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Research article

Ginsenoside profiles and related gene expression during foliation in *Panax ginseng* Meyer

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ABSTRACT

Panax ginseng is one of the most important medicinal plants in Asia. Triterpene saponins, known as ginsenosides, are the major pharmacological compounds in *P. ginseng*. The present study was conducted to evaluate the changes in ginsenoside composition according to the foliation stage of *P. ginseng* cultured in a hydroponic system. Among the three tested growth stages (closed, intermediate, and opened), the highest amount of total ginsenoside in the main and fine roots was in the intermediate stage. In the leaves, the highest amount of total ginsenoside was in the opened stage. The total ginsenoside content of the ginseng leaf was markedly increased in the transition from the closed to intermediate stage, and increased more slowly from the intermediate to opened leaf stage, suggesting active biosynthesis of ginsenosides in the leaf. Conversely, the total ginsenoside content of the main and fine roots decreased from the intermediate to opened leaf stage. This suggests movement of ginsenosides during foliation from the root to the leaf, or vice versa. The difference in the composition of ginsenosides between the leaf and root in each stage of foliation suggests that the ginsenoside profile is affected by foliation stage, and this profile differs in each organ of the plant. These results suggest that protopanaxadiol- and protopanaxatriol (PPT)-type ginsenosides are produced according to growth stage to meet different needs in the growth and defense of ginseng. The higher content of PPT-type ginsenosides in leaves could be related to the positive correlation between light and PPT-type ginsenosides.

1. Introduction

Panax ginseng Meyer is a slowly growing perennial herb belonging to the Araliaceae family. It has been cultivated for its highly valued roots and used in traditional medicine as a natural adaptogen for >1000 yr [1]. Ginseng has numerous pharmacological effects on humans, including anticancer [2–4], antidiabetic [5,6], immunomodulatory [2,7], neuroprotective [2], radioprotective [8], antiamnestic [2], and antistress [9] properties. Most of the medicinal effects of ginseng have been attributed to triterpene saponins, which are referred to as ginsenosides. More than 40 ginsenosides have been isolated and identified from white and red ginseng, showing different biological activities based on their structural differences [10–15]. Two types constitute >80% of the identified ginsenosides: protopanaxadiol (PPD)-type saponins (sugar moieties are attached to the β-OH at C-3 and/or C-20) such as ginsenosides Rb1, Rb2, Rc, and Rd, and protopanaxatriol (PPT)-type saponins (sugar moieties are attached to the α-OH at C-6 and/or β-OH at C-20) such as ginsenosides Re, Rg1, and Rf [16].

The cultivation of *P. ginseng* is difficult due to the long duration (4–6 yr) needed for cultivation, and due to plant diseases such as red skin and root rot. Furthermore, ginseng needs to be cultivated under special conditions to meet its requirements of about 30% full sunlight. High exposure to light (50% solar radiation) decreases the levels of ginsenosides in *Panax pseudoginseng* [17], while exposure to >36% sunlight has been reported to cause photobleaching and leaf death in *P. ginseng* plants [18]. Although there have been many studies on the production of ginsenoside using tissue and cell cultures, the productivity has been low. To meet the demand for safe agricultural products of high quality, the cultivation of ginseng by hydroponics was developed in Korea [19,20]. This technique involves a shorter period by cultivation in a greenhouse in which variables such as light, temperature, moisture, and carbon dioxide content can be controlled. Hydroponic systems can produce

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ginseng roots that are pesticide free and ginseng leaves with high ginsenoside contents [19,20].

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. Although the *P. ginseng* root has been the main component in medicinal uses of ginseng, recent studies have revealed that the leaf and root hair contain higher ginsenoside levels than the root [21]. Ginseng berries contain ginsenoside levels that are 4.8 times higher than the levels in cultivated 4-yr ginseng roots, with the levels of the ginsenoside Re being 28 times higher in the berry than in the root [22,23]. Ginsenoside content in the root and root hair increases with age in *P. ginseng* plants from 1 yr to 5 yr, but it decreases with age in the leaves, except there is no alternation in the 3-yr-old stage [21]. Although several studies have evaluated the ginsenoside content in different parts of the plant at different ages, there have been no studies investigating the ginsenoside profile of plants in different foliation stages. The present study was conducted to investigate the changes in ginsenoside composition in the leaves and root of 3-yr-old ginseng plants cultivated by hydroponics according to their foliation stage.

