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Enhanced Triterpene Accumulation in *Panax ginseng* Hairy Roots Overexpressing Mevalonate-5-pyrophosphate Decarboxylase and Farnesyl Pyrophosphate Synthase

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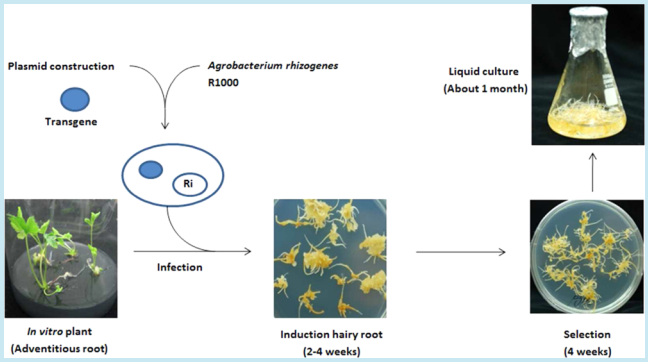
Supporting Information

ABSTRACT: To elucidate the function of mevalonate-5-pyrophosphate decarboxylase (MVD) and farnesyl pyrophosphate synthase (FPS) in triterpene biosynthesis, the genes governing the expression of these enzymes were transformed into *Panax ginseng* hairy roots. All the transgenic lines showed higher expression levels of *PgMVD* and *PgFPS* than that by the wild-type control. Among the hairy root lines transformed with *PgMVD*, M18 showed the highest level of transcription compared to the control (14.5-fold higher). Transcriptions of F11 and F20 transformed with *PgFPS* showed 11.1-fold higher level compared with control. In triterpene analysis, M25 of *PgMVD* produced 4.4-fold higher stigmasterol content (138.95 μg/100 mg, dry weight [DW]) than that by the control; F17 of *PgFPS* showed the highest total ginsenoside (36.42 mg/g DW) content, which was 2.4-fold higher compared with control. Our results indicate that metabolic engineering in *P. ginseng* was successfully achieved through *Agrobacterium rhizogenes*-mediated transformation and that the accumulation of phytosterols and ginsenosides was enhanced by introducing the *PgMVD* and *PgFPS* genes into the hairy roots of the plant. Our results suggest that *PgMVD* and *PgFPS* play an important role in the triterpene biosynthesis of *P. ginseng*.



KEYWORDS: *Panax ginseng, gene expression, overexpression, triterpene, transgenic hairy root*

Isoprenoids are natural compounds that play vital roles in plant metabolism.[1,2] With over 30 000 known compounds, isoprenoids consist of one of the largest structurally varied groups of natural products.[3] Ginsenoside, a major compound present in ginseng, is known to have numerous physiological and pharmacological effects.[4] To date, over 150 ginsenosides have been discovered from different parts of plants,[5] with approximately 40 ginsenoside compounds found in *Panax ginseng*. Several studies have suggested that the main physiological effects of ginsenosides are to influence the central nervous system activity and function as anticancer medicines.[6−11] In addition, ginsenosides have also been reported to have other functions, including anti-inflammatory, antiallergic, anticarcinogenic, immunomodulatory, antidiabetic, antiatherosclerotic, antistress, and antihypertensive effects.[12]

Isoprenoid is synthesized by both the mevalonate pathway and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway.[13,14] Mevalonate-5-pyrophosphate decarboxylase (MVD), which is the last enzyme of the mevalonate pathway, produces isoprenyl diphosphate (IPP). Farnesyl diphosphate (FPP) is then derived from IPP and dimethylallyl diphosphate via a reaction catalyzed by farnesyl diphosphate synthase (FPS).[15] In the first crucial step toward sterol and triterpenoid biosynthesis, FPP acts as a precursor for the synthesis of squalene by the enzyme squalene synthase (SS).[16,17] Enzymes, including squalene epoxidase (SE) and oxidosqualene cyclase, subsequently produce triterpene. Recently, Kim et al.[18] demonstrated that *PgFPS* upregulated triterpene and phytosterol biosynthesis in *Centella asiatica* hairy roots. It was demonstrated that some genes (i.e., *SS*,[19] *SE*,[20] and *DDS* (dammarenediol-II-synthase))[21] are highly correlated to ginsenoside contents in *P. ginseng* (Figure 1).

