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Study on biosynthesis of ginsenosides in the leaf of *Panax ginseng* by seasonal flux analysis

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Abstract Seasonal ginsenoside flux in the leaves of 5-year-old Panax ginseng was analyzed from the field-grown ginseng, for the first time, to study possible biosynthesis and translocation of ginsenosides. The concentrations of nine major ginsenosides, Rg1, Re, Rh1, Rg2, R-Rh1, Rb1, Rc, Rb2, and Rd, were determined by UHPLC during the growth in between April and November. It was confirmed total ginsenoside content in the dried ginseng leaves was much higher than the roots by several folds whereas the composition of ginsenosides was different from the roots. The ginsenoside flux was affected by ginseng growth. It quickly increased to 10.99±0.15 (dry wt%) in April and dropped to 6.41±0.14% in May. Then, it slowly increased to 9.71±0.14% in August and maintained until October. Ginsenoside Re was most abundant in the leaf of *P. ginseng*, followed by Rd and Rg1. Ginsenosides Rf and Ro were not detected from the leaf. When compared to the previously reported root data, ginsenosides in the leaf appeared to be translocated to the root, especially in the early vegetative stage even though the metabolite translocated cannot be specified. The flux of ginsenoside R-Rh1 was similar to the other (20*S*)-PPT ginsenosides. When the compositional changes of each ginsenoside in the leaf was analyzed, complementary relationship was observed from ginsenoside Rg1 and Re, as well as from ginsenoside Rd and Rb1+Rc. Accordingly, ginsenoside Re in the leaf was proposed to be synthesized from ginsenoside Rg1. Similarly, ginsenosides Rb1 and Rc were proposed to be synthesized from Rd.

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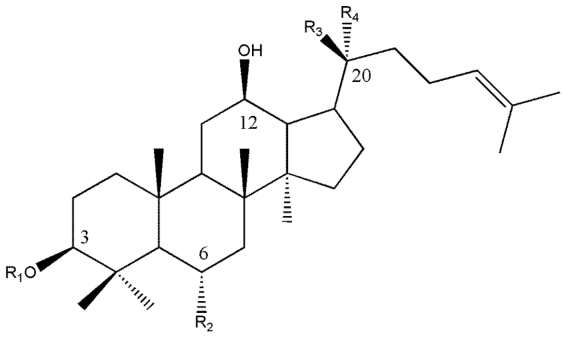
Keywords Biosynthesis · Ginsenosides · *Panax ginseng* · Seasonal flux · Translocation · UHPLC

Introduction

Continuous analysis of secondary metabolites through the plant growth stages provides valuable data, even though it is not an easy task when the samples collected from cultivation field [1]. As a powerful modeling tool, plant metabolite flux can also lead to the biosynthetic pathway elucidation of plant secondary metabolites. In principle, long-term flux change of biosynthetically-related secondary metabolites reflects the changes of the corresponding enzymes activity resulting from the regulation of different gene expression [2]. In the case of ginsenosides, a group of bioactive secondary metabolites in ginseng is presumably biosynthesized from the sequential glycosylation of protopanaxadiol (PPD) and protopanaxatriol (PPT). But, each structurally related ginsenoside has different biological activities. The unique situation of ginsenosides makes metabolic flux investigation meaningful, even without isotope-labelling.

Recently, we have reported seasonal ginsenoside flux change in the root of field-grown *Panax ginseng* to assess this proposition [3]. Apart from the practical finding that the highest ginsenoside content was produced in May not in the harvest season, we have also confirmed that the seasonal ginsenoside flux in the root could be utilized for the biosynthetic pathway elucidation. Encouraged by the results, seasonal variation of ginsenosides in the ginseng leaf was investigated with the same ginseng samples of which roots were analyzed previously (Fig. 1). This study is expected to not only elucidate the ginsenosides biosynthetic pathway in the leaf, but also insinuate translocation of ginsenosides between ginseng leaf and root.

In 2017, more than 23,310 tons of ginseng were harvested in Korea [4], and more than thousands of tons of ginseng leaves



Ginsenoside	R1	R2	R3	R4
Rg1	–H	–*O*–Glc	–*O*–Glc	–CH_3
Re	–H	–*O*–Glc^{2}–^{1}Rha	–*O*–Glc	–CH_3
Rh1	–H	–*O*–Glc	–OH	–CH_3
Rg2	–H	–*O*–Glc^{2}–^{1}Rha	–OH	–CH_3
(20R)-Rh1	–H	–*O*–Glc	–CH_3	–OH
Rb1	–Glc^{2}–^{1}Glc	–H	–*O*–Glc^{6}–^{1}Glc	–CH_3
Rc	–Glc^{2}–^{1}Glc	–H	–*O*–Glc^{6}–^{1}Ara(f)	–CH_3
Rb2	–Glc^{2}–^{1}Glc	–H	–*O*–Glc^{6}–^{1}Ara(p)	–CH_3
Rd	–Glc^{2}–^{1}Glc	–H	–*O*–Glc	–CH_3