2. Materials and methods

2.1. Ginseng materials

Samples were obtained from 3-yr-old ginseng plants hydroponically cultured in perlite and peat moss and grown at 23 ± 2°C under white fluorescent light (60–100 μmol/m^2/s) in a controlled greenhouse (kindly provided by i-farm in Yeoju, Korea). For the ginsenoside analysis and RNA extraction, the plant leaves, main roots, and fine roots were sampled at different stages during foliation (Fig. 1).

2.2. Analysis of ginsenosides by HPLC

First, 0.8 g milled powder from heat-dried leaves, main roots, and fine roots was soaked in 80% methanol at 80°C. After the liquid evaporated, the residue was dissolved in water and extracted with water-saturated *n*-butanol. The butanol layer was then evaporated to produce a saponin fraction. Each sample was dissolved in methanol (1 g/5 mL) and then filtrated through a 0.45-μm filter for HPLC analysis. The HPLC separation was carried out on an Agilent 1260 series HPLC system (Agilent, Palo Alto, CA, USA), equipped with an autosampler and an UV detector using a C18 column (4.6 mm × 50 mm, 1.8 μm; Zorbax Eclipse Plus, Agilent). Gradient elution was used using solvent A (100% acetonitrile) and solvent B (100% water) at 38°C using the following gradient program: 0–4 min, 19% A (isocratic); 4–9 min, 19–25% A; 9–20 min, 25–40% A; 20–25 min, 40–56%; 25–28 min, 56–70% A; 28–29 min, 70–100% A; 29–35 min, 100% A (isocratic); 35–36 min, 100–19% A; 36–42 min, 19% A (isocratic). The flow rate was kept at 1.2 mL/min, the sample injection volume was 5 μL, and UV absorption was measured at 203 nm. Quantitative analysis was performed using a one-point curve method using external standards of authentic ginsenosides.

