Yeo-Ju, Korea) were used for whole plant treatment. Ginseng roots were dipped in water containing 50 mM MJ and were maintained in the dark. After 2 d, fine root, root body (the inner part including xylem and pith), epidermis (the outer surface including cortex), rhizome, stem, and leaf parts were separately used for ginsenoside analysis. For chilling treatment, 1-yr-old ginseng roots were kept at 4°C for 4 wk. For

Fig. 1. Photographs of different organs of a 3-yr-old ginseng plant used for ginsenoside analysis. Three-yr-old ginseng plants hydroponically cultured in perlite and peat moss were used for methyl jasmonate (MJ) treatment. For ginsenoside analysis, different organs were sampled separately: the (A) leaf, stem, (B) rhizome, and fine root were sampled. The main root was divided again into the epidermis (the outer surface including the cortex) and (C) the root body (the inner part including the xylem and pith) by peeling. (D) A horizontal close-up image of the main root. All bars indicate 1 cm.
ginsenoside analysis, rhizome, epidermis, upper and lower root body, and fine root parts were sampled separately.

2.2. Analysis of ginsenosides by high-performance liquid chromatography

Milled powder (0.3–1 g) of freeze-dried adventitious roots, leaves, and roots of ginseng were twice soaked in an 80% (v/v) methanol solution at 70°C for 1 h. The extract was filtered and then evaporated to remove the liquid. The residue was dissolved in distilled water followed by extraction with water-saturated n-butanol. The butanol layer was then evaporated to produce the saponin fraction. Each sample was dissolved in methanol (1 g/5 mL), filtered using a 0.45-μm filter, and then used for high-performance liquid chromatography (HPLC) analysis. The HPLC separation was carried out on an Agilent 1260 series HPLC system (Palo Alto, CA, USA). This experiment employed a C18 (250 mm × 4.6 mm, ID 5 μm) column using distilled water (Solvent A) and acetonitrile (Solvent B) mobile phases, with a flow rate of 1.6 mL/min and the following gradient: A/B ratios of 80.5:19.5 for 0–29 min, 70:30 for 29–36 min, 68:32 for 36–45 min, 66:34 for 45–47 min, 64.5:35.5 for 47–49 min, 0:100 for 49–61 min, and 80.5:19.5 for 61–66 min. The sample was detected at a wavelength of 203 nm. Quantitative analysis was performed via a one-point curve method using external standards of authentic ginsenosides.

2.3. Statistical analyses

The values of the ginsenoside contents and relative gene expression were expressed as mean ± standard deviation (SD). Statistical analyses were carried out using the GraphPad Prism software (GraphPad, San Diego, CA, USA) by one-way analysis of variance (ANOVA). Duncan’s multiple range test was employed to test for significant differences between the treatments at p < 0.05 and p < 0.01.

3. Results

3.1. Response of different ginseng tissues to MJ treatment

The total ginsenoside contents in each tissue of the entire ginseng plant were analyzed. Cultivation of ginseng by hydroponics involves a shorter cultivation period in a greenhouse in which variables such as light, temperature, moisture, and carbon dioxide content can be controlled [30,31]. Therefore, we used hydroponically cultured 3-yr-old ginseng plants (Fig. 1). Fig. 2 shows that ginsenoside accumulations within the aerial parts (leaf and stem) were increased as compared with the control. Total ginsenoside contents in the leaf were higher than other tissues. In addition, total ginsenoside contents within the underground parts (rhizome, root body, epidermis, and fine root) were also increased, except in the epidermis. Total ginsenoside contents of the root body in MJ-treated plants increased by approximately twofold compared with that of the control. This result demonstrates that the increase in ginsenoside contents of the root body is the highest among all tested ginseng organs. In rhizome, total ginsenoside accumulation and its composition was significantly increased after MJ treatment. Total ginsenoside content of fine roots was increased by approximately 6 mg/g compared with the control, which is the most increased content observed in underground parts. In the epidermis, total ginsenoside content was only minimally influenced by MJ treatment.

