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Enhanced Accumulation of Phytosterol and Triterpene in Hairy Root Cultures of *Platycodon grandiflorum* by Overexpression of *Panax ginseng* 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase

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Supporting Information

ABSTRACT: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyzes the rate-limiting step in the mevalonate pathway. To elucidate the functions of HMGR in triterpene biosynthesis, *Platycodon grandiflorum* was transformed with a construct expressing *Panax ginseng* HMGR (PgHMGR). We used PCR analysis to select transformed hairy root lines and selected six lines for further investigation. Quantitative real-time PCR showed higher expression levels of HMGR and total platycoside levels (1.5−2.5-fold increase) in transgenic lines than in controls. Phytosterols levels were also 1.1−1.6-fold higher in transgenic lines than in controls. Among these lines, line T7 produced the highest level of total platycosides (1.60 ± 0.2 mg g^{-1} dry weight) and α-spinasterol (1.78 ± 0.16 mg g^{-1} dry weight). These results suggest that metabolic engineering of *P. grandiflorum* by *Agrobacterium*-mediated genetic transformation may enhance production of phytosterols and triterpenoids.

KEYWORDS: *3-Hydroxy-3-methylglutaryl-coenzyme A reductase, gene expression, overexpression, Platycodon grandiflorum, triterpene*

INTRODUCTION

Platycodi radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), is a well-known medicinal plant in northern China and Korea. It has been used as a food additive and in traditional Oriental medicines to treat coughs, colds, upper respiratory tract infections, sore throats, tonsillitis, and chest congestion.[1] Chemical investigation of *P. grandiflorum* revealed that triterpenoid saponins were the main chemical components. More than 20 types of saponin components, such as platycodin D, have been reported thus far.[2,3] Platycodins, a group of triterpene glycosides, were identified as the principal pharmacological components of Platycodi radix.[4−6] Platycosides have been shown to have many biological activities, such as anti-inflammatory, antiallergy, antitumor, antiobesity, and antihyperlipidemia effects, as well as augmenting immune responses and stimulating apoptosis in skin cells.[7−11] All platycosides are oleanane-type triterpene carboxylic acid 3,28-*O*-bisdesmosides.[2,12]

Terpenoids are synthesized by condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), of which the former is derived from the mevalonate (MVA) pathway (Figure 1). The synthesis of mevalonic acid is catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), which, in higher eukaryotes, is generally recognized to be the rate-limiting enzyme in the triterpene biosynthetic pathway.[13] Sequential action of MVA pathway enzymes then produces IPP.

When the yield of specific metabolites in the plant is too low for commercial purposes, metabolic engineering is a useful tool for enhancing accumulation of specific metabolites. *Agrobacterium rhizogenes*-mediated transformation of hairy roots provides a rapid and simple means to introduce and express foreign genes in plant cells that are capable of synthesizing specific secondary metabolites. It has many advantages that involved high safety, high growth rate, frequent branching, and genetic and biochemical stability. Plenty of studies have demonstrated the feasibility of hairy root culture for scaling up for the industrial exploitation of hairy roots.[14] Secondary metabolite production has been enhanced using hairy root transformation in a number of cases, e.g., tropane alkaloid production in *Hyoscyamus niger* hairy root[15] and *p*-hydroxybenzoic acid (pHBA) glucose ester production in hairy roots of *Beta vulgaris*.[16] Moreover, overexpression of *Panax ginseng* farnesyl diphospate (PgFPS) has been achieved in *Centella asiatica*,[17] while overexpression of squalen synthase (SS) in

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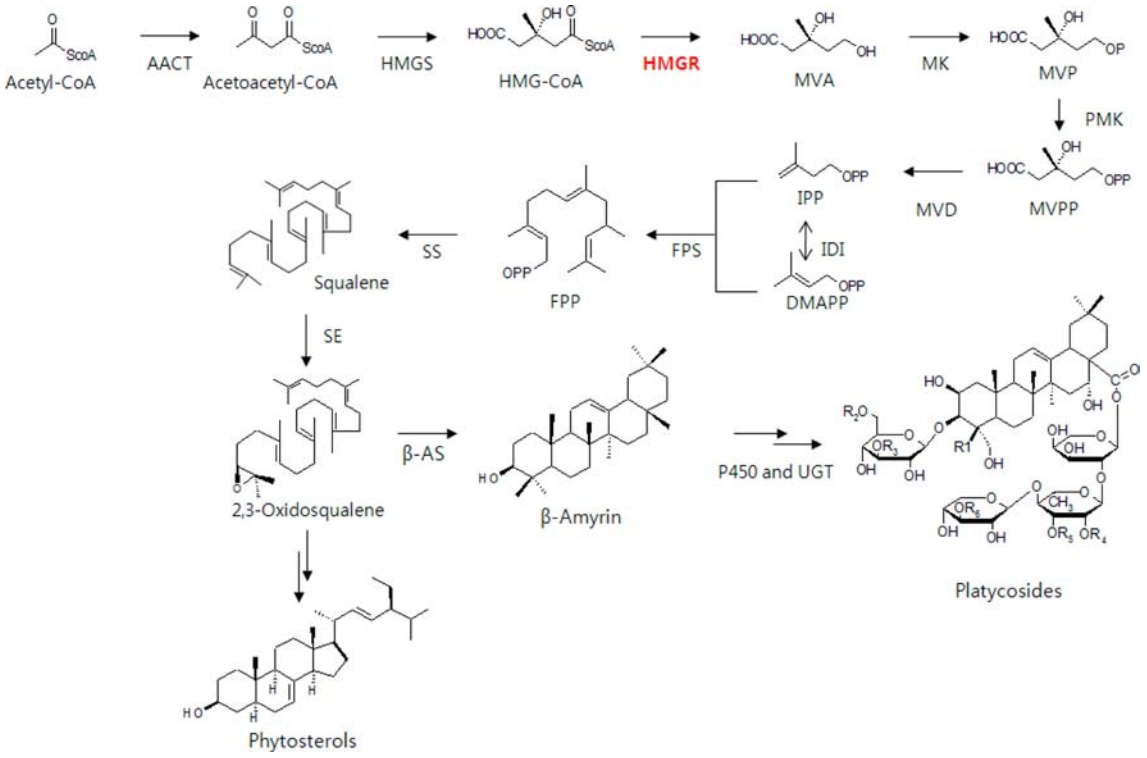

