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Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation

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Abstract

Squalene synthase (SS) catalyzes the first committed step in sterol and triterpenoid biosynthesis. Transgenic *Eleutherococcus senticosus* Rupr. and Maxim. plants were generated by introducing an SS-encoding gene derived from *Panax ginseng* (PgSS1) together with genes expressing hygromycin phosphotransferase and green fluorescent protein (GFP) through *Agrobacterium*-mediated transformation. Early globular embryo clusters developing from the embryogenic callus were used for *Agrobacterium*-mediated transformation. Transformants were selected on Murashige Skoog medium containing 25 mg/L hygromycin. Hygromycin-resistant somatic embryos developed into plants after the cotyledonary embryos were treated with 14.4 μM gibberellic acid. Transformation was confirmed by polymerase chain reaction, Southern, and GFP analyses. The SS enzyme activity of the transgenic plants was up to 3-fold higher than that of wild-type plants. In addition, GC-MS and HPLC analysis revealed that phytosterols (β-sitosterol and stigmasterol) as well as triterpene saponins (ciwujianosides B (**1**), C_1 (**2**), C_2 (**3**), C_3 (**4**), C_4 (**5**), D_1 (**6**) and D_2 (**7**)) levels in transgenic *E. senticosus* were increased by 2- to 2.5-fold. These results suggest that the metabolic engineering of *E. senticosus* to enhance production of phytosterols and triterpenoids by introducing the *PgSS1* gene was successfully achieved by *Agrobacterium*-mediated genetic transformation.

Keywords: *Eleutherococcus senticosus*; Siberian ginseng; Araliaceae; Ciwujianoside; Genetic transformation; Squalene synthase; Triterpene biosynthesis

1. Introduction

Eleutherococcus senticosus Rupr. and Maxim. (=*Acanthopanax senticosus*, Araliaceae) is a woody medical plant popularly known as Siberian ginseng. It is found only in northeast Asia. The cortical root and stem tissues of these plants are used as a tonic and a sedative as well as to treat rheumatism and diabetes (Umeyama et al., 1992; Davydov and Krikorian, 2000). Norolean-ane-type and oleanane-type triterpene saponins isolated from *E. senticosus* have been denoted as ciwujianosides

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; GA_3, gibberellic acid; GFP, green fluorescence protein; HPT, hygromycin phosphotransferase; MS, Murashige and Skoog; NOS, noparine synthase; RT-PCR, reverse transcription polymerase chain reaction; PgSS1, *Panax ginseng* squalene synthase; SS, squalene synthase.

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(Shao et al., 1988; Fig. 1A). They possess important pharmacological activities, including inhibiting histamine release (Umeyama et al., 1992), inducing hypoglycemia (Sui et al., 1994a), and reducing acute myocardial infarct size in ischemic dogs (Sui et al., 1994b). Triterpene saponins exhibit a wide range of structural diversity and biological activity and have been shown to serve in chemical defenses against pathogens and herbivores (Bouarab et al., 2002; Agrelli et al., 2003). Many triterpene saponins are of economical importance as drugs, detergents, sweeteners and cosmetics. As a result, improving the yield and quality of medicinal plants and

