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Enhancement of triterpenoid saponins biosynthesis in *Panax notoginseng* cells by co-overexpressions of 3-hydroxy-3-methylglutaryl CoA reductase and squalene synthase genes

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ABSTRACT

Triterpene saponins from *Panax notoginseng* are widely used in the treatment of cardio-cerebrovascular diseases with increasing demand. Herein, key enzyme genes 3-hydroxy-3-methylglutaryl CoA reductase (*PnHMGR*) and squalene synthase (*PnSS*) involved in the triterpene saponins biosynthetic pathway were introduced into *Panax notoginseng* cells to enhance triterpene saponins productions. Overexpression of *PnHMGR* alone or co-overexpression of *PnSS* and *PnHMGR* could remarkably enhance the accumulation of total saponins compared with the control. The highest yield of total saponins was got from the co-overexpression of *PnSS* and *PnHMGR* cell line, which was 3-fold and 1.5-fold higher than those in control and the cell line overexpressed *PnHMGR* alone, respectively. In addition, the contents of six major monomer saponins (Re, Rb1, Rg1, Rh1, Rh2, and F1) and phytosterols were also increased in *PnHMGR-PnSS* transgenic cell lines. Our results provide a useful genetic engineering strategy to improve the contents of triterpene saponins in many medicinal plants.

1. Introduction

Panax notoginseng (Burk) F. H. Chen has been used as both medicinal herb and food for centuries in China, which was widely utilized for the treatment of different disorders, including cardiovascular diseases [1,2], atherosclerosis [3,4] and myocardial ischemia [5]. *Panax notoginseng* may also possess antioxidant [6,7], liver protection [8]. Moreover, anti-tumor activities of this herb and its major constituents have been reported [9–12]. *Panax notoginseng* saponins (PNS), which belong to dammarane-type tetracyclic triterpenoid saponins, are considered as the major active components of *P. notoginseng* and exhibit multiple biological activities.

It is known that triterpenoid saponins and phytosterols are mainly biosynthesized from mevalonic acid (MVA) through isoprenoid pathway (Fig. 1). Some structural genes in the triterpenoid saponins biosynthetic pathway have been isolated and characterized in *P. notoginseng*, which encode farnesyl-pyrophosphate synthase (FPS), squalene synthase (SS), squalene epoxidase (SE), and dammarenediol-II synthase (DS) [13]. 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) is a key enzyme in MVA-isoprenoid pathway, which catalyzes the conversion of 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) to mevalonic acid [14]. The conversion is the rate-determining reaction in sterol and polyisoprenoid biosynthesis [15,16]. Some *HMGR* genes have been cloned and characterized from medicinal plants including *Eucommia ulmoides, Panax ginseng, Paris fargesii* and *Salvia miltiorrhiza* [14,17–19]. Overexpression of *HMGR* genes significantly increased the accumulations of secondary metabolites such as monoterpenes [20], sesquiterpenes [21], diterpenes [19], triterpene [22,23] and tetraterpenes [24]. In our previous study, *HMGR* of *P. notoginseng*, named *PnHMGR* (GenBank accession number KJ578757), was cloned and characterized, and its expression exhibited a positive correlation with PNS contents.

The synthesis of PNS is controlled and regulated by some important enzymes in *P. notoginseng*. HMG-CoA reductase is generally regarded as a rate-limiting enzyme, which catalyzes HMG-CoA to mevalonic acid in MVA pathway. Mevalonic acid is converted to isopentenyl pyrophosphate (IPP) by pyrophosphorylation and decarboxylation, then IPP is converted to farnesyl diphosphate (FPP) by IPP isomerase, geranyl diphosphate (GPP) synthase, and FPP synthase [25,26]. SS is a key enzyme in triterpenoids synthesis pathway, which catalyzes the condensation of two molecules of farnesyl diphosphate(FPP)to from presqualene diphosphate (PSPP)