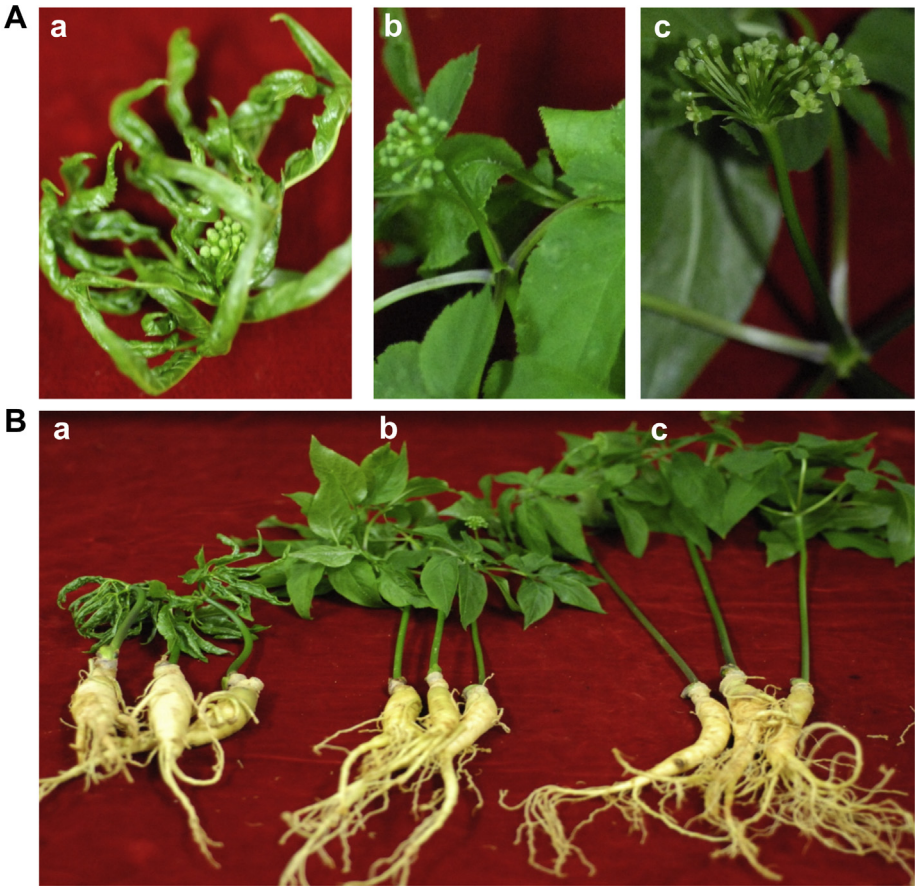

Fig. 1. Phenological growth stage of ginseng during foliation. Three-year-old ginseng plants hydroponically cultured in perlite and peat moss were sampled. For the ginsenoside analysis and RNA extraction, the leaf, main root, and fine root were sampled at different stages during foliation, including the (a) closed, (b) intermediate, and (c) opened leaf stages. (A) Closed-up leaf and inflorescence. (B) Whole plants during foliation stage.

2.3. Quantification of transcript levels

Total RNA was extracted from the frozen samples with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) including the DNase I digestion step. Next, 2 μg total RNA was reverse transcribed with the RevertAid H Minus M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). Real-time quantitative polymerase chain reaction was performed using 100 ng cDNA in a reaction volume of 10 μL using SYBR Green Sensimix Plus Master Mix (Quantace, Watford, UK). The thermal cycler conditions recommended by the manufacturer were used: 10 min at 95°C, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. The fluorescent product was detected during the final step of each cycle. Amplification, detection, and data analysis were carried out on a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). The primers used were 5′-CCT CGC CAG ATT TGG AGT AA-3′ and 5′-GCA CAG AAC CGG AAG ATA GC-3′ for *PgSS* (AB115496); 5′-GAT GTG CCT GGA CAA AAG GT-3′ and 5′-AGG ATG GCG CGC ATA TTG AAA G-3′ for PgSE (AB122078); 5′-GAG AGA TCC GAC ACC TCT GC-3′ and 5′-ATT TTG AGC TGC TGG TGC TT-3′. To determine the relative fold-differences in template abundance for each sample, the Ct values for each of the gene-specific primers were normalized to the Ct value for β-actin (5′-AGA GAT TCC GCT GTC CAG AA-3′ and 5′-ATC AGC GAT ACC AGG GAA CA-3′) and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$.

2.4. Statistical analysis

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

3. Results

3.1. Ginsenoside contents during foliation

The ginsenoside contents of the ginseng leaves and roots were evaluated at different foliation stages. As a perennial herbal plant, ginseng leaves fall and sprout annually, with flowers and berries developing in the 3rd yr of growth. As shown in Fig. 1, we sampled three different stages of leaves, which we referred to as (a) "closed", (b) "intermediate", and (c) "opened", from 3-yr-old ginseng plants cultured by hydroponics. When the ginseng plants sprouted, their leaves appeared closed (Figs. 1 and 2A) and they had an average leaf length of 3 cm, an average shoot height of 7 cm, and an average main root length of 9 cm (Fig. 1Ba). In this early developmental stage, the flower bud was already formed, although the peduncle was short (Fig. 2A). In the intermediate leaf stage (Fig. 2A), the average leaf length was 4.5 cm and the average peduncle length was 4.5 cm. After foliation, the leaves expanded (Fig. 2A) and the flower buds started to bloom (Fig. 1Ac), showing an average leaf length of 6 cm and an average peduncle length similar to that of the intermediate stage. During foliation, the shoot continued to elongate to 10 cm in the intermediate stage (Fig. 1Bb), and grew to 18 cm in the open leaf stage (Fig. 1Bc). The main root did not elongate, but the fine roots grew out sideways during foliation (Fig. 1Bb and Bc).