Agrobacterium rhizogenes-mediated transformation of hairy roots has been found to provide a rapid and simple tool for introducing and expressing foreign genes that are capable of synthesizing specific secondary metabolites in plant cells. This method not only had many advantages, including high safety

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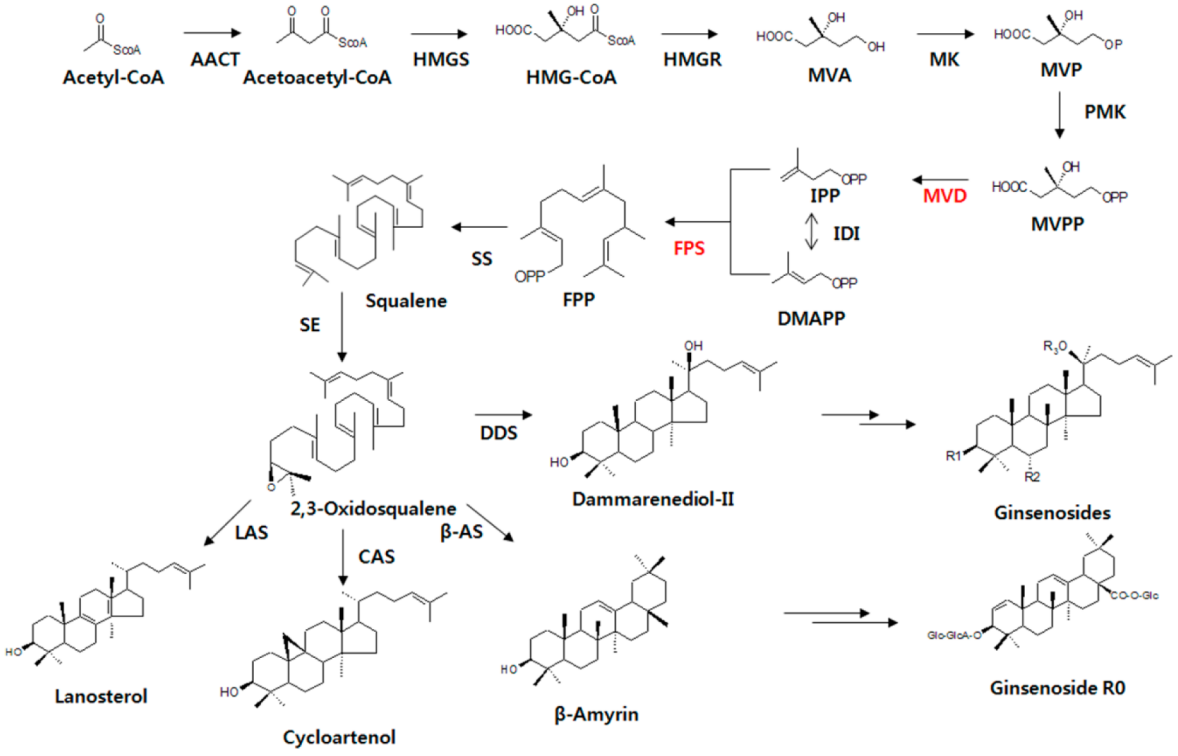

Figure 1. Ginsenoside biosynthetic pathway. AACT, acetyl-coenzyme A (CoA) acetyltransferase; *β*-AS, beta-amyrin synthase; CAS, cycloartenol synthase; DDS, dammarenediol-II synthase; FPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; HMGS, HMG-CoA synthase; IDI, isopentenyl diphosphate isomerase; MVD, mevalonate diphosphate decarboxylase; MK, mevalonate kinase; MPK, mevalonate-5-phosphate kinase; SE, squalene epoxidase; SS, squalene synthase. Red letters indicate overexpressed genes.