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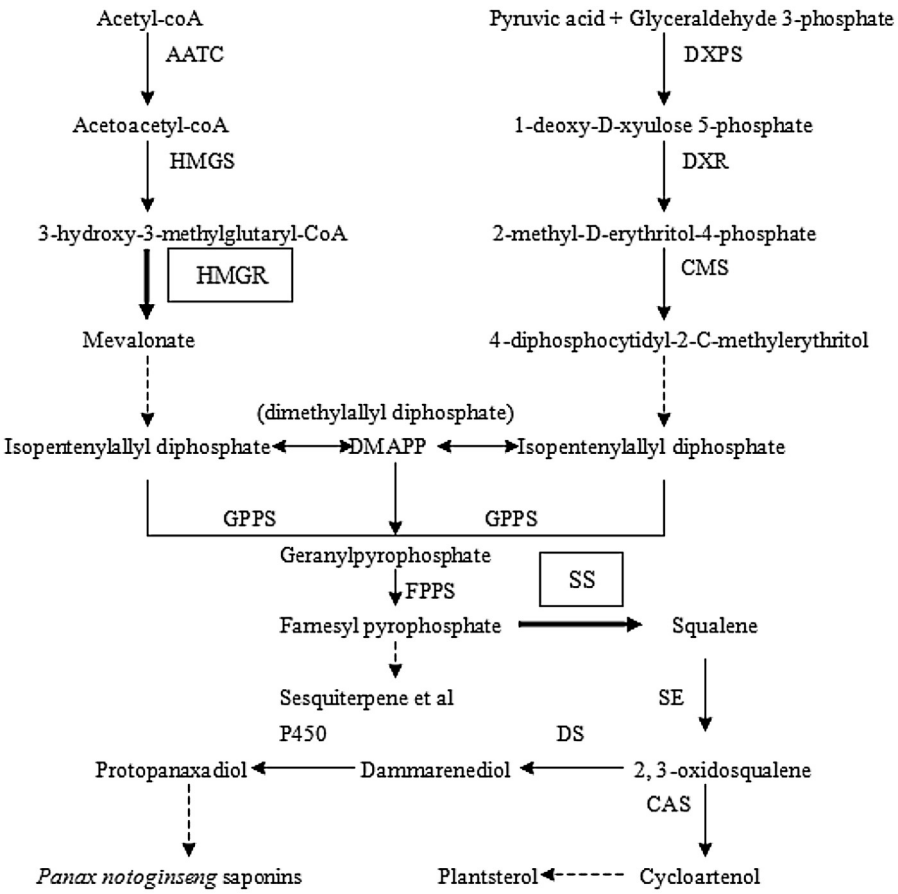

Fig. 1. Biosynthetic pathway of triterpenes and phytosterols in *P. notoginseng*. Arrowheads represent enzymatic steps. The enzymes, HMGR and SS, are discussed in the present work.

and next converts PSPP to squalene (SQ) [27]. SQ is an essential substrate for the biosynthesis of triterpene, cholesterol and steroid [28]. Overexpression of SS gene promote the accumulations of triterpenes, phytosterols and/or steroidal in *Panax ginseng* [29], *Eleutherococcus* senticosus [30] and *Ganoderma lucidum* [31]. In addition, the squalene synthase gene from *P. notoginseng* (*PnSS*) was cloned. the recombinant *PnSS* enzymes catalyzed two-step cyclization reaction of FPP to squalene via PSPP in vitro [32]. The two enzymes, HMGR and SS, have been proposed to play pivotal roles in regulating the carbon flux into end-product. Manipulating multiple biosynthetic genes at several regulatory points is a feasible strategy to produce considerable secondary metabolites [33–35], which will become an attractive approach to increase the yield of triterpenoid saponins by metabolic engineering of *P. notoginseng* cell culture. Co-overexpression of *PnHMGR* and *PnSS* was carried out to enhance the content of triterpenoid saponins in *P. notoginseng* cells. We found that the contents of triterpenoid saponins and phytosterols were increased, as well as corresponding genes transcription levels and enzymes activities were elevated in the transgenic *P. notoginseng* cells. This work provides a useful strategy for promoting the synthesis of triterpenoid saponins in medicinal plants.

2. Materials and methods

2.1. Plant materials and growth conditions

The 3-year-old *P. notoginseng* was obtained from Wenshan, China. *P. notoginseng* callus was induced from the root and cultured on Murashige and Skoog (MS) agar medium supplemented with 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2, 4-D) and 1.0 mg L^{-1} kinetin, pH 5.8 [36], in the dark at 25 °C, and subcultured every 45 days. The *P. notoginseng* calli were used as the experimental materials during the whole research.

2.2. RNA extraction and RT-PCR

Total RNA of *P. notoginseng* was isolated from 0.2 g fresh cells by the guanidine thiocyanate procedure [37,38]. The quality and concentration of total RNA samples were determined by using agarose gel electrophoresis and a UV/visible spectrophotometer (GE, USA). 2.0 μg of total RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega, USA) following manufacturer's instructions.

2.3. Vector construction and transformation

PnHMGR (GenBank accession number KJ578757) and *PnSS* (GenBank accession number KC953033.1) were amplified with specific primers *HM-Sma*I-F, *HM-Xba*I-R, and *SS-BamH*I-F, *SS-Pst*I-R (Table 1), respectively. The PCR mixture was incubated in a 20 μL volume using a DNA thermal cycler (2720 thermal cycler, ABI, USA). The cycling parameters of *PnHMGR* amplification were as follows: 95 °C for 5 min; then 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 2 min; with a final 10 min extension at 72 °C. And the cycling parameters of *PnSS* amplification were as follows: 95 °C for 5 min; then 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90s; with a final 10 min extension at 72 °C. The amplified PCR products were inserted into the pGEM-T-Easy vector (Promega, USA) and sequenced. The amplified *PnSS* coding region digested by