Fig. 1 Molecular structures of ginsenosides analyzed from ginseng leaf

were wasted. Due to high content of ginsenosides, the leaf of *P. ginseng* has been recognized as a promising bioactive material exhibiting anti-fatigue, hypoglycemic, anti-obesity, and anti-cancer activities [5-7]. Although leaves of *P. ginseng* cultivated in Korea has a great potential for the bio-related industry as an alternative of radix ginseng [8], extensive study on the ginsenosides flux is very limited and most studies on ginseng leaf were performed at the vegetative stage.

On the other hand, ginseng leaf harvest is practically only possible at the harvest season, because earlier leaf amputation inhibits ginseng growth. Hence, leaf ginsenoside flux changes through the growth stages will be necessary to overcome the obstacles hindering biotechnological applications of ginseng leaf as a new functional bioactive material. This is the first extensive study on the seasonal ginsenoside flux in the leaf of the field-cultivated 5-year Korean ginseng.

Materials and Methods

Ginsenoside F1, F3, Ra1, Rg1, Re, Rf, Rh1, Rg2, Rg3, Ro, (20R)-Rh1, Rb1, Rc, Rb2, and Rd were purchased from Embo Laboratory (Daejeon, Korea) in Korea and used for reference compounds. Voucher specimen of ginseng leaves harvested throughout the year 2017 were obtained from Phytobean AC. LTD (Yecheon-gun, Korea). HPLC-grade methanol (MeOH), water and acetonitrile (MeCN) were purchased from Honeywell (Morristown, NJ, USA).

Ginseng samples and analyte preparation

The leaves of *P. ginseng* from 20 ginseng plants were collected from the cultivation field at Bongwha, Korea (36°85'2"N; 128°80'63"E; altitude 280 m) in 2017. The leaves were cut at the base near petiole, and dried under shade at room temperature until no changes of weight were observed. The dried leaves were brittle and pulverized by blender. The average dry weight was about 10% of fresh ginseng leaves. For the analysis of ginsenosides by UHPLC (Ultimate 3000, ThermoFisher, Waltham, MA, USA), 50.0 mg of dried powder were added to the 10.0 mL of 80% methanol, and the solution was sonicated with 135 kHz for 2h at 40 °C (5510R-DTH waterbath sonicator, Bransonic, Danbury, CT, USA). The solution was centrifuged for 15 min (2000×g) and the supernatant (8.0 mL) was transferred to 30 mL glass vial in the Micro-Cenvac (NB-503CIR, N-Biotek, Bucheon, Korea). After dryness for 2h at 60 °C, the residue was dissolved in 10.0 mL of methanol. About 3 mL of methanolic solution was filtered through a 0.22-μm cellulose filter to prepare the UHPLC analyte.

General UHPLC Method

A Thermo Ultimate 3000 equipped with a PDA detector and a C18 Phenomenex Kinetex^{TM} (Torrance, CA, USA) column (2.1×100 nm, 1.7 μm), was used for UHPLC analysis. The injection volume was 2 μL and the flow rate was 0.6 mL/min. The mobile phase for the analysis of ginsenosides was composed of 0.001% phosphoric acid in deionized water (solvent A) and 0.001% phosphoric acid in MeCN (solvent B). For the eluent gradient system, solution B started at 18% for 5 min and increased linearly to 45% from 5 min to 25 min, to 90% from 25 min to 30 min, and was held constant at 90% for 2 min before returning to the initial composition. The column was maintained at 40 °C. The chromatograms were generated by 203 nm UV absorption.

Calibration Curves of Ginsenosides

The standard solutions of ginsenosides were diluted to five to nine different concentrations between 1 and 100 μg/mL, depending on the abundance in the ginseng sample. The analytes were then analyzed by UHPLC in triplicate, and the calibration curves were constructed by plotting peak area versus the concentration of ginsenoside (Supplementary material, Table S1).