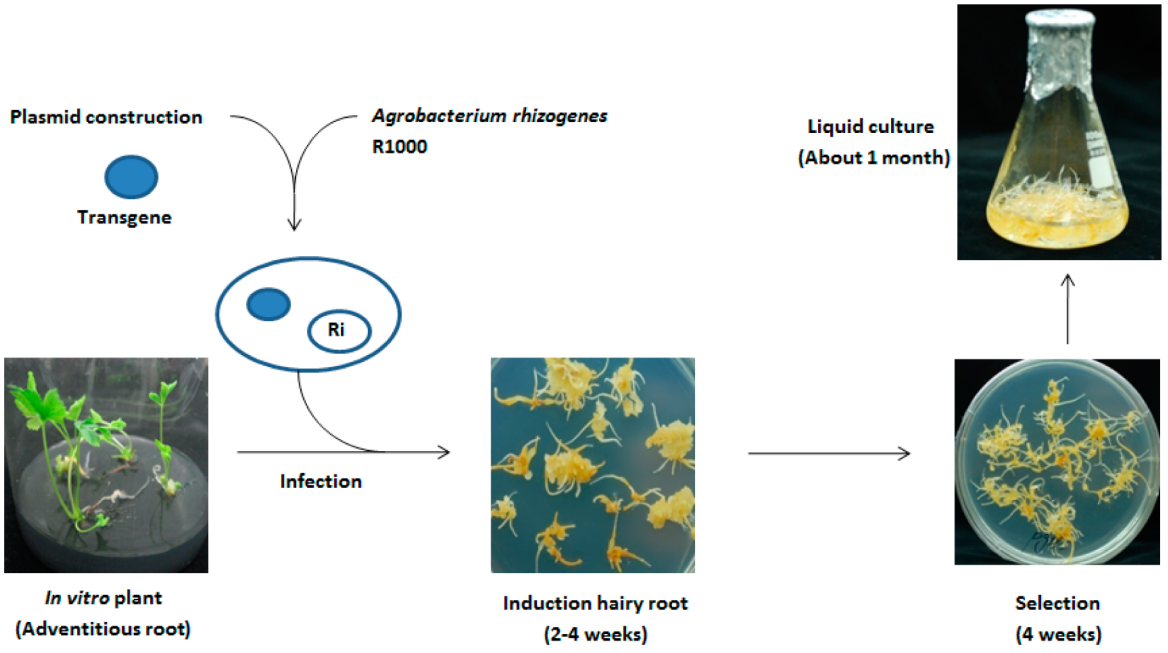

Figure 2. Hairy root induction platform in *P. ginseng*.

level, high growth rate, frequent branching, as well as genetic and biochemical stability,[22] but also enhanced the contents of secondary metabolites for use as cosmetics, pharmaceuticals, and food additives.[22−25]

Metabolic engineering is an important field as the enhancement of cellular activities by transport, manipulating enzymatic, or regulatory functions by recombinant DNA technology. Plant metabolic engineering has been advanced as a very powerful tool for creating improved plants through proper design and construction of genetic engineering. We previously reported that the overexpression of *P. ginseng* 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *Platycodon grandiflorus* increases the production of platycoside and phytosterols efficiently.[26] This successful genetic engineering of a medicinal plant has

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increased prospects for biotechnological applications of secondary metabolite production. Therefore, to examine their function in triterpene biosynthesis, we transformed *PgMVD* and *PgFPS* into the hairy roots of *P. ginseng*, hoping to influence the later steps in the mevalonate pathway and the early steps in the terpenoid pathway, respectively.

RESULTS AND DISCUSSION

Induction of Hairy Roots. The hairy root is a useful system for elucidating gene functions. Because the growth of *P. ginseng* is severely slow in the field, an *in vitro* culture system was selected to obtain adventitious roots. *P. ginseng* adventitious roots genetic transformation with *A. rhizogenes* strain R1000 harboring the *PgMVD-PgFPS* binary vector was carried out, according to a slightly modified transformation protocol described by Choi et al.[29] Transgenic hairy root lines with abnormal phenotypic characteristics, such as short length, brown color, and stagnated growth were discarded. Kanamycin-resistant hairy root lines (approximately 25−30 *PgMVD*- and *PgFPS*-transgenic lines) with the normal lines were selected and used for further analysis (Figure 2).

PCR Analysis. To confirm the integration of *PgMVD* and *PgFPS* genes into the hairy root genome, selected kanamycin-resistant hairy root lines were subjected to the PCR analysis. To discard the interference from *P. ginseng* endogenous genes, both forward primers for amplifying *PgMVD* and *PgFPS* genes landed on the cauliflower mosaic virus (CAMV) 35S promoter sequences. We confirmed the integration of *PgMVD* and *PgFPS* genes through PCR and showed that both the control and transgenic hairy roots contained the *rol A*, *rol B*, and *rol C* genes (Figure 3). It is well-known that each rol gene of the Ri plasmid in *A. rhizogenes* is responsible for the induction of hairy roots from plant species.