Before foliation, the total ginsenoside content in the closed leaves was the lowest among the different leaf stages (10.9 mg/g dry weight). As shown in Fig. 2B, the total ginsenoside content during foliation increased rapidly (by 3 times) from the closed to the intermediate stage (30.8 mg/g dry weight), and then slowly (by 1.2 times) from the intermediate to the opened stage (38.3 mg/g). All individual ginsenosides, with the exception of the ginsenoside Rb1, increased three to four times from the closed to the intermediate leaf stage (Fig. 2C). In contrast, as shown in Fig. 3A and C, the total ginsenoside content in the main root (12.3–12.5 mg/g) and the fine root (18–20.1 mg/g) did not significantly increase from the

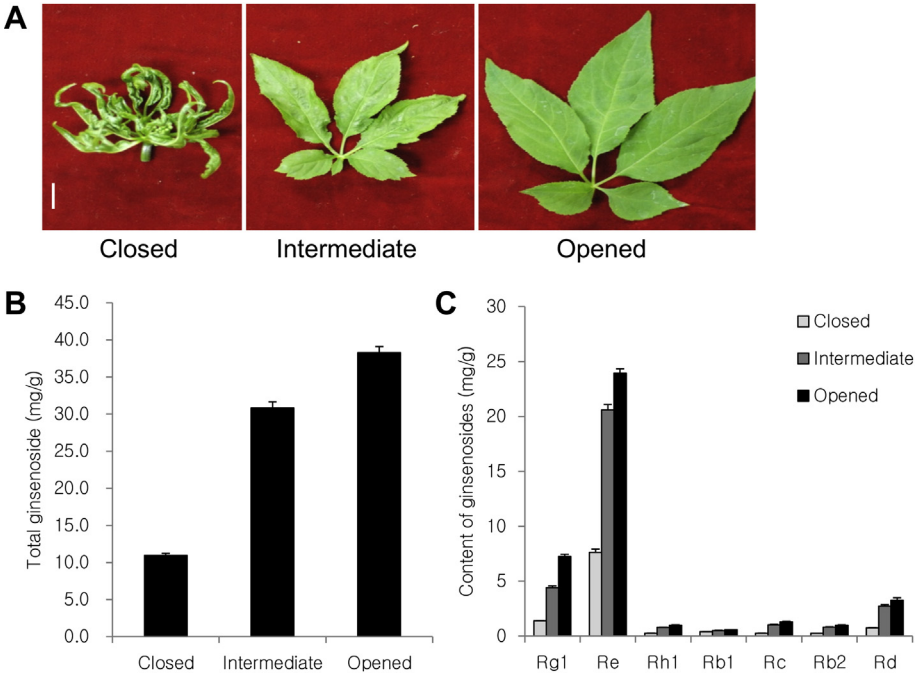

Fig. 2. Ginsenoside contents of leaves from *Panax ginseng* during foliation. (A) Closed, intermediate, and opened leaf stages were considered in the ginsenoside analysis. Bar indicates 1 cm. (B) Total ginsenoside content in the leaves were analyzed. (C) The major individual ginsenosides in the leaves were analyzed. Vertical bars indicate the mean ± standard error from three independent experiments.