Results

Ginsenosides in the leaf of field-cultivated *P. ginseng*

The ginseng leaves collected biweekly in between April and November 2017 from Bongwha, Korea (Supplementary material, Fig. S1) were analyzed by UHPLC (Fig. S2). In the area where the samples collected from, ginseng started to bud in the early April and grew until August. Necrosis of the leaves began in the

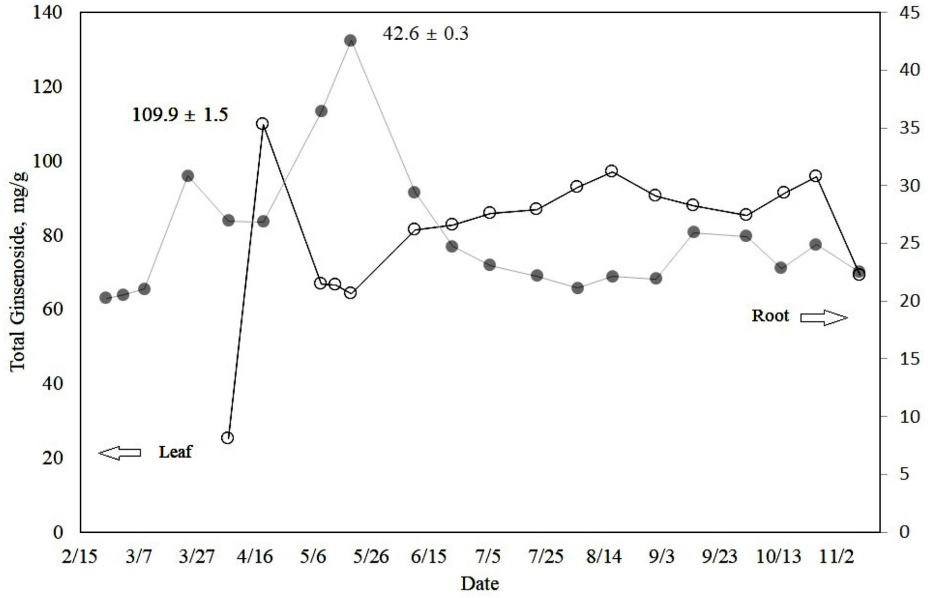


Fig. 2 Total ginsenoside flux (mg/g in dry weight) changes of ginseng leaf and root. The root data was obtained from ref [3]

beginning of September due to temperate climate.

Under the optimized UHPLC conditions that resolve major ginsenosides, the chromatographic separation of Rh1 and Rg2 was not complete. However, ginsenoside Rg2 was minor component and other major ginsenosides could not be resolved with the solvent systems that separate Rh1 and Rg2. Therefore, vertical integration was adopted for the each ginsenoside. Likewise, the concentration of ginsenoside Rc, eluted with ginsenoside F1, was obtained by the vertical integration, too. Along with other major ginsenosides, the presence of ginsenoside (20R)-Rh1 (R-Rh1) in the leaf was confirmed, that was distinctive from all the chromatograms [9]. Interestingly, ginsenosides Rf and Ro characteristic in the ginseng root were not detected from the ginseng leaf samples, even though ginsenoside Rf from the leaf was reported previously [10]. The literature inconsistency of ginsenoside Rf existance in the leaf could be in part due to the different biosynthetic gene expression upon the cultivation years [11].

Seasonal ginsenoside flux in the leaf of *P. ginseng*

In the early vegetative stage (April), the contents of ginsenosides quickly increased with the formation of leaves. After the quick drop in May, total ginsenoside steadily increased from June until November (Fig. 2). The abrupt decrease of leaf ginsenosides could be related with translocation to the root. The highest total ginsenoside content (109.9±1.5 mg/g DW), equal to 11% of dried leaf, was found in the middle of April, but total ginsenosides in the leaves stayed steady at around 90 mg/g in between July and October. In November, the total ginsenoside content decreased to 69.2±1.4 mg/g with leaf senescence. The total ginsenoside content of the fallen leaves collected in December was measured about 3% of total mass (data not shown).

When each ginsenoside was compared, ginsenoside Re, Rd, and Rg1 were major ginsenosides in the leaf (Fig. 3, Table S2). Interestingly, ginsenoside Re and Rg1, but Rd, were major in the leaves of 5-year-old *P. ginseng* cultivated in China [12]. According to Liu et al., ginsenoside Rb3, Rc and notoginsenoside FC were reported as major ginsenosides of *P. notoginseng* leaf [13]. Contrary to ginseng root in which PPD is more abundant by 2.5 times [3], the ginseng leaf showed high content of PPT type ginsenosides. The ratio of PPD/PPT ≤1 was observed, except Apr 19, and gradually decreased through the year (Fig. 4). Therefore, it was found that PPT biosynthesis is more active in ginseng leaf.