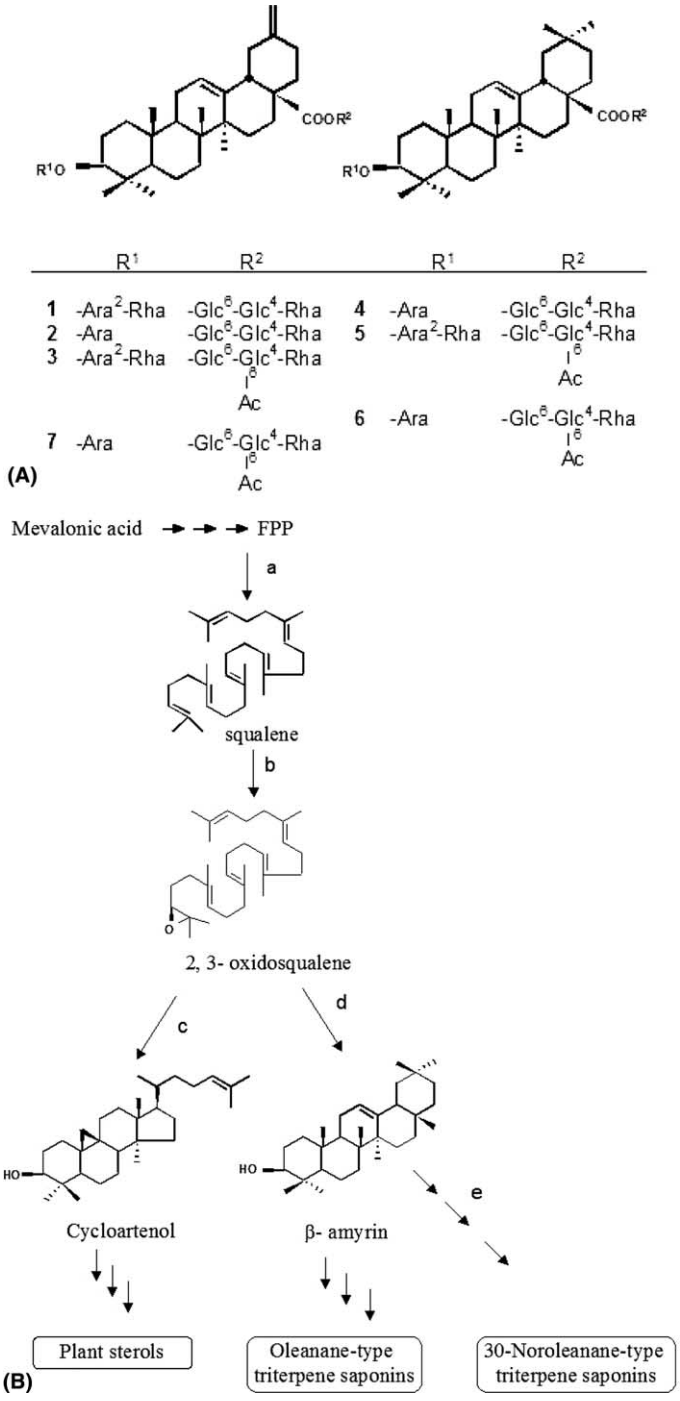

Fig. 1. Ciwujianoside structures and the biosynthetic phytosterol and triterpene pathway. (A) Structure of the ciwujianosides B (**1**), C_1 (**2**), C_2 (**3**), C_3 (**4**), C_4 (**5**), D_1 (**6**) and D_2 (**7**) subjected to the triterpene analysis in this work. Ara, arabinopyranose; Glc, glucopyranose; Rha, rhamnopyranose. (B) The biosynthetic phytosterol and triterpene pathway in *E. senticosus*. Triterpenes undergo oxidation and glycosylation and are converted into triterpene saponins. The indicated enzymes are: (a) squalene synthase (SS), (b) squalene epoxidase (SE), (c) cycloartenol synthase (CAS), (d) β-amyrin synthase (bAS), (e) Ikuta and Itokawa (1989b) proposed that 30-noroleane-type triterpenes in *Stauntonia hexaphylla* callus tissues are derived by oxidation of β-amyrin.

crops that are the source of triterpene saponins has become an important issue. One approach towards achieving this goal has been to modify the triterpene biosynthesis pathway in plants.

Squalene synthase (SS, EC 2.5.1.21) catalyzes the first enzymatic step in sterol and triterpenoid biosynthesis (Abe et al., 1993; Fig. 1B). Since SS acts at a putative key branch point in the isoprenoid biosynthetic pathway, it may be that SS plays a regulatory role in this pathway. This question has been investigated in animals (Guan et al., 1998), yeast (Kennedy and Bard, 2001) and plants (Vögeli and Chappell, 1988; Devarenne et al., 1998, 2002; Wentzinger et al., 2002; Lee et al., 2004). However, despite many reports showing the important roles of the SS gene, it has not yet been shown that the SS has a rate-limiting function in the biosynthesis of triterpene and phytosterols in plants, although we recently found that SS is an important regulatory role in the synthesis of phytosterol and triterpene in *Panax ginseng* (Lee et al., 2004).

We describe here the effect of overexpressing SS in woody *E. senticosus*, which was achieved by introducing the *PgSS1* (*P. ginseng SS1*, Accession No. AB115496) gene. We found that overexpressing *PgSS1* enhanced the biosynthesis of phytosterols and triterpenes in *E. senticosus*.

2. Results and discussion

2.1. Genetic transformation of E. senticosus

We recently established the genetic transformation method for *E. sessiliflorus* that employs *Agrobacterium tumefaciens* (Choi et al., 2004). We tested whether this protocol could also be used for *E. senticosus*. Thus, early globular embryo clusters developed from the embryogenic callus derived from *E. senticosus* zygotic embryos were subjected to *Agrobacterium*-mediated transformation. We used pCAMBIA1302 (Cambia, Australia) as the vector, which enabled us to detect the transformants as GFP-positive cells or plants. In this construct, *PgSS1* is driven by a tandem cauliflower mosaic virus 35S promoter followed by an alfalfa mosaic virus translational enhancer (Fig. 2). Early globular embryos were co-cultivated with *A. tumefaciens* LBA4404 harboring the pCAMBIA1302 construct for three days and then incubated on Murashige and Skoog (MS) agar medium supplemented with 4.4 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and 400 mg/L cefotaxime for 2 weeks. Hygromycin-resistant somatic embryos were obtained on the surfaces of the browned callus (Fig. 3(a)) and GFP signals were used to rapidly screen for the transformants (Fig. 3(b)). GFP-positive embryos were grown to cotyledonary somatic embryos (Fig. 3(c)). Embryos that survived on the selection