Upregulation of *PgMVD* and *PgFPS* Gene Expressions Analyzed Using qPCR. To investigate whether over-expression of each gene contributes to ginsenoside biosynthesis, qPCR was performed. The results show increased accumulation of *PgMVD* and *PgFPS* transcripts in *PgMVD*- and *PgFPS*-transgenic lines, respectively, compared to the wild-type control (Figure 4). Among the transgenic lines of *PgMVD*, M18 and M25 showed the highest expression levels, whereas the other lines showed levels similar to that of the control (Figure 4A).

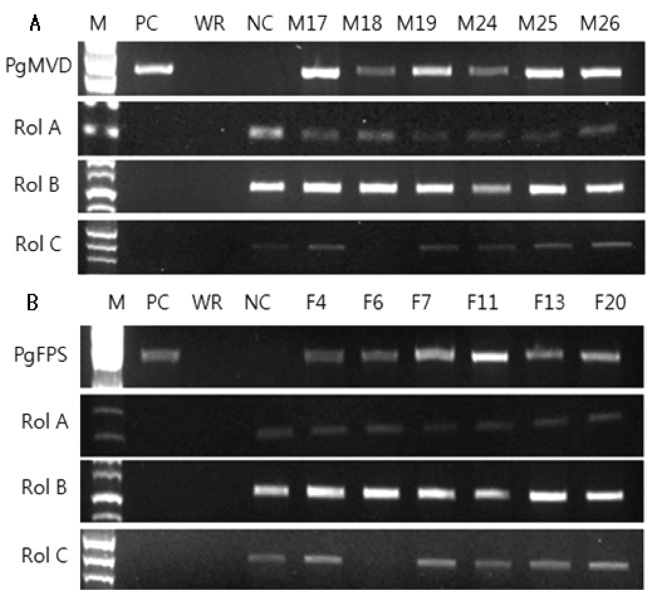

Figure 3. PCR analysis of *PgMVD* (A) and *PgFPS* (B) hairy root lines. PC, positive control (pBI121-*PgMVD* and pBI121-*PgFPS* plasmids, respectively); NC, negative control (pBI121-GUS-overexpressing hairy root line); WR, wild-type root; Mn, *PgMVD*-overexpressing lines; Fn, *PgFPS*-overexpressing lines.

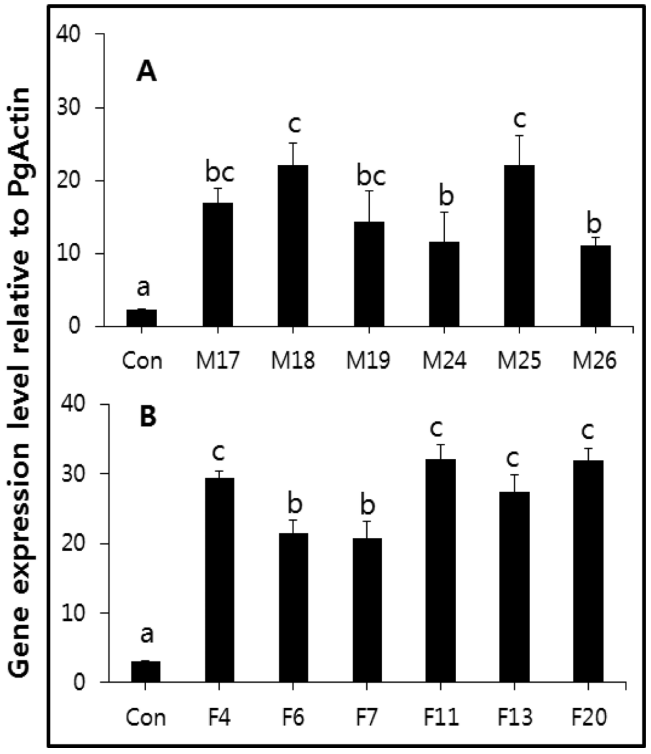

Figure 4. Transcript levels of *PgMVD* (A) and *PgFPS* (B) in hairy root lines. Con, nontransgenic lines; Mn, *PgMVD*-overexpressing lines; Fn, *PgFPS*-overexpressing lines. The *Y*-axis represents the relative gene expression level compared to *PgActin*. Each value is the mean of three replicates with an average of three samples for each replicate, and error bars indicate SDs. The bars with different letters indicate statistical difference by the Duncan's multiple-range test, $p < 0.05$.

