

Overexpression of *PgSQS1* Increases Ginsenoside Production and Negatively Affects Ginseng Growth Rate in *Panax ginseng*

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The medicinal plant *Panax ginseng* (*P. ginseng*) contains various phytosterols and bioactive triterpene saponins (ginsenosides). Squalene synthase catalyzes the first committed step in ginsenoside biosynthesis. Transgenic plants of *P. ginseng* were generated by introducing the squalene synthase gene derived from *P. ginseng*. Adventitious roots of the transgenic ginseng grew best in B5 medium, and 2 g of inoculum secured an optimal growth rate. Two phytohormones, indolebutyric acid and 1-naphthalene acetic acid, increased root growth and decreased ginsenoside production. Treatment with two selected elicitors, chitosan and jasmonic acid, and a precursor of the isoprenoid pathway, mevalonic acid, enhanced ginsenoside production and retarded ginseng growth rate.

Keywords: Elicitor, Ginseng, Ginsenoside, Jasmonic acid, Squalene synthase

INTRODUCTION

Panax ginseng (*P. ginseng*) is one of the most well-known oriental medicinal plants. Triterpene saponins (ginsenosides) extracted from the roots of *P. ginseng* have various kinds of biological activities [1]. Although more than 80 different skeletal types of triterpenes are known to occur naturally, the dammarane-type triterpenes are found only in a few species as major compounds, e.g., *P. ginseng* [2] and *Gynostemma pentaphyllum* (jiaogulan) [3]. Recent research including large-scale gene analysis [4-5] and proteome analysis [6-7] has shown that *P. ginseng* is one of the most suitable systems for studying the biosynthesis of dammarane-type triterpene saponins.

The isoprenoid biosynthetic pathway plays an important role in plant metabolism. The best-known metabolites in this pathway are sterols, gibberellins, carotenoids [8], and terpenes such as mono-, sesqui-, di-, and triterpenes

[9]. Overproduction of phytosterols and triterpenes by metabolic engineering may be an attractive strategy for producing pharmacologically active medicinal plants of superior quality. Squalene synthase (SQS) (EC 2.5.1.21) catalyzes the first enzymatic step in the central isoprenoid pathway toward sterol and triterpenoid biosynthesis [10]. As SQS acts at a putative key branching point in the isoprenoid biosynthetic pathway, it may have a regulatory role in this pathway. SQS has been investigated in animals [11], yeast [12], and plants [13-16].

Elicitor-induced accumulation of secondary metabolites has received much attention, and jasmonic acid has proven to be an effective elicitor [17-19]. Jasmonic acid is believed to be an integral component of signal transduction pathways resulting in the activation of defense responses and secondary metabolism [20,21]. It was reported that exogenously applied elicitors, particularly

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jasmonic acid, stimulate the biosynthesis of many secondary metabolites when the appropriate type of elicitors are chosen in right concentration. In case of ginseng, jasmonic acid significantly improved ginsenoside production in the cultures of hairy roots [22-23]. Here, we report on the optimal media conditions for enhanced ginsenoside production by introducing the squalene synthase gene into ginseng. The effects of phytohormones and effective elicitors are also addressed.

MATERIALS AND METHODS

Vector construction

The entire coding region of the squalene synthase gene (*PgSQS1*) was amplified from *P. ginseng* cDNA, and the resulting 1248-b.p. PCR product was digested with *NcoI* and *BamHI* and ligated into the same restriction sites between the double cauliflower mosaic virus 35S promoter, followed by the alfalfa mosaic virus enhancer and the NOS terminator in pBI524 (NRC Plant Biotechnology Institute, Saskatoon, Canada). The pBI524-carrying *PgSQS1* was digested with *XbaI*, and the resulting fragment was ligated into the same restriction sites within pCAMBIA1302 (Cambia, Canberra, Australia). The final construct was transformed into ginseng using *Agrobacterium tumefaciens* LBA 4404.

Genetic transformation of ginseng

Genetic transformation of *P. ginseng* was carried out as described earlier [24]. Cotyledonary somatic embryos that survived on the selection medium containing 100 mg/L hygromycin and 200 mg/L cefotaxime were detached and transferred to MS medium [25] supplemented with 100 mg/L GA₃ and 100 mg/L hygromycin. The embryos were then germinated and maintained on half-strength MS medium with 2% sucrose. The culture room was maintained at 23°C under a 16/8-h (light/dark) cycle, with light supplied by white fluorescent tubes at an intensity of 24 μmol/m²/s² [24].