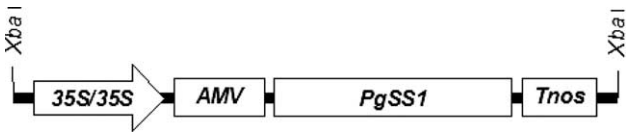

Fig. 2. Schematic representation of the *PgSS1* cassette in pCAMBIA1302. The *PgSS1* cassette was inserted into the *Xba*I site of pCAMBIA1302. *35S/35S*, tandem cauliflower mosaic virus 35S promoter; *AMV*, alfalfa mosaic virus translational enhancer sequence; *PgSS1*, *Panax ginseng* squalene synthase 1 (AB115496); *Tnos*, NOS terminator.

medium were transferred onto MS medium supplemented with 14.4 μM gibberellic acid (GA_3), where they developed into plants (Fig. 3(d)). At about 10 cm in height, the transgenic plants were acclimatized in a plastic box containing peatmoss and pearlite (5:1) for two months, and then transferred to plug soil pots in a glasshouse (Fig. 3(e)).

The somatic embryo-derived *E. senticosus* plants did not grow actively in either the *in vitro* and *ex vitro* conditions. Thus, it was necessary to clonally propagate each line for further analysis. With regard to transgenic *E. sessiliflorus* plants harboring a herbicide-resistant gene, a pure line of embryogenic calli was produced spontaneously on the surfaces of roots of transgenic plants (Choi et al., 2004). However, transgenic *E. senticosus* plants did not produce spontaneous embryogenic cells on their root surfaces. This may be due to the different species involved. Thus, to produce independent transgenic *E. senticosus* lines, cotyledons were detached from the transgenic embryos and cultured on callus induction medium as described by Choi et al. (2002). About 20 independent transgenic cell lines were obtained. The clonal propagation of embryos and plants from the independent transgenic cell lines needed for our further analyses was performed successfully.

2.2. Accumulation of PgSS1 mRNA and SS activity in transgenic E. senticosus

Genomic DNA isolated from wild-type and transgenic plants was digested with *Hin*dIII and subjected to hybridization with a *PgSS1* fragment. Hygromycin-resistant and GFP-positive plants were used for the analysis. Fig. 4 shows the Southern blotting results for a wild-type plant and three independent transgenic lines. In all transgenic lines tested, a hybridization band was detected by the *PgSS1* probe,. which suggests the insertion of a single copy. This result indicates that the *PgSS1*, hygromycin phosphotransferase (*HPT*) and *GFP* genes were successfully introduced into the genome of *E. senticosus*. The different bands in three transgenic lines might be due to integration of introduced genes at variable sites.

The accumulation of *PgSS1* mRNAs in *PgSS1*-transgenic plants was analyzed by reverse transcription-

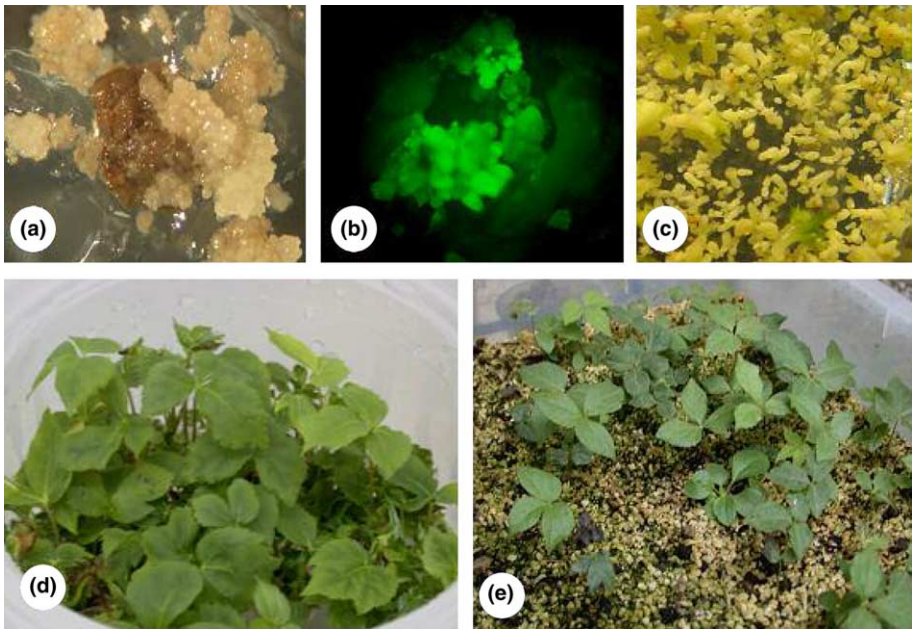

Fig. 3. Putative transgenic embryos and plants. (a) Surviving embryos on the surface of the embryogenic callus on selection medium with 50 mg/l hygromycin and 400 mg/l cefotaxime. (b) GFP-positive somatic embryos. (c) Production of hygromycin-resistant somatic embryos. (d) Plants converted from somatic embryos surviving on selection medium. (e) Transgenic *E. senticosus* plants after their transfer to soil in a glasshouse.