Among PPT group, the concentration of ginsenosides always followed the order of Re > Rg1 > R-Rh1 > Rh1 > Rg2, with the exception of the first measurement on Apr 7. Exception of R-Rh1 of which the absolute configuration on *C*-20 is *R*, the other PPT ginsenosides, Re, Rg1, Rh1, and Rg2, have *S*-configuration on 20-*C*. When the stereoisomers were compared, the ratio of (20*S*)-PPT ginsenosides (*S*-PPT) and R-Rh1 was closed to 10 with exception of the first data on Apr 7 (Fig. S3). Therefore, it was proposed that the biosynthesis of R-Rh1 is correlated to *S*-PPT either enzymatically or non-enzymatically.

As ginseng grew, ginsenoside Rc decreased and ginsenoside Rb1 increased. However, relative distribution of PPD type ginsenosides in the leaf was generally found as Rd > Rb2 ≈ Rc ≈ Rb1 throughout the year (Fig. 3). The flux of ginsenoside Rb2 didn't change much. Interestingly, the most abundant root PPT ginsenoside was reported as Rb1, followed by Rc [3]. Therefore, it is strongly suggested that glycosylation of ginsenoside Rd is less active in ginseng leaf.

To investigate possible biosynthetic pathway of leaf ginsenosides, fluxes of ginsenoside composition in PPT and PPD groups were