Figure 1. The triterpene biosynthetic pathway. AACT, acetoacetyl-coenzyme A (CoA) thiolase; *β*-AS, B-amyrin synthase; FPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl CoA coenzyme A (HMG-CoA) reductase; HMGS, HMG-CoA synthase; IDI, isopentenyl diphosphate isomerase; MVD, mevalonate diphosphate decarboxylase; MK, mevalonate kinase; PMK, mevalonate-5-phosphate kinase; SE, squalene epoxidase; SQS, squalene synthase.

Eleutherococcus senticosus and *P. ginseng* led to marked increases in phytosterol and triterpenoid levels.[18,19]

However, the manipulation of MVA pathway genes that regulate triterpene and phytosterol biosynthesis has yet to be clearly described in *P. grandiflorum*. Moreover, the *P. grandiflorum* isoprenoid biosynthetic pathway and its related genes are not yet fully elucidated. Thus, we here evaluated the consequences of PgHMGR (a construct expressing *P. ginseng* HMGR) overexpression in the hairy roots of *P. grandiflorum* and demonstrate increased production of phytosterols and triterpenoids in the hairy roots.

MATERIALS AND METHODS

Seed Sterilization and Germination. The seeds of *P. grandiflorum* were sterilized with 70% (v/v) ethanol for 30 s and 2% (v/v) sodium hypochlorite solution for 10 min and then rinsed three times in sterilized water. Twenty-five seeds were placed on 25 mL of agar-solidified culture medium in Petri dishes (100 × 15 mm). The basal medium consisted of salts and vitamins of MS medium and was solidified with 0.8% (w/v) agar. The medium was adjusted to pH 5.8 before adding agar and then was sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16 h photoperiod.

Vector Construction. The entire coding region of PgHMGR was amplified with *Pfu* polymerase using primers carrying *Kpn*I and *Sal*I recognition sites. The resulting fragment was inserted into the binary vector pBI121 between the cauliflower mosaic virus 35S promoter and the nopaline synthase (NOS) terminator. The construct was then transformed into *A. rhizogenes* R1000.

Preparation of *A. rhizogenes*. pBI121 was electroporated into the armed *A. rhizogenes* strain R1000. A culture of *A. rhizogenes* was initiated from glycerol stock and grown overnight 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^{-1} kanamycin, to midlog phase (OD_{600} = 0.5). The *A. rhizogenes* cells were collected by centrifugation for 10 min at 2000 rpm and resuspended in liquid inoculation medium (MS salts and vitamins containing 30 g L^{-1} sucrose). The *A. rhizogenes* cell density was adjusted to an OD_{600} of 1.0 for inoculation.

Genetic Transformation of *P. grandiflorum*. Genetic transformation of *P. grandiflorum* was done following the method described by Park et al.[20] Excised leaves of 14-day-old *P. grandiflorum* seedlings were used as the explant material for cocultivation with *A. rhizogenes* R1000. The excised explants were dipped into the *A. rhizogenes* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS medium. After 2 days of cocultivation, the explant tissues were transferred to a hormone-free MS medium containing salts, vitamins, 30 g L^{-1} sucrose, 500 mg L^{-1} cefotaxime, 50 mg L^{-1} kanamycin, and 8 g L^{-1} agar. Putative transgenic hairy roots were observed emerging from the wound sites within 2 weeks.

Isolated putative transgenic roots were transferred to 30 mL of MS liquid medium in 100-mL flasks. After 30 days of culture, each hairy root line was transferred to fresh MS media in the same volume (500 mg FW). Root cultures were 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 μmol s^{-1} m^{-2} and a 16 h photoperiod. The hairy root samples were harvested every 5 days of the 40-day culture. Each experiment was carried out with three flasks per culture condition and repeated twice.