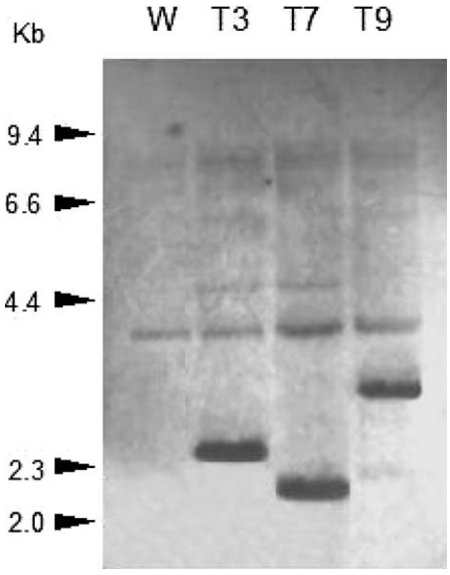

Fig. 4. Southern blot analysis of genomic DNA extracted from three different transgenic *E. senticosus* lines. Lane W, wild-type plants; lanes T3, T7 and T9, independent transformants.

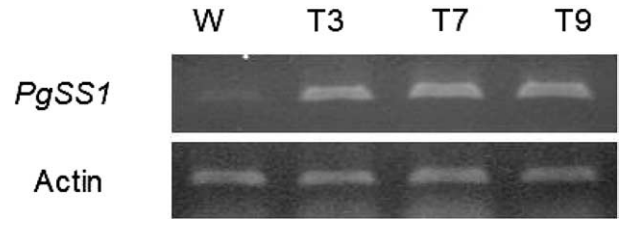

Fig. 5. RT-PCR analysis of *PgSS1* genes in leaves of wild-type and transgenic *E. senticosus*. Total RNAs (2 μg) of leaves of transgenic and wild-type plants were subjected to RT-PCR analysis. The actin gene was used to verify standard RNA loading.

polymerase chain reaction (RT-PCR). Overexpression of the *PgSS1* gene in *E. senticosus* caused the obvious accumulation of *PgSS1* mRNA in the leaves of the transgenic plants (Fig. 5).

SS is an endoplasmic reticulum (ER)-associated enzyme with a predicted carboxy terminus tethering it to the ER membrane (Robinson et al., 1993). Therefore, to determine the level of SS activity in the *PgSS1*-transgenic plants, microsomal fractions were prepared from wild-type and transgenic somatic embryos and plants. The SS activity was 1.5- to 3-fold higher in transgenic embryos than in wild-type embryos (Fig. 6(a)), while the transgenic plants showed 1.2- to 3-fold higher SS activity than wild-type plants (Fig. 6(b)).

All the meristematic zones as well as those undergoing cell expansion would require sterol biosynthesis to support the accompanying membrane biogenesis (Devarenne et al., 2002). We also found that SS enzyme activity was much higher in somatic embryos than plants (Fig. 6), suggesting that the *SS* gene is expressed by actively dividing somatic embryos. Devarenne et al. (2002) have suggested that sterol biosynthesis is localized to the apical meristems and that the apical meristems may be a source of sterols for other plant tissues. Thus, the high enzyme activity in the transgenic somatic *E. senticosus* embryos may have resulted from the meristematic state of somatic embryos, as they possess both shoot and root apical meristems.

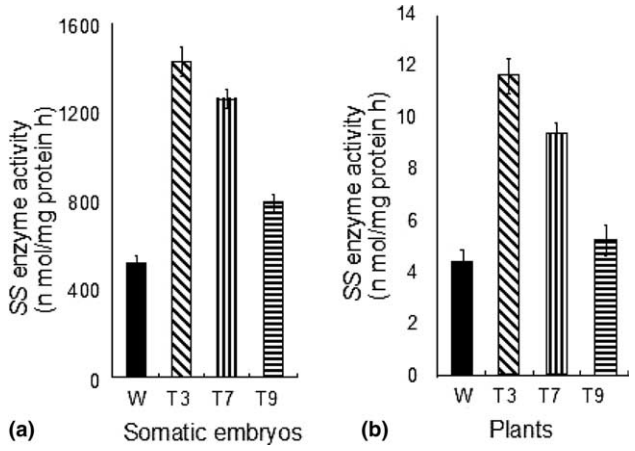

Fig. 6. SS activity in transgenic embryos and plants. Microsomes were isolated from somatic embryos (a) and plants (b) and used to measure SS enzyme activity. W, wild-type; T3, T7 and T9, individual transgenic plants. Vertical bars indicate the mean values ± SE from three independent experiments.