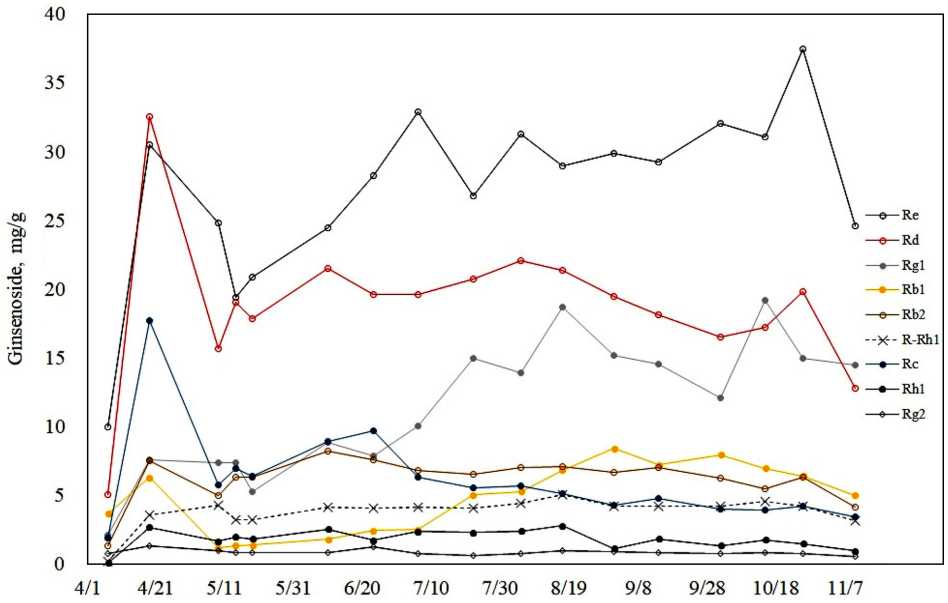


Fig. 3 Ginsenosides flux in the leaf of five-year ginseng throughout the year

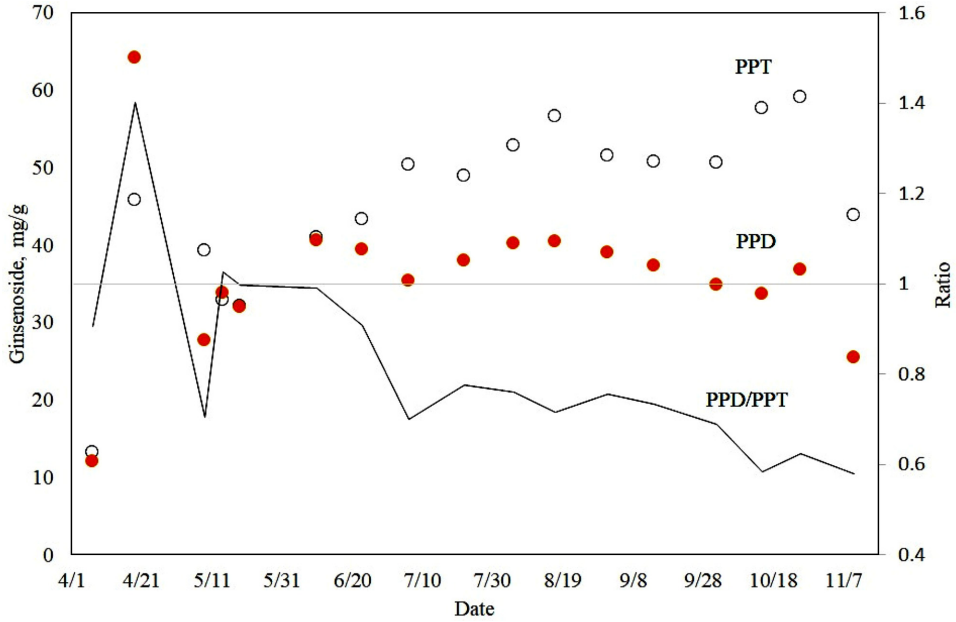


Fig. 4 The flux of PPT and PPD ginsenosides and the ratio of PPD/PPT (line) in the leaf of five-year ginseng. The scales of PPD/PPT ratio are represented at the right Y-axis

analyzed. In the PPT group, percentile flux of each ginsenoside, Rg1, Re, Rh1, and Rg2, in S-PPT ginsenosides was obtained. The percentage of the most abundant ginsenoside Re slowly decreased from 77 to 61% throughout the year. On the contrary, the second major PPT ginsenoside, Rg1, slowly increased from 16 to 36%. Both ginsenosides consituted more than 90% of total S-PPT and steadily increased to 96% as ginseng grew. Interestingly, the percentile fluxes of ginsenoside Rg1 and Re in the growth period were mirror image each other (Fig. 5A). Therefore, it was concluded that ginsenoside Rg1 and Re are the final PPT metabolites in ginseng leaf. The activity of the putative Rg1 rhamnose transferase seems to determine the ratio of Rg1 and Re. The contents of Rg2 and Rh1 were very low through the year, and which implies the putative Rh1 glucose transferase is very active in the leaf.

Percentile flux of each PPD ginsenoside could not be simply correlated (Fig. 5B). After careful simulation, it was found that percentile fluxes of ginsenoside Rd and Rb1+Rc formed mirror