PCR Analysis of Engineered Hairy Root and Established of Hairy Root Line. DNA of selected hairy root lines was isolated for polymerase chain reaction (PCR) analysis. DNA was extracted using a genomic DNA extraction kit (Geneaid). This was used as template in PCR analysis using primers designed to the PgHMGR ORF (reverse primer) and the endogenous CaMV 35S promoter sequence (forward primer). The four primer sets were designed as follows: 35S promoter F, 5′-CGATAAAGGAAAGGCCATCGTT-3′; PgHMGR R, 5′-

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TTAAGATCCAAT-TTTGGAC-3′; Rol AF, 5′-CATGTTTCAG-AATGGAATTA-3′; Rol AR, 5′-AGCCACGTGCGT-ATTAATCC-3′; Rol BF, 5′-TCACAATGGATCCCAAATTG-3′; Rol BR, 5′-TTCAAGTCGGC-TTTAGGCTT-3′; Rol CF, 5′-ATGGCTGAAG-ACGACCTGTGT-3′; Rol CR 5′-TTAGCCGAT-TGCAAACTTG-CA-3′.

The amplification cycle consisted of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. After 30 repeats of the thermal cycle, and a final extension at 72°C for 5 min, amplification products were analyzed on 1% agarose gels. Gels were stained with ethidium bromide and visualized with UV light.

Gene Expression by Quantitative Real-Time PCR. Total RNA was isolated from adventitious roots of a wild-type and hairy roots of three transgenic *P. grandiflorum* lines and then synthesized single-stranded cDNA. Gene-specific primer sets were designed for quantitative real-time PCR (qRT-PCR) as follows: HMGR-qF, 5′-CTAACCAACGGCATTTTCTTCAC-3′; HMGR-qR, 5′-AGATGAGGATTGGACGAAATCA-3′. PCR reactions were carried out in triplicate on a Mini opticon (Bio-Rad) using the Qiagen Quantitect SYBR Green PCR system according to the manufacturer's recommendations. After a denaturation step at 95°C for 15 min, amplification occurred in three steps, for a total of 40 cycles: 15 s of denaturation at 95°C, 15 s of annealing at 55°C, and 20 s of extension at 72°C.

Total Saponins Extraction and HPLC Analysis. Samples (50 mg) were extracted with 10 mL of 100% MeOH for 30 min in sonicator. The 4 mL of extract was concentrated in speed vacuum. The extract was resuspended with 200 μL of MeOH and directly injected to the HPLC system. The seven platycosides (deapioplatycoside, platycoside, platycodin D, platycodin D_3, platyconic acid, polygalacin D_2, and polygalacin D) were analyzed using HPLC system of a model NS-4000 (Futecs Co., Daejeon, Korea) with ELSD (evaporation light scattering detector). The separation was performed on a Chemcopak (4.6 × 150 mm, 3 μm, 100, Chemco) with a flow rate of 0.7 mL min^{-1}. The seven platycosides were separated by linear gradient between solution A (50 mM ammonium acetate:acetonitrile:methanol = 75:20:5) and B (50 mM ammonium acetate:acetonitrile:methanol = 69:26:5). The gradient was as follows: 100% A, 1−15 min; 48% B, 15−28 min; B 50%, 28−33 min; 50% B, 33−60 min; 60% B, 60−73 min; 100% B, 73−88 min.[21] Identification and quantification of saponins was carried out by comparing the retention times and the peak areas respectively with standard or by direct addition of standard into the sample (spike test). Sample aliquots were filtered through a 0.45 μm poly(tetrafluoroethylene) filter prior to injection. All samples were run in triplicate.

Extraction and Derivatization of the Samples. Extraction of sterol was performed according to a slightly modification of the method by Du and Ahn.[22] Sterol components were released from the powdered samples (0.1 g) by addition of 3 mL of ethanol containing 0.1% ascorbic acid (w/v) and 0.05 mL of 5α-colestane (10 μg mL^{-1}), mixed by vortexing for 20 s, and placed in a water bath at 85°C for 5 min. After removal from the water bath, 120 μL of potassium hydroxide (80%) was added, and the samples were vortexed for 20 s and returned to the water bath for 10 min. The samples were immediately placed on ice, and deionized water (1.5 mL) was added. Each sample then received 1.5 mL of hexane and was vortexed for 20 s and centrifuged (1200 *g*, 5 min). The upper layer was pipetted into a separate tube, and the pellet was re-extracted using hexane. The hexane fraction was dried in a centrifugal concentrator (CVE-2000, Eyela). For derivatization, 30 μL of MSTFA and 30 μL of pyridine were added and incubated at 60°C for 30 min at a mixing frequency of 1200 rpm using a thermomixer comfort (Eppendorf AG model 5355).

GC−TOF MS Analysis. Each derivatized sample (1 μL) was injected into the Agilent 7890A gas chromatograph by an Agilent 7683B autosampler (Agilent, Atlanta, GA) with a split ratio of 5 and separated on a 30 m × 0.25 mm i.d. fused-silica capillary column coated with 0.25-μm CP-SIL 8 CB low bleed (Varian Inc., Palo Alto, CA). The injector temperature was 290°C. The helium gas flow rate through the column was 1.0 mL/min. The temperature program was set at 250°C, followed by a 10°C/min oven temperature ramp to 290°C and a 10-min heating at 290°C. The column effluent was later introduced into a Pegasus HT TOF mass spectrometer (LECO, St. Joseph, MI). The transfer line and the ion-source temperatures were 280 and 230°C, respectively. The scanned mass range was 50−800 *m*/*z*, and the detector voltage was set at 1800 V.