Specifically, the transcript levels in M18 and M25 lines were 14.5-fold higher than that in control, whereas M17, M19, M24, and M 26 had 11.2-, 9.5-, 7.7-, and 7.3-fold higher expression, respectively, compared to control. Notably, the lines with the highest expression, M18 and M25, showed almost double the expression of M26, the line with the lowest expression. The trend of the *PgFPS* expression in transgenic lines was slightly different. In particular, the transcript levels in all the transgenic lines were higher than that of control (Figure 4B). The transgenic lines, F11 and F20, showed almost similar transcript levels, which were the highest among all *PgFPS*-transgenic lines. The transcript levels in F11 and F20 were 11.1-fold higher than that in control, whereas the transcript levels in F4, F13, F6, and F7 were 10.2-, 9.5-, 7.4-, and 7.2-fold higher, respectively, compared to control. Together, these results show the accumulation of both *PgMVD* and *PgFPS* transcripts in transgenic hairy roots, confirming that the hairy root lines overexpressing *MVD* and *FPS* were successfully generated.

HPLC Analysis of Saponin. To investigate the effect of *PgMVD* and *PgFPS* overexpression on triterpene biosynthesis, extracts obtained from hairy roots cultured for 30 days were analyzed by HPLC for the ginsenoside content. We found that

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the total ginsenoside levels in *PgMVD*-transgenic lines did not vary significantly from that in control and that only M18 showed a slightly higher amount of ginsenosides (Figure 5A).

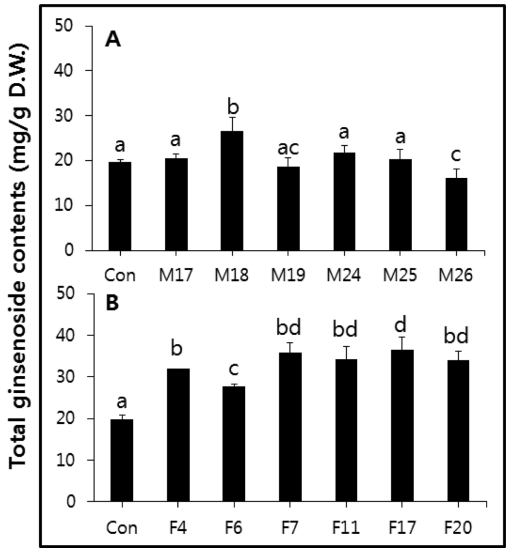

Figure 5. Analysis of total ginsenosides in *PgMVD* (A) and *PgFPS* (B) hairy root lines. Con, nontransgenic lines; Mn, *PgMVD*-overexpressing lines; Fn, *PgFPS*-overexpressing lines. Each value is the mean of three replicates with an average of three samples for each replicate, and error bars indicate SDs. The bars with different letters indicate statistical difference by the Duncan's multiple-range test, $p < 0.05$.

On the other hand, all the *PgFPS*-transgenic lines achieved higher amounts of total ginsenosides, compared to both controls (Figure 5B). The variation among the total ginsenoside contents in these *PgFPS*-transgenic lines was very small. Among them, F17 showed the highest total ginsenosides content (36.4 mg/g dry weight (DW); 2.4-fold higher than control 3).

Gas Chromatography–Mass Spectrometry Analysis of Triterpenes. Among the transgenic lines, M25 of *PgMVD* produced the highest amount of stigmasterol and β-sitosterol (138.9 μg/100 mg DW and 443.6 μg/100 mg DW, respectively); the former was 4.4-fold higher compared to control and the latter was 1.6-fold higher than that of control, according to our gas chromatography–mass spectrometry (GC-MS) analysis. The cholesterol content among all lines, including the control, was almost the same; M17 of *PgMVD* produced slightly higher levels of cholesterol and campesterol than that by the control (Table 1).

The trend of the phytosterol and β-amyrin contents in *PgFPS*-overexpressing lines was similar to the triterpene content in *PgMVD*-overexpressing lines. In most cases, transgenic lines contained a higher amount of phytosterols than that in the control, whereas the amount of β-amyrin was higher in the control (Table 2). Among the transgenic lines, F6 contained the highest amount of campesterol, stigmasterol, and β-sitosterol (1.6-, 4.5-, and 1.4-fold higher, respectively, than the control).