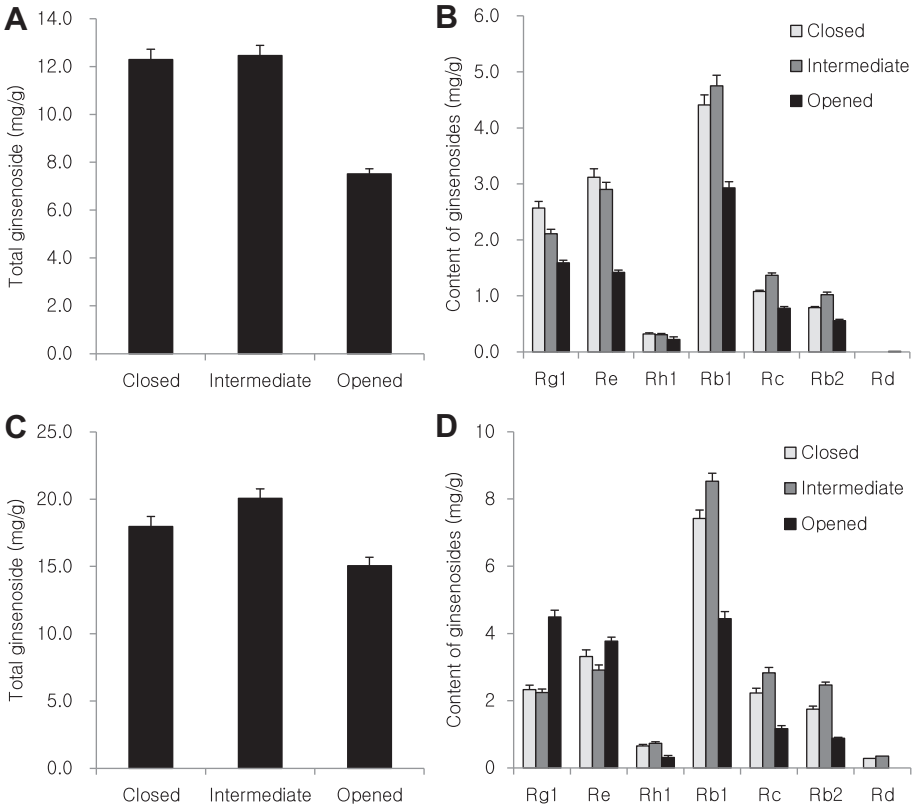

Fig. 3. Ginsenoside contents of roots from *Panax ginseng* during foliation. The total ginsenoside content in the (A) main root and (C) fine root was analyzed according to the closed, intermediate, and opened leaf stages in Fig. 1. The major individual ginsenosides in the (B) main root and (D) fine root were analyzed. Vertical bars indicate the mean ± standard error from three independent experiments.

closed to the intermediate stage. After the leaf opened, the total ginsenoside contents decreased by about 0.7 times in both the main root (7.5 mg/g) and the fine root (15.1 mg/g).

3.2. Ginsenoside profiles during foliation

The ratio of PPD-type ginsenosides (Rb1, Rb2, Rc, and Rd) to PPT-type ginsenosides (Rg1, Re, and Rh1) changed during foliation (Table 1). In the transition from the closed to the intermediate stage, this ratio increased in the main and fine roots. In particular, PPT-type ginsenosides such as Rg1 and Re decreased, while PPD-type ginsenosides increased in the main and fine roots of plants in the intermediate leaf stage, with the exception of the PPT-type ginsenoside Rh1. Interestingly, the PPD/PPT ratio decreased in the fine roots after foliation. The levels of the ginsenosides Rg1 and Re increased by 1.2 and 2 times, respectively, while all other ginsenoside levels decreased.

The ratios of the main ginsenosides Rb1, Re, and Rg1 also changed in different organs during foliation (Table 1). In leaves, the percentage of the ginsenosides Re and Rb1 decreased, although their absolute contents increased during foliation. However, the percentage of Rg1 among the total ginsenosides increased. In the main roots, the ratio of Re to the total ginsenosides decreased during foliation, while the ratio of Rb1 to the total ginsenosides increased. The fine roots showed a similar ginsenoside pattern to that of the main roots in the closed and the intermediate stage, but showed a different pattern in the opened leaf stage. The ratios of the PPT-type ginsenosides Re and Rg1 to the total ginsenoside content increased.