RESULTS

Generation of Hairy Roots Overexpressing PgHMGR. Hairy roots were transformed with *A. rhizogenes* strain R1000 harboring the PgHMGR binary vector. The kanamycin-resistant root lines were subcultured in fresh medium every 2 weeks. More than 30 lines were selected by kanamycin resistance. Transgenic hairy root lines with abnormal phenotypic characteristics, such as short, brown, and aged, during growth stagnation were ignored. Six hairy root lines with a normal phenotype (Figure 2C) were selected for further analysis.

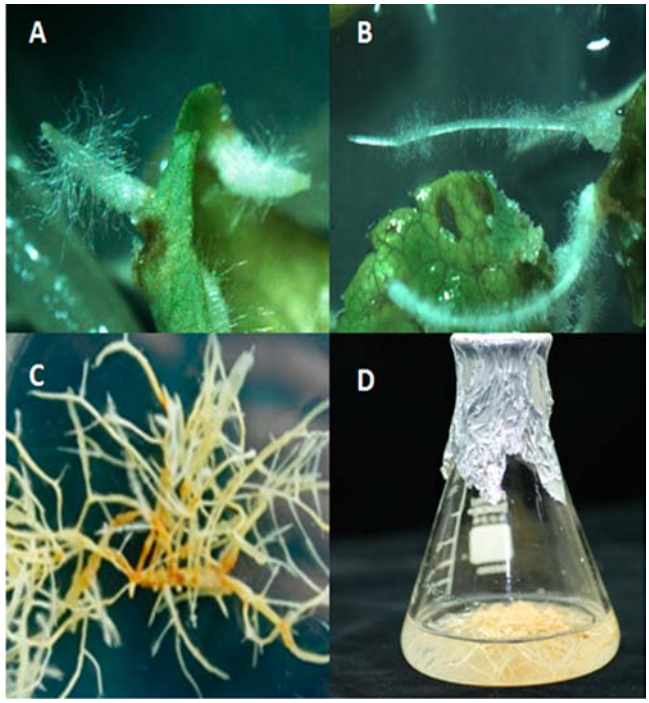

Figure 2. Hairy root induction. (A) Hairy root induction after transformation of *P. grandiflorum* leaf explants, after (B) 1 week of culturing, (C) 1 month of culturing, and (D) 1 month of culturing in liquid medium.

For further selection, PCR analyses were performed for the presence of PgHMGR, Rol A (304 bp), Rol B (797 bp), and Rol C (550 bp) genes (Figure S1 in Supporting Information). A constructed plasmid was used as a positive control (PC), as well as DNA of a wild-type root (WT). The selected hairy root lines showed all PCR bands, suggesting that the HMGR gene was successfully inserted into the *P. grandiflorum* hairy root genome.

Hairy root growth and total platycoside production increased with increasing time up to 30 days; even after 30 days of liquid medium culture, the total platycoside production continued to increase and only began to decline at 40 days of liquid medium culture in all transformed lines (Figure 3). At 30 days after commencing culture, the highest amount of hairy root culture (10 g L^{-1}) was achieved (Figure 3A) and the highest total

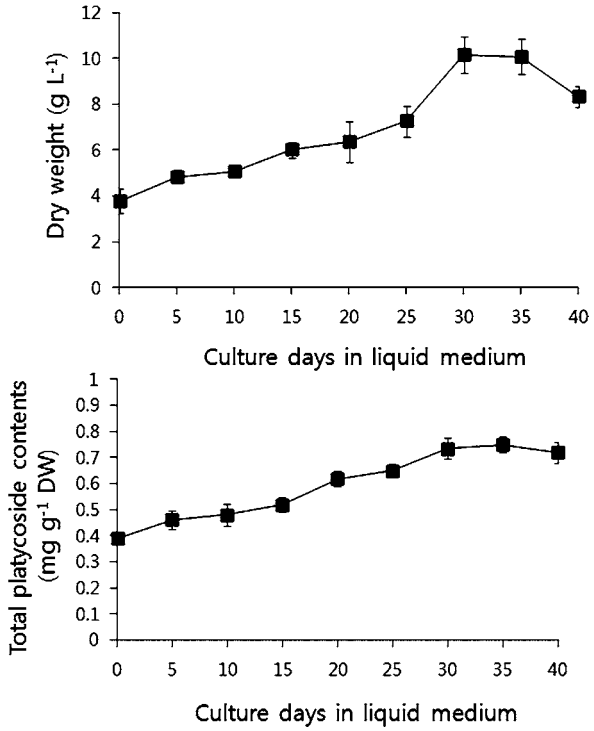

Figure 3. Hairy root growth during 40 days of liquid medium culture: (A) dry weight of hairy root and (B) total platycoside content.

platycoside levels [(0.71 mg g^{-1} dry weight (DW)] was found at 35 days after commencing culturing (Figure 3B). Hairy root was harvested and used for further experiments (qRT-PCR, HPLC, and GC−MS analysis).

Upregulation of HMGR Gene Expression Using qRT PCR. To determine whether overexpression of the HMGR gene contributed to platycoside biosynthesis, qRT-PCR was used to compare overexpression lines with WT. In *P. grandiflorum* transgenic lines, HMGR transcripts accumulated to a greater extent in the hairy roots than in WT (Figure 4), further indicating that the PgHMGR-overexpressing hairy root lines had been generated successfully. The transcript levels of T7 line were the highest, followed by the T9 line, while transcription of T11, T8, and T4 lines were similar (Figure 4).