We suggest that *PgFPS* overexpression might upregulate PgCAS and PgDDS, whereas *PgMVD*-overexpression might upregulate only PgCAS (Figure 4, Tables 1 and 2).

The *PgMVD*- and *PgFPS*-overexpressing lines in our study showed mixed results in triterpene biosynthesis. In the case of *PgMVD* overexpression, the concentrations of metabolites (including those of campesterol, stigmasterol, and β-sitosterol) increased, whereas the levels of ginsenoside and β-amyrin decreased (Figure 4, Table 1). In contrast, the ginsenoside levels were high in the *PgFPS*-transgenic lines (Figure 4).

In any case, the amount of any compound that can be produced by the engineering of a single gene is limited. Our suggestion is to use the current multigene transformation methods to increase secondary metabolites. Coexpression of *FPS* and *MVD* genes or combinations with other genes governing rate-limiting enzymes may produce a positive cooperative effect to stimulate accumulation. Multigene transformation may be a reasonable strategy to further improve the ginsenoside accumulation in *P. ginseng* hairy roots.

Our current study provides a good foundation and helpful information for large-scale commercial production of ginsenosides and phytosterols via the *P. ginseng* root system. We believe that hairy root transformation system will be a powerful tool for elucidating gene function involved in terpenoid biosynthesis pathways. In addition, this tool will also be helpful in achieving the desired objective of metabolic engineering for generating plants that produce pharmaceutically important phytosterols or triterpene saponins. Overall, we conclude that *P. ginseng* hairy root samples could provide valuable information regarding the regulation of the pathways by overexpressing a target gene involved in triterpene biosynthesis. Moreover, the transgenic hairy-root systems of *PgMVD* and *PgFPS* would be helpful for enhancing triterpene content in hairy root cultures.

METHODS

Plant Materials. Ginseng plants were obtained from the ginseng field of Chungnam National University. Adventitious roots induced by 3 mg/L IBA and 0.1 mg/L kinetin on SH medium with 0.8% agar were used as explant materials. The culture room was maintained at 24°C with a 16 h photoperiod of 24 lmol m^{-2} s^{-1} provided by a cool white fluorescent tube.

Vector Construction. Full-length cDNA of *PgMVD* (GenBank accession no. GU565096) and *PgFPS* (GenBank

Table 1. Phytosterol and β-Amyrin Content in *PgMVD*-overexpressing Lines

μg/100 mg	con	M17	M18	M19	M24	M25	M26
cholesterol	0.13 ± 0.01^{ab}	0.14 ± 0.02^{b}	0.13 ± 0.01^{abc}	0.11 ± 0.01^{c}	0.12 ± 0.00^{ac}	0.13 ± 0.00^{abc}	0.12 ± 0.01^{ac}
campesterol	9.43 ± 0.90^{a}	14.15 ± 0.85^{b}	13.68 ± 0.74^{b}	12.89 ± 1.20^{b}	13.14 ± 0.50^{b}	13.28 ± 0.35^{b}	13.45 ± 0.24^{b}
stigmasterol	45.21 ± 14.49^{a}	113.25 ± 8.64^{b}	118.06 ± 7.05^{b}	120.42 ± 9.37^{b}	131.37 ± 5.36^{b}	138.95 ± 2.01^{b}	135.25 ± 7.29^{b}
β-sitosterol	307.62 ± 43.62^{a}	427.58 ± 6.95^{b}	431.22 ± 31.25^{b}	421.23 ± 15.64^{b}	387.25 ± 28.94^{ab}	443.6 ± 21.93^{b}	392.87 ± 20.32^{ab}
β-amyrin	6.38 ± 1.71^{a}	0.92 ± 0.1^{b}	0.96 ± 0.08^{b}	1.02 ± 0.09^{b}	1.14 ± 0.01 ^{b}	1.42 ± 0.12^{b}	1.25 ± 0.00^{b}

Values are presented as mean ± SD (each value is the mean of three replicates with an average of three samples for each replicate). Different letters (a, b, c) represent significant differences between means according to ANOVA, followed by individual comparison using Duncan's multiple-range test at $p < 0.05$.