Fig. 3 shows the accumulation of individual ginsenosides in different tissues. The content of ginsenoside Re in aerial parts (leaf and stem) of the ginseng plant was the highest. In leaf, ginsenoside Re and Rd contents were mainly enhanced. The ratio between PPD-type and PPT-type ginsenosides was significantly changed in the stem. The content of ginsenoside Rd was increased more than other ginsenosides; therefore, the ratio of PPD-type ginsenoside was increased. In rhizome, the ratio of PPD-type ginsenoside was also increased due to accumulated ginsenoside Rd, although the content of ginsenoside Rg1 in the rhizome was the highest. The greatest increase of ginsenoside level was shown in the root body. All individual ginsenoside contents were increased. Levels of ginsenosides Rb1 and Rg1 were doubled as compared with the control. Although the content of ginsenoside Rg1 in the rhizome was the highest, ginsenoside Rd was enhanced fivefold. In addition, ginsenoside Rc and Rb2, which was not detected in the control, accumulated after MJ treatment, showing in the increased ratio of PPD-type ginsenoside. In fine root, all individual ginsenosides were also increased. Fine roots contained mostly ginsenoside Re, but the ratio of ginsenoside Rb1 was enhanced upon MJ treatment. Therefore, the ratio of PPD-type ginsenoside was increased to greater than that of the PPT-type ginsenoside. In the epidermis, all individual ginsenoside accumulation was similar to the control.

3.2. Response of different ginseng tissues to chilling treatment

As a representative treatment for abiotic stress, we treated chilling stress to root and analyzed ginsenoside contents. Total ginsenoside contents of chilled ginseng were analyzed in different organs from 1-yr-old root (rhizome, epidermis, upper root body, lower
Fig. 3. Individual ginsenoside contents by methyl jasmonate (MJ) elicitation in different ginseng plant tissues. Three-yr-old ginseng plants were treated with 50μM of MJ for 2 d. Individual ginsenoside contents in the leaf, stem, rhizome, epidermis, root body, and fine root were analyzed. Protopanaxadiol (PPD) indicates the sum of ginsenoside Rb1, Rb2, Rc, and Rd, and protopanaxatriol (PPT) indicates the sum of ginsenoside Re, Rg, Rg1, and Rg2. Vertical bars indicate the mean value ± standard deviation from three independent experiments. *p < 0.05 and **p < 0.01 denote significant difference from the control, respectively.

As shown in Fig. 4, total ginsenoside levels of all tissues were increased. In particular, total ginsenoside contents of the lower root body after removing the epidermis increased approximately eightfold as compared with the control. Total ginsenoside contents of the lower root body showed the highest increased level of approximately 14 mg/g compared with the control (Fig. 4C). Total ginsenoside contents of the upper root body after removing the epidermis were increased threefold as compared with the control. The ratio of ginsenoside accumulation in the upper root body to lower root body in the control was 1:2. After chilling treatment, this ratio was changed to 1:5. In addition, the ratio of ginsenoside accumulation in the lower root body to fine root in the control was 1:13. After chilling treatment, a 1:2 ratio was noted.

We also analyzed the contents of individual ginsenosides in different organs upon chilling treatment (Fig. 5). In the epidermis, the contents of ginsenosides Re, Rb1, and Rg2 were significantly increased after chilling. By contrast, ginsenoside Rg1 was decreased in root rhizome and epidermis. Ginsenoside Re increased to the highest level in the epidermis as compared with the control (~6.6 mg/g) and was not significantly increased in rhizome (Fig. 5). In fine root, ginsenoside Re content was increased, whereas ginsenoside Rg1 content was essentially unchanged, and ginsenosides Rb1, Rc, and Rb2 were reduced. Ginsenoside Re content was increased to the highest ratio and level compared with the control in fine root. The upper and lower roots of the body both showed increased ginsenoside accumulation of most ginsenosides. In the upper root, ginsenosides Rc and Rb2 were not detected but were present after chilling treatment. Ginsenoside Rd content significantly increased by approximately 14-fold. All individual ginsenosides in the lower root body highly increased after chilling treatment (Fig. 5). Ginsenosides Re, Rb2, and Rd were dramatically enhanced. The ratio of ginsenosides Re and Rb2 was increased more than 20-fold. The ratio of PPT-type ginsenosides was increased in all tissues except the rhizome, which showed a static level.
Cortex and xylem of the ginseng root [33]. Histochemical studies showed that ginsenosides are located in the periderm and outer cortex regions of the root, and root hairs contain higher ginsenoside contents than main roots [35,36].