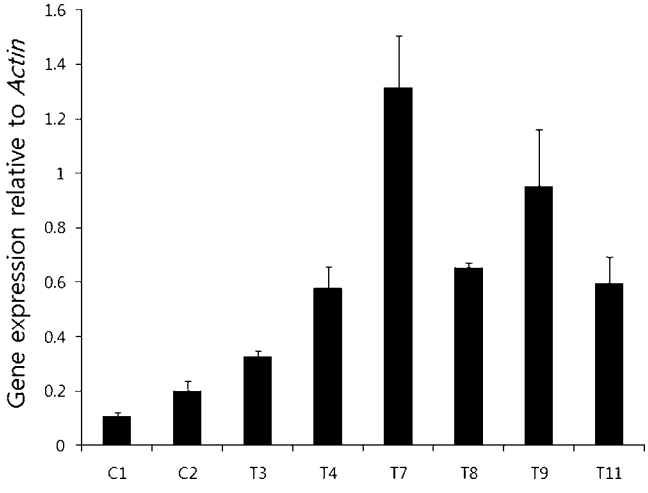

Figure 4. Quantitative real-time PCR analysis of PgHMGR-overexpression lines: C, wild-type root (control); T, PgHMGR overexpression transgenic lines. The height of the bars and the error bars indicate the mean and standard deviation (n = 3), respectively.

HPLC Analysis in Saponin. To analyze the effect of overexpression of PgHMGR on terpene biosynthesis, extracts from 30-day-old cultured hairy roots were analyzed by HPLC for platycosides. The selected hairy root lines produced total platycoside levels 1.6−2.5-fold higher than that of the controls (Figure 6A). The highest level of total platycosides (1.60 ± 0.2 mg g^{-1} DW) were detected in the T7 line.

GC−MS Analysis in *β*-Amyrin and Phytosterols. To demonstrate the phytosterol content of the transgenic lines, we performed GC−MS as well as HPLC, using the same samples. Using GC−MS, we detected *β*-amyrin and *α*-spinasterol, which are the main phytosterols in *P. grandiflorum*. The identification of *β*-amyrin and *α*-spinasterol is shown in Figure 5. *α*-Sinasterol was increased 1.1−1.6-fold compared to the control (Figure 6B); in the T7 line this compound accumulated to a level of 1.78 ± 0.16 mg g^{-1} DW. However, the *β*-amyrin content of these lines was slightly decreased (0.3−0.8-fold) compared to control levels (Figure 6C).

DISCUSSION

Our study showed that PgHMGR could be successfully introduced into *P. grandiflorum* hairy roots, as confirmed by PCR analysis (Figure S1 in Supporting Information) and qRT-PCR. Six selected transgenic hairy root lines increased amounts of platycosides and phytosterols. *P. grandiflorum* hairy roots are considered a good model for assessing triterpene biosynthesis. In higher plants, HMGR is well-known rate-limiting enzyme in the mevalonate pathway. Several papers have noted that overexpression of the HMGR gene resulted in sterol overproduction.[23,24] Treatment of specific inhibitors of the MVA pathway (lovastatin) significantly decrease the sterol production.[25] These findings may indicate that HMGR plays a role in regulation, not only of phytosterol production but also of triterpene biosynthesis. Similarly, our study shows that upregulation of the PgHMGR also can contribute to production of triterpenes. Thus, *P. grandiflorum* hairy root samples can provide us with valuable information regarding the regulation of triterpene biosynthesis by overexpression of a target gene in this pathway.

While platycosides and *α*-spinasterol increased in concentration upon PgHMGR overexpression, *β*-amyrin levels were slightly decreased (Figure 6). This may be due to increased expression of hydroxylases and sugar transferases that lead to changes in platycosides and phytosterols. In *P. ginseng*, heterologous expression of dammarenediol synthase (DDS) in hairy root resulted in minimal levels of the aglycon of ginsenoside because of the high expression of hydroxylases and sugar transferases.[26]

In some cases, overexpression of single genes may lead to unintended consequences. When phytoene synthase was overexpressed in tomato to increase lycopene content, lower rather than higher levels of lycopene were observed, along with dwarf plants; the increase in phytoene synthase caused a decrease in the flux through the gibberellin pathway, a competing pathway and an important plant hormone.[27] Thus, the increase in phytoene synthase disrupted the hormone balance of the transgenic plant, causing unintended consequences. In contrast, targeting the expression of phytoene synthase to canola seeds led to a significant increase in carotenoid concentrations.[28]