Adventitious root culture

Adventitious roots (0.5 g) were grown in a 100-mL Erlenmeyer flask with 40 mL of B5 [26] medium (pH 5.7) supplemented with indolebutyric acid (IBA) or 1-naphthalene acetic acid (NAA). The mother inoculum was subcultured every 30 days. The ginseng cell lines were grown in three media, namely MS (20.6 mM NH₄⁺ ion), B5 (2.0 mM NH₄⁺ ion), and WPM [27], with 0.25, 0.5, 1, and 2 g/L inoculum (fresh weight). The grown roots were used for further studies. The culture room

was maintained at 23°C under a 16/8-h (light/dark) cycle, with light supplied by white fluorescent tubes at an intensity of 24 μmol/m²/s² [24].

Elicitor and mevalonic acid treatment

Adventitious roots developed around the excised edges of the segments were detached from the maternal explants and subcultured in B5 medium with 3% sucrose and 2 mg/L IBA. The culture room was maintained at 23°C under a 16/8-h (light/dark) cycle, with light supplied by white fluorescent tubes at an intensity of 24 μmol/m²/s² [24]. The elicitors, chitosan, jasmonic acid, and mevalonic acid, were added at different concentrations once the cultures reached the vigorous growth phase (after 4 weeks).

Ginsenoside analyses by TLC and HPLC

The ginsenosides were extracted twice using a mixture of water and n-butyl alcohol (1:1). The solvent was evaporated *in vacuo*. The residue redissolved in methanol was detected by thin layer chromatography (TLC) using a Silica gel plate 60 F-254 (Merck, Darmstadt, Germany). CHCl₃-MeOH-H₂O (65:35:10, v/v, lower phase) was used as the developing solvent, and bands were detected by spraying 10% H₂SO₄ on TLC plates, followed by heating at 110°C. HPLC-grade acetonitrile and water were purchased from SK chemicals (Ulsan, Korea). The reaction mixture was extracted with *n*-butanol saturated with H₂O, which was evaporated *in vacuo*. The residue dissolved in CH₃OH was applied to HPLC analysis. HPLC was performed with a C18 (250×4.6 mM, ID 5 μm) column, acetonitrile (solvent A), and distilled water (solvent B) at A:B ratios of 15:85, 21:79, 58:42, 90:10, 90:10, 15:85, and 15:85, with running times of 0 to 5, 5 to 25, 25 to 70, 70 to 72, 72 to 82, 82 to 84, and 84 to 100 min, respectively, at a flow rate of 1.6 mL/min. The detection of ginsenoside was done at 203 nm [28].

RESULTS AND DISCUSSION

Genetic transformation of *P. ginseng* and induction of adventitious roots

P. ginseng was transformed following the previously described method [24]. Hygromycin-resistant somatic embryos were developed on the surfaces of brown explants during the subculture on the selection medium. Selected cotyledonary embryos were germinated on MS medium supplemented with 100 mg/L GA₃ and 50 mg/L hygromycin for 1 month. No morphological differ-

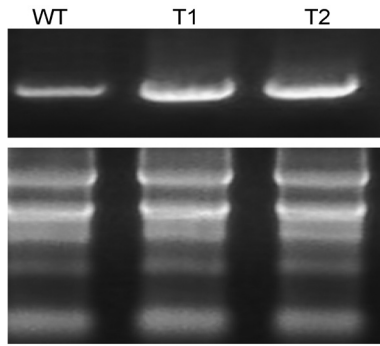


Fig. 1. RT-PCR results showing increased mRNA expression of squalene synthase gene (*PgSQS1*) in transgenic ginseng. Total RNA was used as the loading control (lower panel). The transcripts of *PgSQS1* were greater in the transgenic lines (T1 and T2) than in non-transgenic wild-type ginseng (WT, upper panel).

ences were observed between transgenic and non-transgenic *P. ginseng* plants. The integration of *PgSQS1* and hygromycin phosphotransferase (HPT) genes into the hygromycin-resistant plantlets was confirmed by PCR. The PCR product showed clear bands for the *PgSQS1* (1,248 b.p.) and HPT genes (700 b.p.) (data not shown). RT-PCR analysis showed increased gene expression from the transgenic plants (Fig. 1).