Recent, multicenter matrix-assisted laser desorption/ionization mass spectrometry imaging confirmed that ginsenosides were more highly concentrated in the cortex and the periderm than that in the medulla of a lateral root, and localization of ginsenosides in the root tip is higher than that in the pith of the root [38]. In addition, a quantitative difference was detected between localizations of PPD-type ginsenosides (Rb1, Rb2, or Rc) and the PPT-type ginsenoside (Rf) in the root [38]. As saponins are known to be distributed to the root epidermis [34], we confirmed the accumulation of ginsenosides in the epidermis rather than the root body (J. Y. Oh et al, unpublished). However, our data in other work showed that ginsenoside biosynthesis genes are expressed in the root vasculature, such as phloem [18,39]. This controversial distribution and biosynthesis information led us to hypothesize that ginsenosides are produced in the root vasculature and then transported to the epidermis for a defensive role. To confirm this hypothesis, we selected MJ, known as a strong effective elicitor, to stimulate the biosynthesis of ginsenoside in vivo.

To improve the metabolite contents, some elicitors have been used to increase the expression and activities of key enzymes in the rate-limiting step of the biosynthetic pathway. MJ is a key signaling compound involved in the elicitation process, which leads to the accumulation of secondary metabolites [40]. Because ginsenosides are secondary metabolites in ginseng, the accumulation of these compounds is also controlled by the treatment of elicitors such as MJ and salicylic acid [41,42]. The total ginsenoside content increases approximately fourfold following MJ treatment in suspension cultured adventitious roots [6]. Depending on the timing of MJ application, the adventitious roots appear to show different growth effects and ginsenoside production. It was shown from previous reports that the addition of MJ at the early phase of Panax ginseng growth inhibits adventitious root growth [43]. Jasmonic acid (JA) also strongly inhibited ginseng hairy root growth [23]. To prevent a reduced biomass of adventitious roots, mostly 10µM of MJ was used in adventitious root cultures of P. ginseng 4 wk after inoculation. Higher MJ concentration and extended cultivation time also showed effects on root growth [43,44].

According to previous studies, this elicitation effect of ginsenosides is attributable to an MJ-induced expression of ginsenoside biosynthetic genes [6,29]. In our experiment using ginseng adventitious root, the expression of PgHMGR1, which plays a role in the production of mevalonic acid (MVA), was increased threefold (J. Y. Oh et al, unpublished). The expression of PgDDS, which is involved in the dammarenediol backbone for ginsenoside, was strongly upregulated, whereas PgCAS was decreased (data not shown). This expression pattern is similar to a previous report wherein MJ induced changes of triterpene saponins in ginseng hairy root [29]. Exposure to MJ at 100µM in hairy roots of P. ginseng induced the expression of genes involved in ginsenoside biosynthesis, such as PgSS, PgSE, and PgDDS, with a slight decrease of PgCAS [29,45], suggesting that MJ as a signal transducer may stimulate ginsenoside production by activation of the enzymes in the MVA pathway to dammarenediol and may also inactivate enzymes for sterol production.

Our present and previous results confirmed MJ as an effective elicitor of ginsenoside synthesis in ginseng adventitious roots. However, until now, it was not clear if MJ had the same effect on the whole ginseng plant. In this study, we tested the effect of MJ as an elicitor of ginsenoside accumulation in whole ginseng plants. When we analyzed the ginsenoside contents of the whole ginseng plant after exposing the ginseng root to MJ for 2 d, a pronounced increase of the ginsenosides was observed in the leaf, stem, root body, and fine root, with the greatest increase noted in the root body. An interesting observation was that most accumulation was observed in the root body, not the epidermis, which is known to have a high ginsenoside content. Rather, the epidermis did not show any alteration, indicating that ginsenoside biosynthesis actively occurs in the root body. After production in the root cortex, ginsenosides may be transported to the epidermis to play a defensive role. Ginsenosides can be synthesized in vasculature tissue such as phloem [46] and then be transported for storage or play a defensive role. Saponin glycosides can be stored in vacuoles through the fusion of endoplasmic reticulum-derived vesicles [47] or transported by the ATP-binding cassette transporter [48] or multidrug and toxic compound extrusion transporters [49,50]. Further studies...
on the ginsenoside transporter will provide more detail regarding ginsenoside transport.