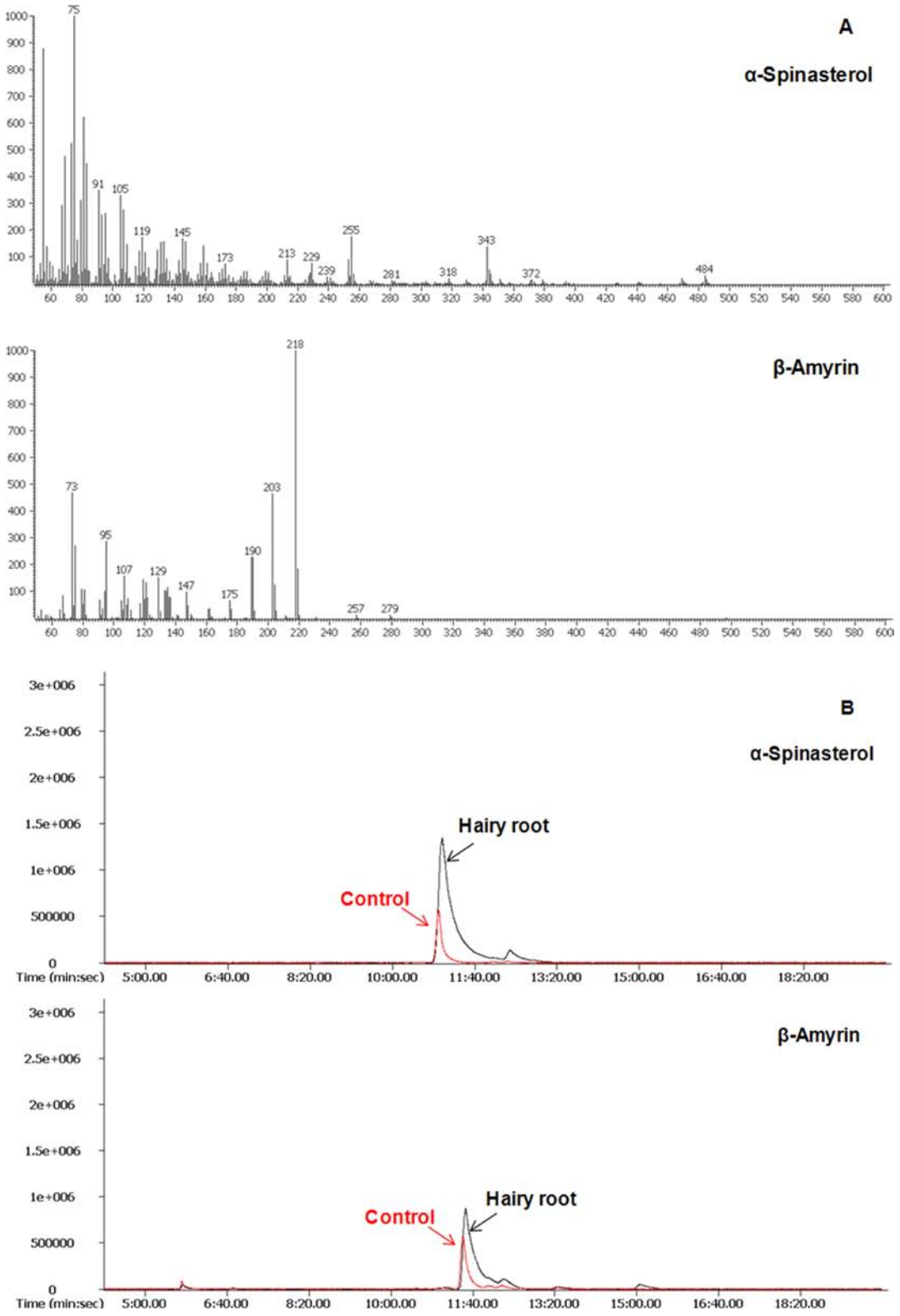

Figure 5. Mass spectra (A) and selected ion chromatograms (B) of α-spinasterol and β-amyrin extracted from a *P. grandiflorum* roots. The peaks correspond to the following: α-spinasterol (retention time, 10:55 min; selective ion, m/z 343) and β-amyrin (retention time, 11:27 min; selective ion, m/z 218).

An approach for overcoming this type of difficulty is engineering the enzyme to be insensitive to feedback signals. Anthranilate synthase (AS) is feedback-inhibited by tryptophan when feedback-insensitive AS was overexpressed in rice, soybean, and *Catharanthus roseus*, and an increase in the concentration of tryptophan within the plant was observed.[29−32] While overexpression of single genes may increase flux through the pathway, overexpression of multiple genes may be necessary to achieve significant gains in product accumulation.

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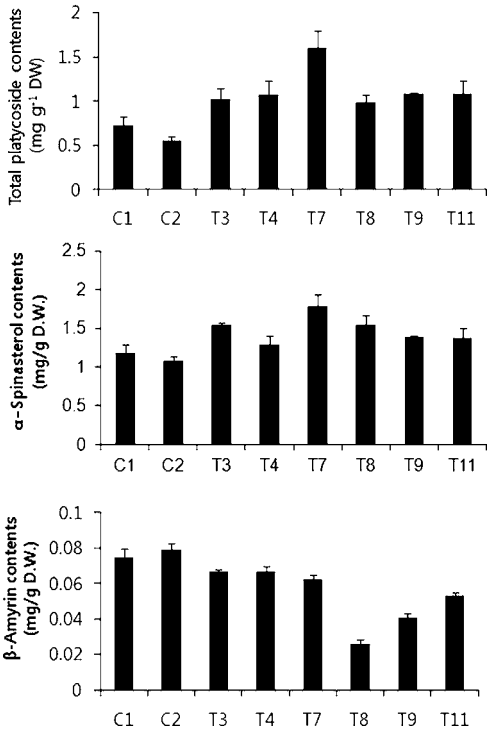

Figure 6. Metabolite analysis in a PgHMGR overexpression lines: (A) total platycoside content, (B) α-spinasterol content, (C) β-amyrin content; C, wild-type root (control); T, *P. ginseng* HMGR overexpression transgenic lines. The height of the bars and the error bars indicate the mean and standard deviation (n = 3), respectively.