Because ginseng grows extremely slowly in the field, roots cultured *in vitro* were used to analyze the characteristics of the transgenic plants. Leaves and stem segments of both transgenic and wild-type plants were cultured for 1 month to induce adventitious roots. The cultures were maintained by subculturing every 4 weeks. Adventitious roots derived from independent transgenic ginseng plants were regarded as independent lines.

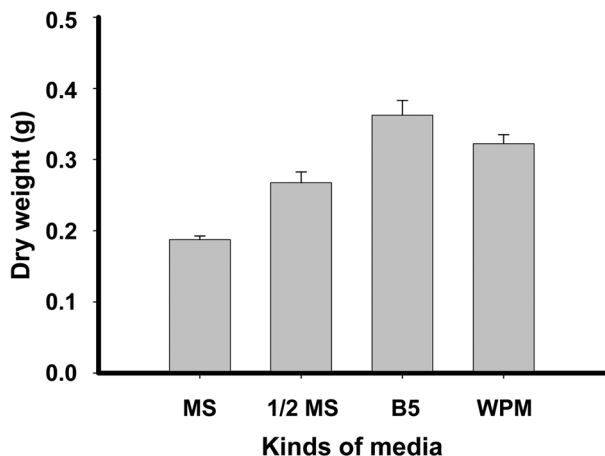


Fig. 2. Effect of different media on the growth of adventitious roots. Vertical bar indicates mean values±standard error ($n=5$).

Optimization of media conditions for ginsenoside production

Growth of transgenic adventitious roots reached a maximum in various media (0.36 g dry weight in B5 medium, 0.19 g dry weight in MS medium, 0.27 g dry weight in 1/2 medium, and 0.32 g dry weight in WPM medium) by 30 days. The best result was observed on B5 medium (Fig. 2). The media differed in the concentration of NH_4^+ ions, which was 20.6 mM in MS and 2.0 mM in B5. The high concentration of NH_4^+ ions in MS medium seemed to inhibit the growth of ginseng roots.

The size of the inoculum was also investigated, and the results are shown in Fig. 3. Net growth (total weight - inoculum weight) during the culture increased with increasing inoculum weight up to 2 g.

Effect of *PgSQS1* overexpression on growth rate

The growth rate of adventitious roots overexpressing *PgSQS1* (*PgSQS1ox*) cultured in B5 liquid media supplemented with auxins was compared to that of the wild-type control (Fig. 4). Good proliferation of transgenic roots was observed in the media supplemented with IBA and NAA in the first 30 days. The dry weight of transgenic roots and wild type roots cultured with IBA and NAA increased rapidly until day 30, after which the growth curves plateaued. The maximum growth of adventitious roots by day 30 varied with inoculum (transgenic adventitious roots, 0.27 and 0.24 g dry weight after treatment with 5 mg/L IBA and 5 mg/L NAA, respectively; non-transgenic adventitious roots, 0.3 and 0.34 g dry weight after treatment with 5 mg/L IBA and 5 mg/L NAA, respectively). The non-transgenic adventitious roots grew faster than the transgenic roots. The

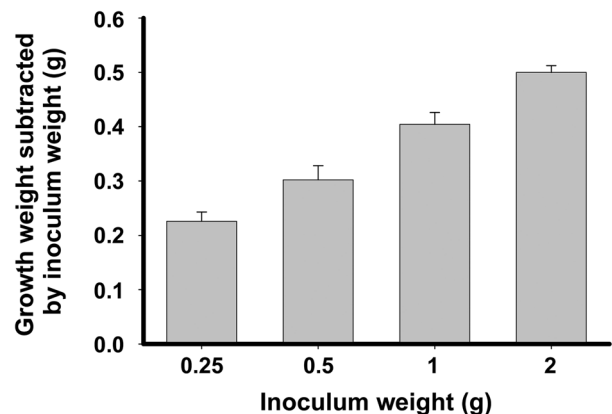


Fig. 3. Growth of adventitious transgenic ginseng root tips after 30 days of culture and for different amounts of inoculum. Vertical bars indicate mean values±standard error ($n=5$).