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Table 2. Phytosterol and β-Amyrin Content in *PgFPS*-overexpressing Lines

μg/100 mg	con	F4	F6	F7	F11	F17	F20
cholesterol	0.14 ± 0.02[a]	0.11 ± 0.01[a]	0.11 ± 0.005[a]	0.11 ± 0.012[a]	0.13 ± 0.015[a]	0.12 ± 0.001[a]	0.11 ± 0.001[a]
campesterol	9.42 ± 1.44[a]	12.70 ± 0.82[ab]	13.90 ± 1.30[b]	11.00 ± 1.21[ab]	11.68 ± 1.00[ab]	12.92 ± 1.9[ab]	13.29 ± 0.25[ab]
stigmasterol	45.30 ± 17.23[a]	122.01 ± 7.36[bc]	139.86 ± 1.26[c]	105.8 ± 1.54[b]	106.15 ± 2.05[b]	117.65 ± 3.21[bc]	127.33 ± 1.08[bc]
β-sitosterol	307.62 ± 48.86[a]	383.32 ± 10.21[a]	385.46 ± 35.56[a]	361.11 ± 29.24[a]	325.91 ± 25.00[a]	357.42 ± 13.27[a]	378 ± 12.66[a]
β-amyrin	6.38 ± 1.77[a]	1.52 ± 0.20[b]	1.00 ± 0.10[b]	1.05 ± 0.05[b]	1.05 ± 0.12[b]	1.95 ± 0.1[b]	1.74 ± 0.21[b]

Values are presented as mean ± SD (each value is the mean of three replicates with an average of three samples for each replicate). Different letters (a, b, c) represent significant differences between means according to ANOVA, followed by individual comparison using Duncan's multiple-range test at $p < 0.05$.

accession no. DQ087959) was isolated as follows. The entire coding region of both genes was amplified with Pfu polymerase by using primers carrying *Kpn*I/*Sac*I and *Kpn*I/*Sal*I sites, respectively. The resulting fragment was inserted in pBI121 between the CAMV 35S promoter and the nopaline synthase terminator, and the construct was subsequently transformed into *A. rhizogenes* R1000, as described below.

RNA Isolation and cDNA Synthase. Total RNA was isolated using Tri-Reagent (MRC, U.S.A.) and the RNase Plant Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. Next, total RNA (5 μg) of each organ was reverse transcribed according to the manufacturer's instruction by using the Superscript II First Strand Synthesis Kit (Life Technologies, U.S.A.) with an oligo $(dT)_{20}$ primer.

Gene Expression by Quantitative Real-Time PCR. For quantitative real-time PCR (qPCR), gene-specific primer sets of each gene were designed using the Primer3 melting temperature (Tm) calculator program (http://bioinfo.ut.ee/primer3-0.4.0/) (Table S1, Supporting Information). The qPCR assay was performed in a total volume of 20 μL, containing 10 μL of 2× SYBR Green Real-time PCR Master Mix (Qiagen, Germany), 0.5 μM (each) of specific primers, and 5 μL of cDNA, which was then diluted 20-fold. The thermal cycling conditions were as follows: denaturation at 95 °C for 15 min; 15 s of denaturation at 95 °C; 15 s of annealing at 55 °C; 20 s of extension at 72 °C, with 40 cycles. The PCR reactions were performed in triplicate on a MiniOpticon (Bio-Rad, U.S.A.) by using the QuantiTect SYBR Green PCR system (Qiagen, Germany) according to the manufacturer's instructions. The reaction was carried out in triplicate. *PgActin* (GenBank accession no. AY907207) was used as a reference gene, and the transcript levels were calculated relative to *PgActin*.

Preparation of *A. rhizogenes*. *A. rhizogenes* cultures initiated from the glycerol stock were grown overnight to the midlog phase (OD_{600} = 0.5) at 28 °C with shaking (180 rpm) in liquid Luria−Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) containing 50 mg/L kanamycin. Cells were collected by centrifugation for 10 min at 2000 rpm and resuspended in liquid inoculation medium containing MS salts, vitamins, and 30 g/L sucrose. The cell density was then adjusted to an A_{600} of 1.0 for inoculation.