The establishment of hairy root transformation systems is a powerful tool for elucidating terpenoid skeletal diversity and the biosynthetic pathways, and *P. grandiflorum* hairy roots offer a good model for assessing triterpene biosynthesis. These techniques will aid metabolic engineering of plants for overproduction of pharmaceutically important phytosterols and triterpene saponins.

■ ASSOCIATED CONTENT

Supporting Information

PCR analysis used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MVA, mevalonate; FPS, farnesyl diphospate; SS, squalen synthase; PCR, polymerase chain reaction; AS, anthranilate synthase; qRT-PCR, quantitiative real-time reverse transcription.

■ REFERENCES

(1) Lee, E. B. Pharmacological studies on *Platycodon grandiflorum* A DC. IV. A comparison of experimental pharmacological effects of crude platycodin with clinical indications of platycodi radix (author's transl.). *Yakugaku Zasshi* **1973**, *93*, 1188−1194.

(2) He, Z.; Qiao, C.; Han, Q.; Wang, Y.; Ye, W.; Xu, H. New triterpenoid saponins from the roots of *Platycodon grandiflorum*. *Tetrahedron* **2005**, *61*, 2211−2215.

(3) Li, W.; Xiang, L.; Zhang, J.; Zheng, Y. N.; Han, L. K.; Saoto, M. A new triterpenoid saponin from the roots of *Platycodon grandiflorum*. *Chin. Chem. Lett.* **2007**, *18*, 306−308.

(4) Ahn, K. S.; Moon, K. Y.; Lee, J.; Kim, Y. S. Downregulation of NF-kappaB activation in human keratinocytes by melanogenic inhibitors. *J. Dermatol. Sci.* **2003**, *31* (3), 193−201.

(5) Kim, J. Y.; Kim, D. H.; Kim, H. G.; Song, G. Y.; Chung, Y. C.; Roh, S. H.; Jeong, H. G. Inhibition of tumor necrosis factor-α-induced expression of adhesion molecules in human endothelial cells by the saponins derived from roots of *Platycodon grandiflorum*. *Toxicol. Appl. Pharmacol.* **2006**, *210*, 150−156.

(6) Zhao, H. L.; Cho, K. H.; Ha, Y. W.; Jeong, T. S.; Lee, W. S.; Kim, Y. S. Cholesterol-lowering effect of platycodon D in hypercholesterolemic ICR mice. *Eur. J. Pharmacol.* **2006**, *537* (1−3), 166−73.

(7) Lee, J. Y.; Yoon, J. W.; Kim, C. T.; Lim, S. T. Antioxidant activity of phenylpropanoid esters isolated and identified from *Platycodon grandiflorum* A DC. *Phytochemistry* **2004**, *65*, 3033−3039.

(8) Wang, C.; Schuller; Levis, G. B.; Lee, E. B.; Levis, W. R.; Lee, D. W.; Kim, B. S.; Park, S. Y.; Park, E. Platycodin D and D3 isolated from the root of *Platycodon grandiflorum* modulate the production of nitric oxide and secretion of TNF-α in activated RAW 264.7 cells. *Int. Immunopharmacol.* **2004**, *4*, 1039−1049.

(9) Ahn, K. S.; Noh, E. J.; Zhao, H. L.; Jung, S. H.; Kang, S. S.; Kim, Y. S. Inhibition of inducible nitric oxide synthase and cyclooxygenase II by *Platycodon grandiflorum* saponins via suppression of nuclear factor-κB activation in RAW 264.7 cells. *Life Sci.* **2005**, *76*, 2315−2328.

(10) Kim, J. Y.; Hwang, Y. P.; Kim, D. H.; Han, E. H.; Chung, Y. C.; Roh, S. H.; Jeong, H. G. Inhibitory effect of the saponins derived from roots of *Platycodon grandiflorum* on carrageenan-induced inflammation. *Biosci. Biotechnol. Biochem.* **2006**, *70* (4), 858−64.

(11) Zhao, H. L.; Sim, J. S.; Shim, S. H.; Ha, Y. W.; Kang, S. S.; Kim, Y. S. Antiobese and hypolipidemic effects of platycodin saponins in diet-induced obese rats: Evidences for lipase inhibition and calorie intake restriction. *Int. J. Obes. (London)* **2005**, *29*, 983−990.

(12) Nikaido, T.; Koike, K.; Mitsunaga, K.; Saeki, T. Triterpenoid saponins from root of *Platycodon grandiflorum*. *Nat. Med.* **1998**, *52*, 54−59.

(13) Bach, T. J. Some new aspects of isoprenoid biosynthesis in plants: A review. *Lipids* **1995**, *30*, 191−202.

(14) Georgiev, M. I.; Agostini, E.; Ludwig-Müller, J.; Xu, J. Genetically transformed roots: From plant disease to biotechnological resource. *Trends Biotechnol.* **2012**, *30* (10), 528−37.

(15) Zhang, L.; Ding, R.; Chai, Y.; Bonfill, M.; Moyano, E.; Oksman-Caldentey, K. M.; Xu, T.; Wang, Z.; Zhang, H.; Kai, G.; Liao, Z.; Sun, X.; Tang, K. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (17), 6786−6791.