Upon exposure of hairy roots or roots of *P. ginseng* to MJ, both tissues showed increased PPD-type ginsenoside content, whereas PPT-type ginsenoside content changed only slightly compared with controls [6,29]. JA also improved the accumulation of PPD ginsenosides much more than PPT ginsenosides [23], indicating that JA and its MJ elicitor might have triggered the synthesis of PPD-type ginsenosides. Similarly, different tissues in our experiment showed more accumulation of PPD-type ginsenosides, especially in the stem, root body, and fine root (Fig. 3). The recent discovery of protopanaxadiol synthase (PPDS), a cytochrome P450 (P450) for production of PPD ginsenosides much more than PPT ginsenosides [23], indicating that JA and its MJ elicitor might have triggered the synthesis of PPD-type ginsenosides. Similarly, different tissues in our experiment showed more accumulation of PPD-type ginsenosides, especially in the stem, root body, and fine root (Fig. 3). The recent discovery of protopanaxadiol synthase (PPDS), a cytochrome P450 (P450) for production of PPD ginsenosides much more than PPT ginsenosides [23], indicating that JA and its MJ elicitor might have triggered the synthesis of PPD-type ginsenosides. Similarly, different tissues in our experiment showed more accumulation of PPD-type ginsenosides, especially in the stem, root body, and fine root (Fig. 3). The recent discovery of protopanaxadiol synthase (PPDS), a cytochrome P450 (P450) for production of PPD ginsenosides much more than PPT ginsenosides [23], indicating that JA and its MJ elicitor might have triggered the synthesis of PPD-type ginsenosides. Similarly, different tissues in our experiment showed more accumulation of PPD-type ginsenosides, especially in the stem, root body, and fine root (Fig. 3).

Overall, PPT-type ginsenoside accumulation was enhanced by chilling treatment (Fig. 5). When we performed chilling treatment as an abiotic stress, PPT-type ginsenosides were increased in the epidermis, upper and lower root body, and fine root, differently than with MJ treatment. This implies that ginseng roots are capable of differentially activating distinct defense pathways. The production of various secondary metabolites, including ginsenosides, is usually associated with defense responses to stresses [53]. JA and its methyl ester MJ are signaling compounds that modulate various physiological processes in plants, such as root growth, senescence, and the defense response against pathogens and insect attack [54]. In addition, MJ induces or increases the biosynthesis of many secondary metabolites that play important roles in the adaptation of plants to particular environments [21,55]. MJ treatment actually mimics a pathogen or herbivorous attack [56]. Saponins also have

[Fig. 5. Individual ginsenoside contents upon chilling stress treatment in different ginseng plant tissues. One-yr-old ginseng plants were subjected to chilling for 4 wk. Individual ginsenoside contents in rhizome, epidermis, upper root body, lower root body, and fine root, as shown in Fig. 4, were analyzed. Protopanaxadiol (PPD) indicates the sum of ginsenoside Rb1, Rb2, Rc, and Rd, and protopanaxatriol (PPT) indicates the sum of ginsenoside Re, Rg, Rg1, and Rg2. Vertical bars indicate the mean value ± standard deviation from three independent experiments. *p < 0.05 and **p < 0.01 denote significant difference from the control, respectively.]
potent antifungal activities, as has been shown for avenacins, which are oleane-type triterpene saponins found in oats [24]. However, although there is less known about the physiological role of ginsenoside, it is also considered to have a defensive role [12]. Different elicitation effects of MJ and chilling on individual ginsenosides might be explained by the differentiated defense strategy of ginsenosides (Fig. 6), correlated with the different biological activities of different ginsenosides.

The best of our knowledge, this study is the first to report on the evaluation of individual ginsenoside levels in separate epidermis and root body. Moreover, this is the first study to provide a detailed profile of ginsenoside composition in different organs of whole ginseng plants following elicitor treatment, although further studies of gene expression in different tissues of ginseng will be required.

Conflict of interest

All contributing authors declare no conflicts of interest.

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