effects of NAA and IBA were similar. The ginsenoside levels of the transgenic adventitious roots were assayed by TLC (Fig. 5). Both transgenic and non-transgenic adventitious roots, as well as ginsengs in the field, showed similar levels of ginsenosides from Rb1 to Rg1. The adventitious roots of transgenic and non-transgenic plants assayed using TLC were subjected to HPLC. The levels of nine major ginsenoside compounds (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2, and Rg3) were determined by HPLC (Table 1). Ginsenosides Rb1, Rc, and Rd increased 2-, 3-, and 17-fold in the transgenic lines. The effect of auxins on ginsenoside production differed between transgenic and non-transgenic plants. In non-transgenic plants, auxin generally seemed to boost plant

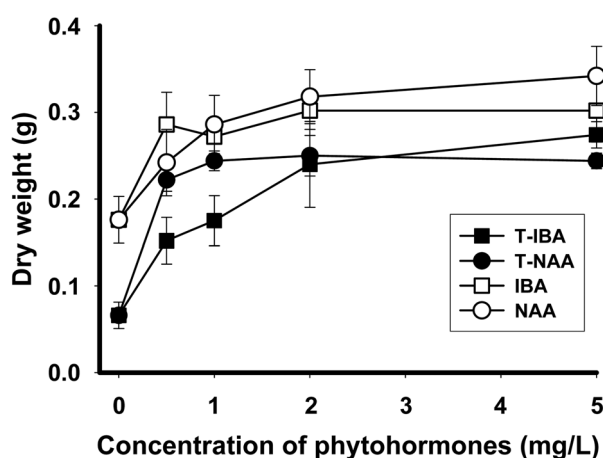


Fig. 4. *In vitro* growth of ginseng roots on media containing phytohormones. T-IBA, transgenic roots with indolebutyric acid (IBA); T-NAA, transgenic roots with naphtalene acetic acid (NAA); IBA, non-transgenic roots with IBA; NAA, non-transgenic roots with NAA. Vertical bars indicate mean values \pm standard error ($n=5$).

growth rate and inhibit total ginsenoside production. However, *PgSQS1ox* seemed to suppress the negative effects of auxin. This result led to the hypothesis that increased production of ginsenoside retards plant growth rate and results in decreased total dry weight, as shown in Fig. 4. These results suggest the possible interaction of phytohormones and ginsenoside production, which requires further experimental confirmation.

Effective elicitors of ginsenoside production

The growth rate of transgenic adventitious roots of *P. ginseng* and ginsenoside accumulation by overexpression of the *PgSQS1* gene may be influenced by different elicitors and by the biosynthesis of mevalonate. The dry weight of roots of untreated transgenic cultures averaged 0.27 g per flask (Fig. 6), indicating that the tested elicitors drastically affected growth rate, resulting in decreased dry weight. But no significant effect of mevalonic acid was observed (Fig. 6). Total production

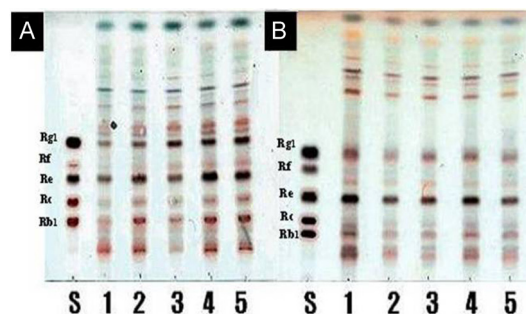


Fig. 5. Thin layer chromatography patterns of ginsenoside in several lines of *Panax ginseng*. (A) transgenic adventitious roots, (B) non-transgenic adventitious roots. S, standard.