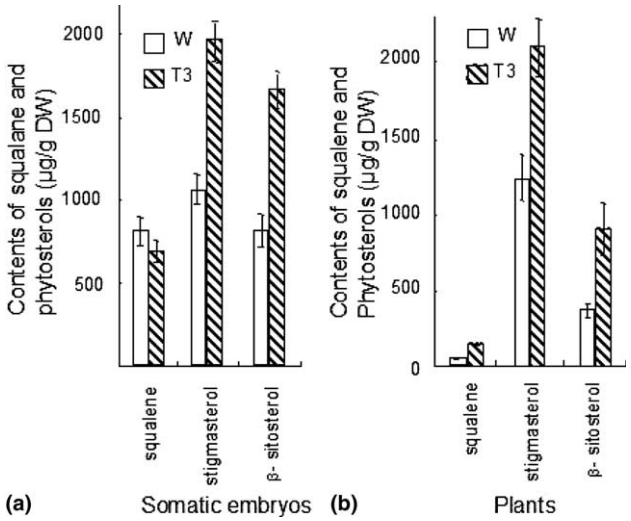

Fig. 7. Squalene and phytosterol levels in transgenic embryos and plants. Wild-type (WT) and transgenic (T3) cotyledonary somatic embryo cells (a) and plants (b) were analyzed. Vertical bars indicate the mean values ± SE from three independent experiments.

2.3. Enhanced production of squalene, phytosterols and triterpene saponins by transgenic *E. senticosus*

The squalene and phytosterol (β-sitosterol, stigmasterol) levels in wild-type and transgenic somatic embryos and plants were analyzed by GC-MS. The squalene levels were similar in transgenic and wild-type cells. However, transgenic cells and plants both had significantly enhanced levels of β-sitosterol and stigmasterol (Fig. 7). This result reveals that SS is an important enzyme in the production of phytosterols, and that these are overproduced in transgenic embryogenic cells and plants expressing *PgSS1*. Similarly, sterol overproduction has been reported in transgenic tobacco expressing the *Hevea brasiliensis* gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGR*), which operates upstream of the *SS* gene (Schaller et al., 1995). In *HMGR* transgenic tobacco (Schaller et al., 1995) as well as in sterol overproducing mutant tobacco (Gondet et al, 1994), most of the overproduced sterols were detected as steryl-esters and were likely to be stored in cytoplasmic lipid body, probably because free-sterols might disturb the membrane integrity.

Sterols a membrane components and as such regulate membrane fluidity and permeability of phospholipid bilayers (Schaller, 2003). Plant membranes consist of a variable mixture of several sterols, with sitosterol usually predominating (Hartmann, 1998). Certain sterols, such as campestrol in *Arabidopsis thaliana*, are precursors of brassinosteroids acting as growth hormones (Yokota, 1997). Brassinosteroid hormones play critical roles in cellular and developmental processes (Yokota, 1997). In this paper, both β-sitosterol and stigmasterol were enhanced clearly in transgenic *E. senticosus* overexpressing *PgSS1* gene. The soil transferred all three lines of transgenic plants showed better size of leaves and looked healthier than wild-type plants during the first-year growth (data not shown).

Ciwujianosides are the triterpene saponins isolated from leaves of *E. senticosus* (Shao et al., 1988). Ciwujianosides are categorized into two types on the basis of their aglycone moiety, namely, noroleandienoic acid glycosides (**1**, **2**, **3**, **7**), and oleanolic acid glycosides (**4**, **5**, **6**) (Fig. 1A). Extracts from transgenic and wild-type plants were subjected to reversed-phase HPLC analysis. Seven different ciwujianosides isolated previously from leaves by Shao et al. (1988) were identified as follows: ciwujianosides B (**1**, Rt 16.5 min), C_1 (**2**, Rt 19.4 min), C_2 (**3**, Rt 18.3 min), C_3 (**4**, Rt 21.3 min), C_4 (**5**, Rt 22.5 min),

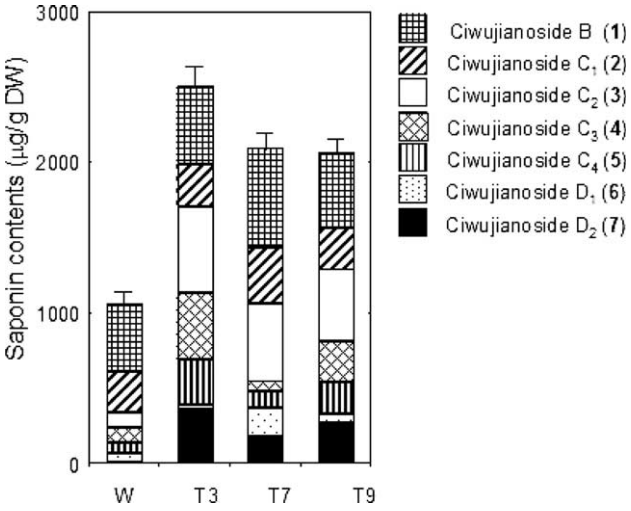

Fig. 8. Triterpene saponin levels in wild-type (WT) and transgenic plants (T3, T7, T9). Vertical bars indicate the mean values ± SE from three independent experiments.