(16) Rahman, L.; Kouno, H.; Hashiguchi, Y.; Yamamoto, H.; Narbad, A.; Parr, A.; Walton, N.; Ikenaga, T.; Kitamura, Y. HCHL expression in hairy roots of *Beta vulgaris* yields a high accumulation of *p*-hydroxybenzoic acid (pHBA) glucose ester, and linkage of pHBA into cell walls. *Bioresour. Technol.* **2009**, *100*, 4836−4842.

(17) Kim, O. T.; Kim, S. H.; Ohyama, K.; Muranaka, T.; Choi, Y. E.; Lee, H. Y.; Kim, M. Y.; Hwang, B. Upregulation of phytosterol and triterpene biosynthesis in *Centella asiatica* hairy roots overexpressed

ginseng farnesyl diphosphate synthase. *Plant Cell Rep.* **2010**, *29* (4), 403−11.
(18) Lee, K. J.; Choi, C. Y.; Chung, Y. C.; Kim, Y. S.; Ryu, S. Y.; Roh, S. H.; Jeong, H. G. Protective effect of saponins derived from roots of *Platycodon grandiflorum* on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity. *Toxicol. Lett.* **2004**, *147*, 271−282.
(19) Seo, J. W.; Jeong, J. H.; Shin, C. G.; Lo, S. C.; Han, S. S.; Yu, K. W.; Harada, E.; Han, J. Y.; Choi, Y. E. Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. *Phytochemistry* **2005**, *66*, 869−877.
(20) Park, N. I.; Pham, A. T.; Li, X. H.; Kim, Y. K.; Yang, T. J.; Park, S. U. An efficient protocol for genetic transformation of *Platycodon grandiflorum* with *Agrobacterium rhizogenes*. *Mol. Biol. Rep.* **2011**, *38*, 2307−2313.
(21) Yoo, D. S.; Choi, Y. H.; Cha, M. R.; Choi, C. W.; Kim, M. R.; Yon, G. H.; Hong, K. S.; Lee, B. H.; Kim, E. J.; Cho, S. W.; Kim, Y. S.; Ryu, S. Y.; Kang, J. S. Variation of saponin content in the decoctions of Platycodi radix (II). *Kor. J. Pharmacogen.* **2010**, *41*, 147−152.
(22) Du, M.; Ahn, D. U. Simultaneous analysis of tocopherols, cholesterol, and phytosterols using gas chromatography. *J. Food Sci.* **2002**, *67*, 1696−1700.
(23) Schaller, H.; Grausem, B.; Benveniste, P.; Chye, M. L.; Tan, C. T.; Song, Y. H.; Chua, N. H. Expression of the *Hevea barsiliensis* 3-hydroxy-3-methylglutaryl-coenzyme A reductase in tobacco results in sterol overproduction. *Plant Physiol.* **1995**, *109*, 761−770.
(24) Hey, S. J.; Powers, S. J.; Beale, M. H.; Hawkins, N. D.; Ward, J. L.; Halford, N. G. Enhanced seed phytosterol accumulation through expression of a modified HMG-CoA reductase. *Plant Biotechnol. J.* **2006**, *4*, 219−229.
(25) Laule, O.; Furholz, A.; Chang, H. S.; Zhu, T.; Wang, X.; Heifetz, P. B.; Gruissem, W.; Lange, M. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 6866−6871.
(26) Tansakul, P.; Shibuya, M.; Kushiro, T.; Ebizuka, Y. Dammarenediol-II synthase, the first dedicated enzyme for ginsenoside biosynthesis, in *Panax ginseng*. *FEBS Lett.* **2006**, *580*, 5143−5149.
(27) Fray, R. G.; Wallace, A.; Fraser, P. D.; Valero, D.; Hedden, P.; Bramley, P. M.; Grierson, D. Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellins pathway. *Plant J.* **1995**, *8*, 693−701.
(28) Shewmaker, C. K.; Sheehy, J. A.; Daley, M.; Colburn, S.; Ke, D. Y. Seed-specific overexpression of phytoene synthase: Increase in carotenoids and other metabolic effects. *Plant J.* **1999**, *20*, 401−412.
(29) Li, J.; Last, R. L. The *Arabidopsis thaliana trip5* mutant has a feedback-resistant anthranilate synthase and elevated soluble tryptophan. *Plant Physiol.* **1996**, *110*, 51−59.
(30) Dubouzet, J. G.; Ishihara, A.; Matsuda, F.; Miyagawa, H.; Iwata, H.; Wakasa, K. Integrated metabolomic and transcriptomic analyses of high-tryptophan rice expressing a mutant anthranilate synthase alpha subunit. *J. Exp. Bot.* **2007**, *58*, 3309−3321.
(31) Hong, S. B.; Peebles, C. A.; Shanks, J. V.; San, K. Y.; Gibson, S. I. Expression of the *Arabidopsis* feedback-insensitive anthranilate synthase holoenzyme and tryptophan decarboxylase genes in *Catharanthus roseus* hairy roots. *J. Biotechnol.* **2006**, *122*, 28−38.
(32) Inaba, Y.; Brotherton, J. E.; Ulanov, A.; Widholm, J. M. Expression of a feedback insensitive anthranilate synthase gene from tobacco increases free typtophan in soybean plants. *Plant Cell Rep.* **2007**, *26*, 1763−1771.