Genetic Transformation of *P. ginseng*. Adventitious roots of *P. ginseng* were used as explant materials for cocultivation with *A. rhizogenes* R1000. Excised roots were dipped into *A. rhizogenes* cultures in liquid inoculation medium for 15 min, blotted dry on sterile filter papers, and incubated in the dark at 25 °C on agar-solidified MS medium. After 2 days of cocultivation, roots were transferred to 1/2 SH containing 3 mg/L IBA, 0.1 mg/L kinetin, 30 g/L sucrose, 50 mg/L kanamycin, and 500 mg/L cefotaxime. Putative hairy roots emerging from wounded sites were observed within a month after selection (50 mg/L kanamycin). Isolated putative transgenic roots (100 mg) were transferred to 30 mL of Murashige and Skoog (MS) liquid medium in 100 mL flasks, subcultured every 4 weeks, and maintained at 25 °C on a gyratory shaker (100 rpm) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 $\mu mol \cdot s^{-1} \cdot m^{-2}$ and a 16 h photoperiod. Each experiment was repeated twice with three flasks per culture condition.

PCR Analysis. Genomic DNA of *P. ginseng* hairy roots for PCR analysis was extracted using genomic Plant DNA Extraction Mini Kit (Intron, Korea). *PgMVD* and *PgFPS* ORF, *Rol A* (304 bp), *Rol B* (797 bp), and *Rol C* (550 bp) gene-specific primers were used in PCR analysis (Table S2, Supporting Information). The amplification of cycles consisted of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension 72 °C for 1 min. After 30 repeats of the thermal cycle and final extension 72 °C for 5 min, amplified products were analyzed on 1% agarose gels.

Ginsenoside Analysis in HPLC. To determine the ginsenoside content of *P. ginseng*, high-performance liquid chromatography (HPLC) was carried out using the model NS-400 system (Futecs Co., Daejeon, Korea), equipped with an Evaporative Light Scattering Detector 300 s (SofTA, Thornton, Co, U.S.A.). The separation method, detailed in Kim et al.,[27] utilized a PRONTOSIL NC (250 × 4.6 mm) column with a flow rate of 0.8 mL/min. The conditions were optimized using a solvent gradient system.[26] Identification and quantification of ginsenosides were performed by either comparing their retention times and peak areas with those of ginsenoside standards or directly adding ginsenoside standards into samples (spike test). A standard mixture of ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, and Rh1 was purchased from Canfo Chemical, China.

Extraction and Derivatization of Triterpene. Triterpene extraction was carried out according to a method described by Du and Ahn[28] with a slight modification. Briefly, to release triterpene components, powdered samples (100 mg) were mixed with 3 mL of ethanol containing 0.1% (w/v) ascorbic acid and 0.05 mL of 5α-colestane (10 μg/mL) by vortexing for 20 s, and the mixtures were then placed in a water bath at 85 °C for 5 min. Next, samples were removed from the water bath, mixed with 120 μL of potassium hydroxide (80%) by vortexing for 20 s, and returned to the water bath. After 10 min, samples were immediately placed on ice and mixed with deionized water (1.5 mL). Subsequently, 1.5 mL of hexane was added to each sample, which was then vortexed for 20 s, followed by centrifugation (1200*g*, 5 min). The upper layers were pipetted into separate tubes, and pellets were re-extracted using hexane. Hexane fractions were dried in a centrifugal concentrator (CVE-2000; Eyela, Japan). For derivatization, 30 μL of each MSTFA and pyridine were added and incubated at 60 °C for 30

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min using the Thermomixer Comfort (Eppendorf AG model 5355) at a mixing frequency of 1200 rpm.

Gas Chromatography with Time-of-Flight Mass Spectrometry (GCTOF) Analysis. Phytosterol quantification was carried out using selected ions. Chroma-TOF software was used to assist peak location. Peak identification of each compound was carried out using direct comparison of the sample mass chromatogram with those of commercially available standard compounds obtained using similar derivatization method and GC with time-of-flight (TOF)-MS analysis. The quantitative calculations of all analytes were based on the peak area ratios relative to that of the internal standard (IS).

Statistical Analysis. The data are shown as the means ± standard deviation (SD). The statistical significance was evaluated using ANOVA, followed by individual comparison using Duncan's multiple-range test at $p < 0.05$, by using SPSS statistical software (version 12.0 for Windows, SPPS Inc., Chicago, IL, U.S.A.).

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S.U. Park and S.-U. Kim designed the experiments and analyzed the data. Y.K. Kim, Y.B. Kim, M.R. Uddin, and S. Lee wrote the manuscript, carried out the experiments, and analyzed the data.

Notes

The authors declare no competing financial interest.

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