Table 1
The ginsenoside compositions of the leaf, main root, and fine root during foliation in closed, intermediate, and opened leaf stage

Ginsenosides	Leaf			Main root			Fine root		
	Closed	Intermediate	Opened	Closed	Intermediate	Opened	Closed	Intermediate	Opened
Total ginsenosides[1)]	10.92 ± 0.30	30.82 ± 0.82	38.28 ± 0.80	12.29 ± 0.43	12.46 ± 0.43	7.51 ± 0.22	17.97 ± 0.75	20.06 ± 0.70	15.06 ± 0.62
PPD[2)]	1.65 ± 0.05	5.04 ± 0.18	6.12 ± 0.20	6.28 ± 0.19	7.14 ± 0.24	4.28 ± 0.13	11.68 ± 0.45	14.18 ± 0.46	6.49 ± 0.29
PPT[3)]	9.27 ± 0.28	25.78 ± 0.58	32.16 ± 0.51	6.01 ± 0.25	5.32 ± 0.19	3.23 ± 0.10	6.29 ± 0.34	5.88 ± 0.27	8.57 ± 0.34
PPD/PPT	0.18	0.20	0.19	1.04	1.34	1.33	1.86	2.41	0.76
Rb1/total ginsenoside	0.04	0.02	0.01	0.36	0.38	0.39	0.41	0.43	0.29
Re/total ginsenosides	0.70	0.67	0.63	0.25	0.23	0.19	0.18	0.15	0.25
Rg1/total ginsenoside	0.13	0.14	0.19	0.21	0.17	0.21	0.13	0.11	0.30

All values are expressed as mean ± standard error ($n = 3$).
PPD, protopanaxadiol; PPT, protopanaxatriol.
[1)] Sum of individual ginsenosides content.
[2)] Rb1 + Rb2 + Rc + Rd.
[3)] Re + Rg1 + Rh1.

3.3. Biosynthesis of ginsenoside-related genes during foliation

As shown in Fig. 4D, ginsenoside is biosynthesized in ginseng by the mevalonic acid pathway. To investigate ginsenoside biosynthesis, we conducted a real-time polymerase chain reaction to analyze the expression of squalene synthase (*PgSS*), squalene epoxidase (*PgSE*), and dammarenediol synthase (*PgDDS*) genes in leaves during foliation. Expression of *PgSS* and *PgSE* increased about 1.5 times in the intermediate and opened leaf stage (1.48- and 1.65-fold, respectively, for *PgSS*; 1.53- and 1.62-fold, respectively, for *PgSE*). The transcript levels of *PgDDS* under conditions of intermediate stage and opened stage were 4.2- and 4.6-fold higher, respectively, than that of the closed leaf stage.

4. Discussion

In this study, we used 3-yr-old hydroponic-cultured ginseng for ginsenoside analysis. Ginseng grown with this method has a different ginsenoside composition compared with that of soil-cultivated ginseng, as shown in a study of 1-yr-old ginseng by Kim et al [20]. First, the leaves and roots of hydroponic ginseng contain the ginsenoside Rh1, which is not detected in soil-cultivated ginseng roots [19]. Rh1 has been reported to possess antiallergic and anti-inflammatory activities [24]. Second, hydroponic-cultured leaves contain a lower ratio of PPD/PPT (0.19) compared with soil-cultivated ginseng leaves (0.35), as shown by Han et al [25]. In particular, the percentage of the ginsenoside Re in hydroponic-cultured ginseng leaves (about 60%) was about three times higher than in its root (about 20%). Soil-cultivated ginseng leaves also contain the highest amount of Re compared with the other ginsenosides, but this amount is only 40–50% of the total ginsenoside content [21]. Re is well known to be a physiologically active substance with anti-inflammatory effects [26] and antidiabetic activities [27]. The levels of this ginsenoside can reach up to 60% in ginseng berries [23]; the highest amount found in the ginseng plant. Based on these findings, hydroponic culturing of ginseng leaves can be used to produce Re. These data confirm that the composition of individual ginsenosides may differ depending on the cultivation system [20]. The higher content of PPT-type ginsenosides in leaves could be related to the positive correlation between light and PPT-type ginsenosides, which corresponds with the observation that high light transmission increased PPT-type ginsenosides in the leaves of ginseng plants [19].