D_1 (**6**, Rt 28.7 min) and D_2 (**7**, Rt 20.6 min). The total ciwujianoside contents in the *PgSS1*-overexpressing transgenic plants were 2- to 2.5-fold higher than in the wild-type plants. In particular, the levels of **3** and **7** were increased by 5- to 10-fold compared to the levels in wild-type plants (Fig. 8). The production of triterpene saponins by the analyzed transformants clearly correlates with the enhanced SS activity in these plants. Interestingly, the left side of the chromatogram of the transgenic lines indicates the presence of many unidentified fractions whose production is also enhanced compared to their levels in the wild-type plant (Fig. 9). It is not clear whether these compounds are from the stem or the root since the HPLC profiles were obtained using whole plant materials.

Thus, the overexpression of the *PgSS1* gene in *E. senticosus* enhanced not only phytosterol production but also the synthesis of triterpenes.

2.4. Conclusion

The present study demonstrated that the *SS* gene plays an important regulatory role in the biosynthesis of ciwujianosides in *E. senticosus*.

Although natural triterpene products encompass more than 200 different skeletal types (Xu et al., 2004), natural sources of 30-noroleanane-type triterpenes are rare and limited thus far to *E. senticosus*, the stem bark of *Guaiacum officinale* (Ahmad et al., 1986), the roots of *Pfaffia glomerata* (Shiobara et al., 1993), and the callus tissues of *Akebia quinata* and *Stauntonia hexaphylla* (Ikuta and Itokawa, 1989a,b). Araliaceae plants are rich in bioactive triperpenes (Shibata, 2001; Zhu et al., 2004). At present, the biosynthetic pathway of the triterpenes awaits determination. Previously, we reported the genetic transformation of *E. sessiliflorus* by using *A. tumefaciens* (Choi et al., 2004) and here demonstrate that this transformation technique could also be used with another Araliaceae woody plant. The establishment of such cell culture and transformation systems will be a powerful tool in helping to elucidate terpenoid skeletal diversity and the biosynthetic pathways involved. Moreover, these techniques will aid the desired objective of metabolic engineering that leads to plants that overproduce pharmaceutically important phytosterol and triterpene saponins.

3. Experimental procedures

3.1. Plant materials

Seeds of *E. senticosus* were collected on Mt. Hambek, Kangwon-do, Korea. The embryogenic callus was derived from zygotic embryos of *E. senticosus* as described by Choi et al. (2002). Zygotic embryos were maintained in MS medium (Murashige and Skoog, 1962) containing 1% sucrose, 4.4 μM 2,4-D and 0.8% agar, pH 5.8. The culture room was maintained at 24 °C with a 16-h photoperiod of 24 μmol m^{-2} s^{-1} provided by a cool white fluorescent tube.

3.2. Vector construction

A full-length cDNA clone of *PgSS1* (Accession No. AB115496) was isolated from expressed sequence tags of a ginseng leaf cDNA library (Lee et al., 2004). The entire coding region of *PgSS1* was amplified with *Pfu* polymerase using primers carrying *Nco*I and a *Bam*HI sites. The resulting 1260 bp fragment was inserted in the *Nco*I and *Bam*HI sites of pBI524 (NRC Plant Biotechnology Institute, Canada) between the double cauliflower mosaic virus 35S promoter (which is followed by the alfalfa mosaic virus enhancer) and the noparine synthase (NOS) terminator. The *PgSS1*-bearing pBI524 plasmid was then digested with *Xba*I and the expression cassette (Fig. 2) was ligated into pCAMBIA1302 (Cambia, Australia). The construct was transformed into *A. tumefaciens* LBA 4404.

3.3. Genetic transformation of E. senticosus

Genetic transformation was carried out according to the reported method reported used to transform *E. sessiliflorus* (Choi et al., 2004). The embryogenic callus was derived from zygotic embryos and the early globular embryos clusters were co-cultivated with *A. tumefaciens* for 3 days and then incubated on MS agar medium supplemented with 4.4 μM 2,4-D and 400 mg/l cefotaxime for 2 weeks. Expression of the transgene in the somatic embryos was confirmed by analysis of GFP signals using an epifluorescence stereomicroscope (Zeiss SV11) equipped with a 100 W mercury bulb light source, a 480 ± 30 nm excitation filter, and a 515 nm long-pass emission filter (Chroma Technology, Brattleboro, Vt., USA). Hygromycin-resistant somatic GFP-positive embryos were grown into cotyledonary somatic embryos. The explants were then transferred into hormone-free MS medium containing 30 mg/l hygromycin and 200 mg/l cefotaxime. The transgenic somatic embryos were then transferred to MS medium supplemented with 50 mg/l hygromycin to induce strong selection during the subculture on selection medium. To produce independent transgenic lines, cotyledons was detached from transgenic embryos and cultured on callus induction medium as described by Choi et al. (2002). About 20 independent transgenic cell lines were obtained. Clonal propagation of embryos and plants from independent transgenic cell lines for additional analyses was successfully performed.