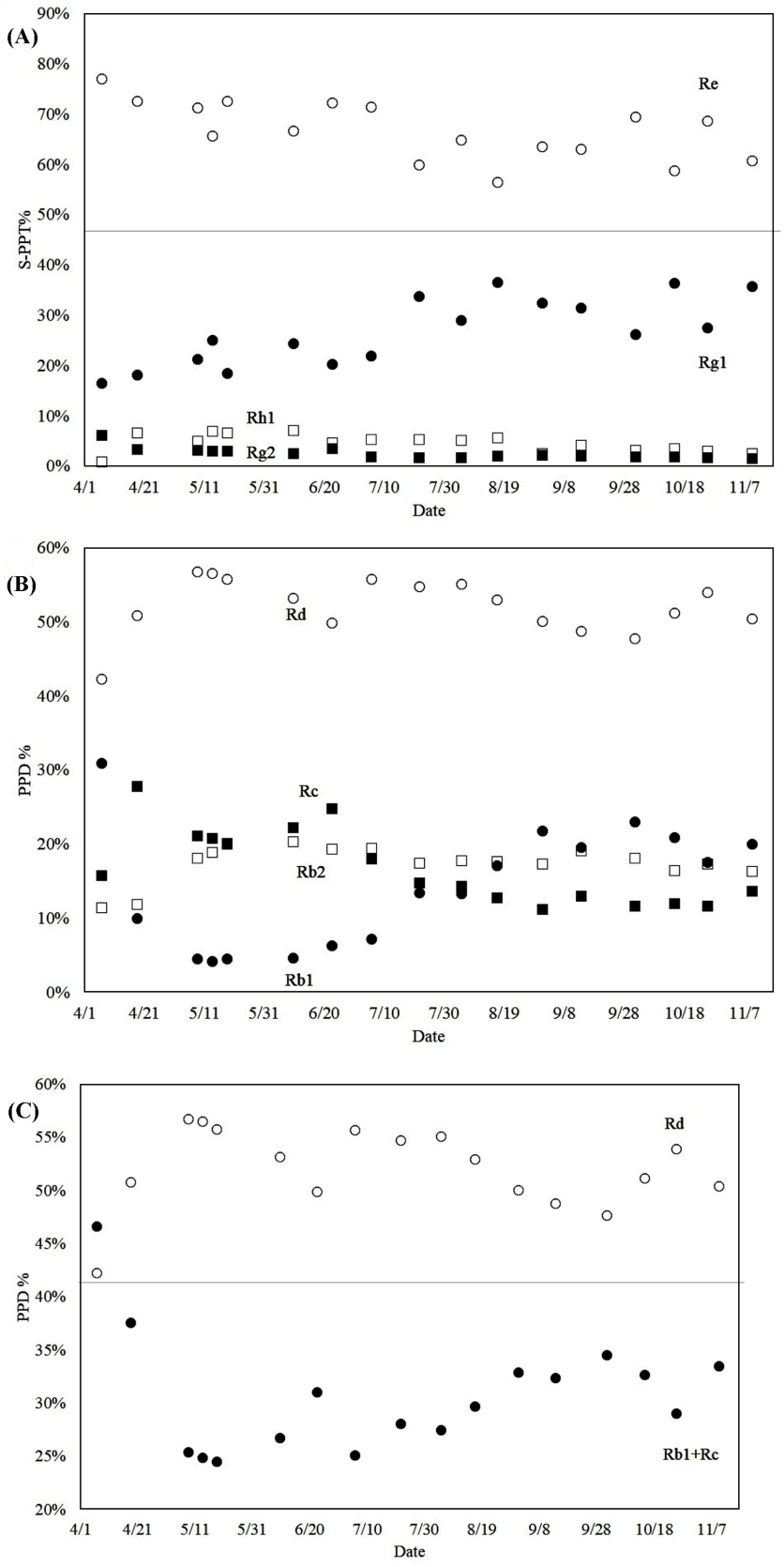


Fig. 5 Percentile flux of S-PPT (A) and PPD (B), as well as percentile fluxes of ginsenosides Rd and the sum of ginsenosides Rb1 and Rc (C)

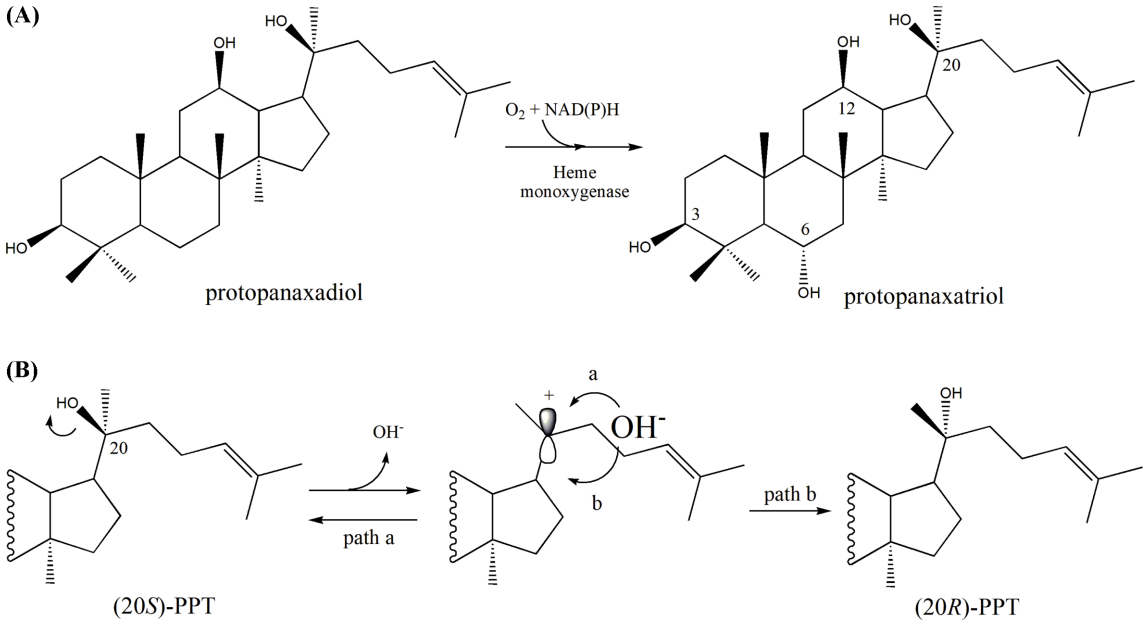


Fig. 6 Plausible mechanism of protopanaxatriol biosynthesis (A) and the racemization of (20*S*)-PPT. Protopanaxatriol synthesis is not related to the racemization on *C*-20 (A), and glucosidation on 20-OH will prevent the racemization on *C*-20 (B)

image as shown in Fig. 5C. Therefore, ginsenoside Rd was proposed as a common biosynthetic precursor of ginsenoside Rb1 and Rc in ginseng leaf.