To the best of our knowledge, information about the changes in ginsenoside content in the leaves and roots of ginseng during its different foliation stages has not been reported. During foliation, the production and composition of ginsenosides changes in leaves and roots (summarized in Fig. 5). The total ginsenoside content decreased in the roots (Fig. 3) and increased in the leaves (Fig. 2), with an increased accumulation of genes related with ginsenoside biosynthesis (Fig. 4) observed when the shoots elongated and the leaves opened. After sprouting, the metabolites already stored in the roots from the last season might be transported to parts of the plant above ground. During photosynthesis, the main sugar products are synthesized in the leaves and are transported to the roots for storage. After the leaves open, PPT-type ginsenosides might preferentially be synthesized and stored in the leaves, while PPD-type ginsenosides seem to be preferentially stored in the

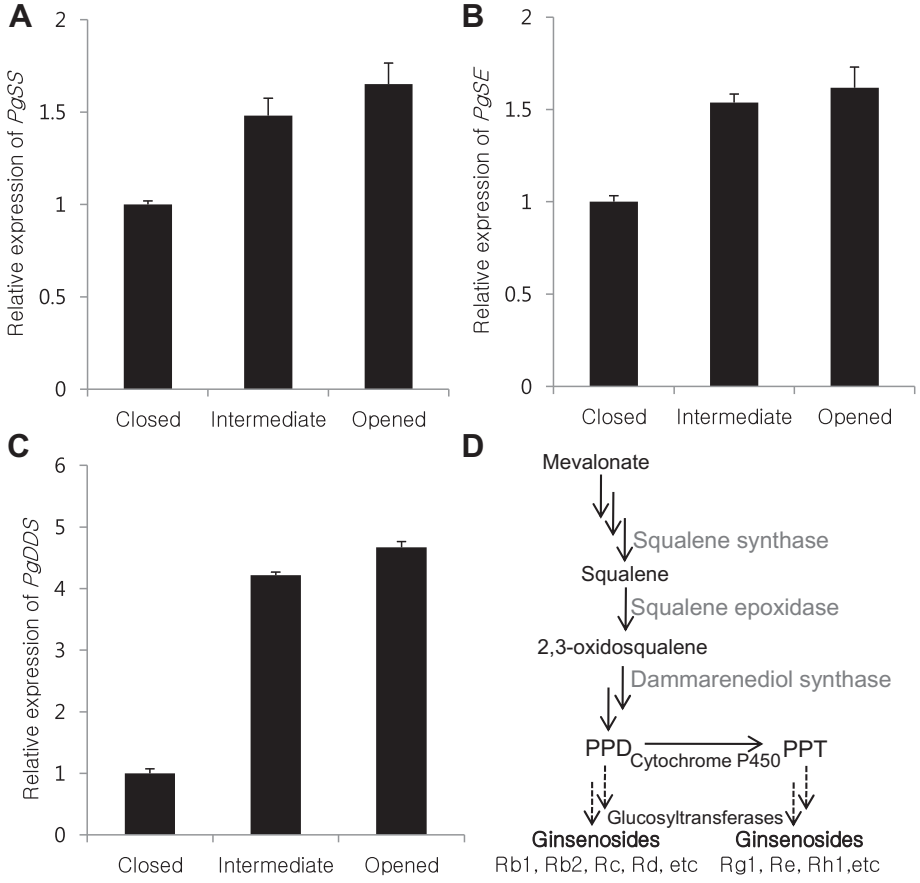

Fig. 4. Expression of genes related to ginsenoside biosynthesis in leaves during foliation. The relative expression of (A) *PgSS*, (B) *PgSE*, and (C) *PgDDS* genes in closed, intermediate, and opened leaves was analyzed by real-time polymerase chain reaction. Vertical bars indicate the mean ± standard error from three independent experiments. (D) The ginsenoside biosynthetic pathway in ginseng. PPD, protopanaxadiol; PPT, protopanaxatriol.