Hygromycin-resistant embryos were transferred onto MS medium containing 14.4 μM GA_3 to induce germi-

nation. Plants were maintained on 1/2-strength MS medium with 2% sucrose.

The small plants (ca. 5 cm in height) were transferred to $10 \times 10 \times 10$ plastic bowls containing 1/2 strength MS medium with 2% sucrose. When plants with both shoots and roots had grown to about 10 cm in height, they were transferred to a glasshouse in plastic pots containing soil and sand (1:1 v/v). Plants were covered with glass beakers for 3 weeks to permit their acclimatization before being exposed to the full glasshouse conditions.

3.4. Southern blot analysis

Genomic DNA from both wild-type and transgenic *E. senticosus* plants was isolated using a Pure-Gene DNA Isolation kit (Gentra Systems, USA). The genomic DNA (30 μg) was digested with *Hin*dIII (TaKaRa, Japan) and then separated by electrophoresis on a 0.8% agarose gel and blotted onto a Hybond-N^+ nylon membrane (Amersham Bioscience, UK). The 0.5 kb *PgSS1* gene fragment that served as a

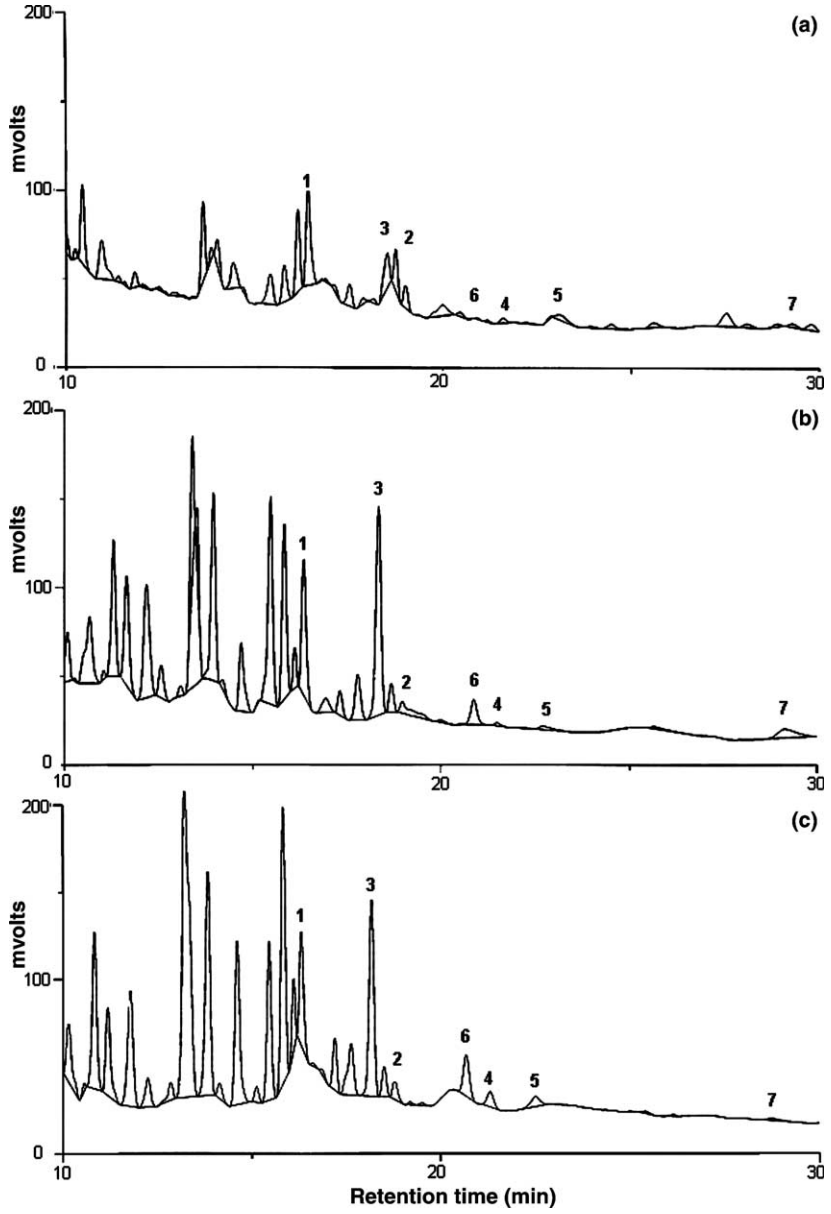

Fig. 9. HPLC profiles of the extracts from wild-type and transgenic plants. The horizontal line indicates the retention time. (a) Wild-type plant. (b) Transgenic plant (T7). (c) Transgenic plant (T9). Peak for ciwujianoside B (**1**, Rt 16.5 min); ciwujianoside C_1 (**2**, Rt 19.4 min); ciwujianoside C_2 (**3**, Rt 18.3 min); ciwujianoside C_3 (**4**, Rt 21.3 min); ciwujianoside C_4 (**5**, Rt 22.5 min); ciwujianoside D_1 (**6**, Rt 28.7 min); and ciwujianoside D_2 (**7**, Rt 20.6 min) are labeled on the chromatogram.

probe was amplified and then purified using a Geneclean II kit (BIO 101, Vista, USA). The amplification primers were 5′-AAG CTC TTC CAT GCC TCT GGG G-3′ and 5′-GTT GTC AAC CTT GGA CTT CAG C-3′. The fragment was labeled with the non-radioactive AlkPhos direct system (Amersham Bioscience, UK) and hybridization signals were detected using Hyper film-ECL (Amersham Bioscience, UK).