Discussion

Leaf of *P. ginseng* has a great potential for the application of bioactive natural product. However, study on ginsenosides in the leaf is scarcer than the root, and the results are very diverse. For examples, Rh1 and Rb3 were reported as the most abundant ginsenosides in the leaf of 6-year-old *P. ginseng* (Chunpoong cultivar) grown in Korea [14]. Besides, Ro and Rf were consistently found in significant amounts from the leaf of *P. ginseng* grown in China [12]. It is estimated that different sampling time of ginseng leaves would have resulted in different results. In this work, we have investigated seasonal ginsenoside flux in the leaf of 5-year-old field grown *P. ginseng*. The results are expected to provide extensive data on ginsenoside compositional changes during the growth period, as well as biosynthetic pathway of leaf ginsenosides through the metabolic flux analysis. In this work, both diastereomers of ginsenoside Rh1, (20*S*)-Rh1 and (20*R*)-Rh1, were also analyzed to investigate the origin of (20*R*)-ginsenosides.

The UHPLC analysis of ginseng leaves did not detect ginsenoside Rf and Ro (Fig. S1), similar to the leaves of hydroponically grown ginseng [15]. The dried leaf samples showed high contents of total ginsenoside (Fig. 2), even more than ginseng roots [3]. However, it should be mentioned that total ginsenosides content of leaf and root were similar when fresh samples were compared. When two data were compared in a graph, the decrease of leaf ginsenosides in May was correlated clearly to the increase of root ginsenosides (Fig. 2). Therefore, ginsenoside translocation from leaf to root was suggested. Even though translocation of ginsenosides from the leaf to the flower is possible, it was ruled out because flowers are removed in the conventional ginseng cultivation practice.

The ginsenoside composition in the leaf was different among different *Panax* species. Ginsenoside Re was the most abundant ginsenoside in the leaf of *P. ginseng*, whereas Rb3 was most abundant in *P. notoginseng* and *P. quinquefolius* [13,16]. When the ginsenoside composition in the leaf was compared to the root samples, interesting point was found. The abundancy of Re > Rd > Rg1 was found in the leaf, whereas the order of Rb1 > Rc > Rg1 was found in the root of ginseng [3]. It seems transglycosylation of PPT type Re is more active in ginseng leaf while transglycosylation of PPD type Rd is more active in ginseng root.

Biosynthesis of metabolites in ginseng is known to be tissue specific. For example, polyacetylenes exhibiting important biological activity are only found from ginseng root [17]. Therefore, it is strongly suggested the activity of the enzymes involved in ginsenoside biosynthesis is quite different between leaf and root. The ratio of PPD/PPT in ginseng leaf was found at the range of 0.6 and 1.4, very different from the root (PPD/PPT ≈ 2.5), throughout the year (Fig. 4). The high contents of PPT ginsenosides in the leaf are probably due to the more available molecular oxygen which is the other substrate of CYP type monoxygenase involved in the conversion of PPD to PPT [18] (Fig. 6A). It was also found that flux change of ginsenoside R-Rh1 was related to that of S-PPT