Table 1. Production of ginsenosides in adventitious roots of transgenic and non-transgenic *Panax ginseng*

Cell line	Treatment	(mg/L)	Ginsenoside content (mg/g dry weight)									Total
			Rb1	Rb2	Rc	Rd	Rg1	Rg2	Rg3	Rf	Re	
Non-transgenic	Non-treatment	0.0	0.45	0.19	0.07	0.01	0.20	0.19	0.00	0.02	1.25	2.36
	NAA	1.0	0.29	0.32	0.03	0.03	0.12	0.11	0.00	0.01	0.88	1.76
		5.0	0.23	0.15	0.02	0.01	0.10	0.03	0.00	0.00	0.62	1.15
	IBA	1.0	0.46	0.36	0.04	0.03	0.18	0.13	0.00	0.03	1.41	2.62
		5.0	0.26	0.28	0.03	0.01	0.16	0.13	0.00	0.01	0.90	1.75
Transgenic	Non-treatment	0.0	0.96	0.24	0.23	0.17	0.16	0.12	0.00	0.03	1.24	3.13
	NAA	1.0	1.78	0.47	0.47	0.31	0.27	0.16	0.00	0.03	1.69	5.17
		5.0	2.10	0.41	0.33	0.53	0.67	0.17	0.03	0.04	1.69	5.94
	IBA	1.0	2.28	0.62	0.49	0.59	0.57	0.36	0.01	0.05	2.69	7.65
		5.0	2.51	0.68	0.44	0.58	0.67	0.10	0.01	0.01	1.77	6.75

Each value is presented as mean value of two experiments ($p<0.003$). NAA, naphtalene acetic acid; IBA, indolebutyric acid.

Table 2. Overproduction of ginsenosides by elicitors and a precursor in the transgenic roots

Elicitor	Ginsenoside (mg/g dry weight)									
	Rb1	Rb2	Rc	Rd	Rg1	Rg2	Rg3	Rf	Re	Total
Non-transgenic	1.00	0.47	0.29	0.15	0.54	0.46	0.00	0.06	4.08	7.04
Transgenic	6.77	1.29	1.07	1.08	1.05	0.54	0.01	0.08	5.68	17.57
Chitosan (50 mg/L)	10.10	2.03	1.27	0.83	0.69	0.43	0.03	0.09	3.47	18.92
Jasmonic acid (10 mg/L)	32.74	13.27	11.21	9.81	1.28	2.02	0.24	0.43	13.48	84.46
Mevalonic acid (50 mg/L)	9.52	2.53	1.47	1.55	2.26	1.04	0.03	0.19	8.93	27.51

Each value is presented as mean value of two experiments ($p < 0.003$).

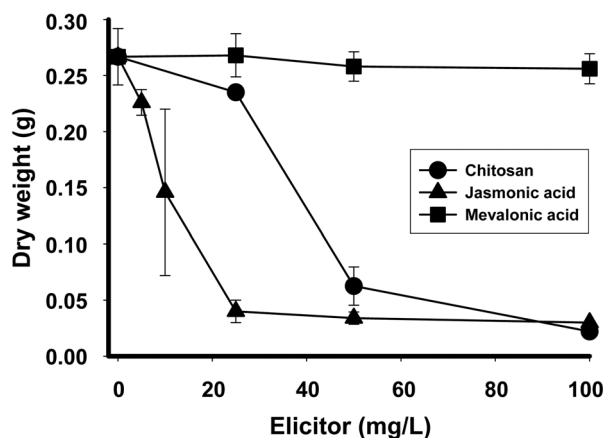


Fig. 6. Effects of elicitors and the precursor (mevalonic acid) on the growth of transgenic adventitious roots. Roots were cultured in B5 medium (pH 5.7) supplemented with 2 mg/L IBA in 100-mL flasks, each containing 40 mL of the medium, on a rotary shaker shaking 100 rpm at 0.5 g and subcultured every 30 days. Vertical bars indicate mean values \pm standard error ($n=5$).

of ginsenosides increased markedly with the addition of elicitors and the precursor (Table 2). Ginsenoside analysis by HPLC revealed that the total ginsenoside content in the transgenic *P. ginseng* roots after 30 days of culture was maximized (84.46 mg/g dry weight) by the addition of jasmonic acid; this level was 12 times higher than that of the wild type (Table 2). In conclusion, overexpression of *PgSQS1* resulted in increased production of ginsenosides, as reported previously [16]. The optimal growth medium for increased ginsenoside production was found to be a B5 medium. Interestingly, the amount of ginsenosides in a ginseng plant seems to be negatively modulated by phytohormones and positively modulated by elicitors, which results in increased growth rates from phytohormones and reduced growth rates from elicitors. Therefore, the effects of the physiological interaction of growth-regulating hormones and growth-retarding elicitors on ginsenoside levels will require future study.

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