3.5. RT-PCR analysis

Total RNAs were isolated from adventitious roots of wild-type plants and three transgenic lines of *E. senticosus* and reverse-transcribed by using the Im-Prom-II Reverse Transcription System (Promega, USA). The first-strand DNAs were used as a template for RT-PCR analysis, performed as follows: 96 °C for 5 min, followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final 10-min extension at 72 °C. The primers used for *P. ginseng PgSS1* (AB115496) were 5′-ATG GGA AGT TTG GGG GCA ATT CT-3′ 5′-GTT CTC ACT GTT TGT TCA GTA GTA GGT T-3′. RT-PCR analysis of β-actin was used to control for RNA integrity and accuracy of loading.

3.6. Microsomal fraction preparation and the squalene synthase enzyme assay

A crude microsomal fraction used to measure *PgSS1* enzyme activity was prepared as described previously (Vögeli and Chappell, 1988). Essentially, 1 g of frozen embryogenic cells or plants was homogenized in 2 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 250 mM sucrose, 4 mM $MgCl_2$, and 5 mM β-mercaptoethanol. The homogenates were filtered through Miracloth (Calbiochem, USA) and centrifuged for 10 min at 100,000 g. 20–30 μg of the microsomal proteins were added to a reaction mixture (total 200 μL) containing 2.5 mM NADPH, 400 mM Tris–HCl (pH 7.5), 20 mM β-mercaptoethanol, 32 mM $MgCl_2$, and 8 nmol of [^{3}H] farnesyl diphosphate (FPP) (87 μCi/μmol, Sigma, USA), and incubated for 30 min at 35 °C. The enzyme assay is based on the conversion of [^{3}H] FPP to squalene, which can be quantified by thin layer chromatography and subsequent scintillation counting of the area corresponding to squalene. Protein levels were measured by the Bradford method (Bradford, 1976).

3.7. Quantitative analysis of squalene and phytosterols by GC-MS

Quantitative analysis of squalene and phytosterols (β-sitosterol and stigmasterol) was performed by GC-MS. Freeze-dried somatic embryos (500 mg) were extracted with ethyl acetate at 100 rpm on a gyratory shaker (20 ml, twice, 25 °C for 6 h). The acidic compounds were removed with aqueous 5% KOH (10 ml, thrice) followed by the removal of the basic compounds with aqueous 5% HCl (10 ml, twice). The organic fraction, which contains the neutral compounds, was washed with water (10 ml, twice) and then dried with anhydrous sodium sulfate. The solvent was evaporated and the residue was dissolved in hexane (2 ml) and then centrifuged for 10 min at 6000 rpm to remove the suspended particles.

An aliquot (2 μL) of the solution was analyzed by GC-MS equipped with the capillary column HP-1 (25 m × 0.25 mm, film thickness 0.33 μm methylpolysiloxane cross-linked capillary column, Hewlett-Packard, USA), carrier gas: He (4 ml min^{-1}), column temperature: 100–250 °C (20 °C min^{-1}). The squalene and phytosterol contents were calculated from the ratios of the peak areas of the relevant compounds to those of the standards. Authentic squalene and phytosterols were obtained from Sigma (USA).

3.8. Quantitative analysis of triterpene saponins by HPLC

The dried powder of plants (0.3 g) was twice extracted with hot EtOH–H_2O (7:3, 50 ml). The aq. EtOH extracts were filtered through Cameo Nylon Syringe Filters (Osmonics, USA), evaporated in vacuo and dissolved in (50 ml) of HPLC grade water. The solution was washed twice with the same volume of Et_2O. The water phase was evaporated and dissolved in H_2O (7:3, 3 ml) EtOH and filtered through a 0.45 μm PTEF filter (Gelman, USA) to yield the saponin fraction. The saponin fraction was analyzed by HPLC (Gilson 321 system, UV 151 detector). The separation was performed on a Cosmosil C18 column (5 μm, 4.6 × 250 mm, Waters, USA) employing the following gradient system: 0 min, 75% eluent A (water) and 25% eluent B (CH_3CN), 13 min, 65% eluent A; 40 min, 60% eluent A; 41 min, 0% eluent A (flow rate 0.8 ml/min, injection volume 20 μm). The saponins were monitored at a wavelength of 203 nm. The authentic saponin ciwujianosides (Shao et al., 1988) were kindly provided by Prof. R. Kasai, Hiroshima International University, Japan.

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