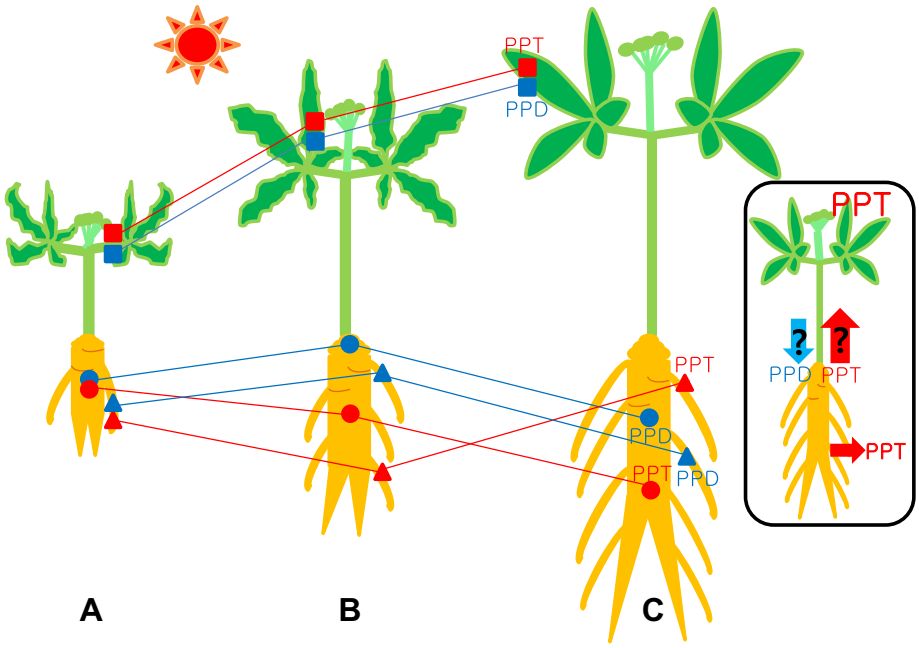

Fig. 5. Summary of the changes in ginsenoside composition in the leaf (■), main root (●), and fine root (▲) during foliation as divided into the closed (A), intermediate (B), and opened (C) leaf stage. The black-boxed picture suggests the possibility for ginsenoside movement. PPD, protopanaxadiol; PPT, protopanaxatriol.

roots, suggesting the possibility of the movement of ginsenosides between the roots, shoots, and leaves. Although there have not been any studies investigating the movement of ginsenosides in ginseng, there is evidence for this phenomenon. One recent study showed that the ginsenoside Rb1 is localized in the chloroplasts, peroxisomes, and cytoplasm of leaf parenchyma, but not the vacuoles. However, Rb1 is localized in the vascular bundles as well as the vacuoles in the leaf stem and the root parenchymal cells [28]. Leaf cells do not seem to be the storage site of Rb1, therefore, the authors suggest that Rb1 can be biosynthesized in both peroxisomes and chloroplasts and then transported to the roots through the phloem.

During the growth of the ginseng plant, ginsenoside composition changed in the leaves and roots. The ginsenosides Re and Rb1 were especially prevalent in the leaf and root, respectively. These results suggest that individual ginsenosides have different roles in the growth and defense systems of ginseng. For example, fine roots increase in number and length during ginseng growth and contain increased PPT-type ginsenosides, especially Rg1, which might play defensive or antioxidant roles in the plants [29]. Each ginsenoside has been shown to have different pharmacological effects, such as anti-aging [30], anti-diabetes [27], anti-inflammatory [31], and anticancer such as the inhibition of tumor-induced angiogenesis [32–34], anti-tumor activity and the prevention of tumor invasion and metastasis [35,36]. Generally, saponins have been suggested to be involved in plant defense against pathogens and pests [37]. However, the physiological roles of saponin in ginseng plants have not been investigated, despite many studies on the effects of ginsenoside on the human body. One study showed that ginsenoside has an important allopathic effect on the ginseng plant [38]. In addition, PPT-type ginsenosides (but not PPD-type ginsenosides) showed stimulatory effects on the radicle length of ginseng seedlings [38]. More research is needed to evaluate the effects of individual ginsenosides on ginseng plant growth and defense in order to better understand the physiological role of ginsenosides in the ginseng plant.

Conflicts of interest

All authors have no conflicts of interest to declare.

Acknowledgments

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