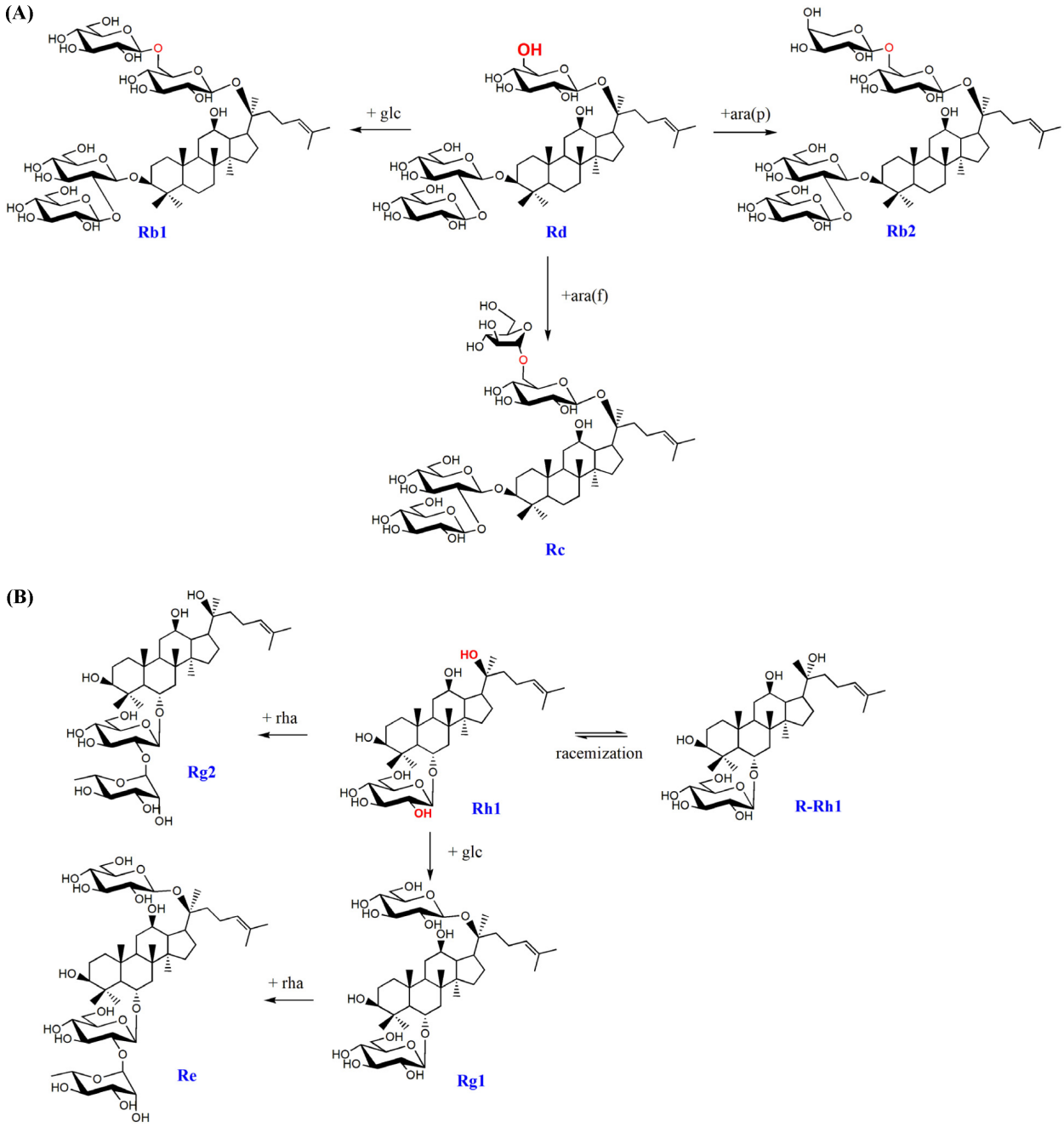


Fig. 7 Possible biosynthetic pathway for the selected PPD (A) and PPT (B) in the leaf of *P. ginseng*

ginsenosides, and the ratios of S-PPT/R-Rh1 were found at around 10 throughout the year, except the first data on Apr 7 (Fig. S3). The occurrence of (20*R*)-ginsenoside stereoisomers, such as R-Rh1, is considered due to the intrinsic lability of tertiary alcohol at *C*-20 which facilitates tertiary carbocation formation (Fig. 6B). Since *C*-20 is a labile tertiary alcohol closed to equatorial 12-OH, the isomerization between ginsenoside Rh1 and R-Rh1 is considered feasible non-enzymatically. At the same time, direct glucosidation at 6-OH of PPT, resulting in both Rh1 and R-Rh1, cannot be ruled out. However, following biosynthetic steps of 20*O*-glucosidation will prohibit such racemization.

The complementary relationship of certain ginsenosides in PPT and PPD groups was found as mirror images of percentile flux as shown at Fig. 5A and C, respectively. In ginseng leaf, metabolic fluxes of Rd and Rb1+Rc was suggested to use the same PPD metabolic pool (Fig. 5C) while that of Rb2 didn't show any correlation with other PPD ginsenosides. The simplest interpretation would be that the glycosylation of Rd catalyzed by the enzyme

which utilize glucosyl and arabiofuranosyl groups equally (Fig. 7A). It is also suggested that different enzyme involves in the Rb2 synthesis, and the catalysis is relatively slow compared to the biosynthesis of Rb1 and Rc. In PPT biosynthesis, it was clear that metabolic fluxes of Re and Rg1 were related and the transfer of rhamnosyl group to Rg1 would produce Re. The glucose transfer at the 20-OH of Rh1 seemed to be faster than the racemization of Rh1 or the arabinose transfer (Fig. 7B). Because fluxes of Re and Rg2 cannot correlated in any ways, it is proposed that different enzymes catalyze the rhamnose transfer to Rg1 and Rh1, respectively.

In conclusion, major ginsenosides flux was measured in the leaf of 5-year-old field-grown *P. ginseng* by UHPLC. The high content of ginsenosides in the leaf proved that it can be a potential bioactive material for the industrial applications. The changes of total ginsenoside content strongly implied the possible translocation from leaf to root, but the flux change alone could not specify the specific metabolite being translocated. Percentile fluxes of PPD and PPT ginsenosides suggested that the synthesis of Rb1 and Rc may be catalyzed by the same enzyme and the synthesis of Rg2 and Re may be catalyzed by different enzymes.

It was confirmed that the seasonal flux changes of structurally related metabolites could provide valuable information for the investigation of biosynthetic pathway. Future biochemical study on the specific ginsenoside biosynthetic metabolism would confirm the conclusions.

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