Overexpression of Panax ginseng sesquiterpene synthase gene confers tolerance against Pseudomonas syringae pv…

Article *in* Physiology and Molecular Biology of Plants · October 2016

DOI: 10.1007/s12298-016-0384-9

CITATIONS
0

READS
44

10 authors, including:

Altanzul Khorolragchaa
Mongolian Academy of Sciences
18 PUBLICATIONS **116** CITATIONS
SEE PROFILE

Sathiyamoorthy Subramaniyam
Theragen Bio Institute
59 PUBLICATIONS **264** CITATIONS
SEE PROFILE

Deok-Chun Yang
Kyung Hee University
538 PUBLICATIONS **3,334** CITATIONS
SEE PROFILE

Physiol Mol Biol Plants
DOI 10.1007/s12298-016-0384-9

RESEARCH ARTICLE

Overexpression of *Panax ginseng* sesquiterpene synthase gene confers tolerance against *Pseudomonas syringae* pv. *tomato* in *Arabidopsis thaliana*

Sung-Joo Yoon[1] · Johan Sukweenadhi[2] · Altanzul Khorolragchaa[1] · Ramya Mathiyalagan[2] · Sathiyamoorthy Subramaniyam[1] · Yeon-Ju Kim[1] · Ho-Bin Kim[3] · Mi-Jung Kim[3] · Yu-Jin Kim[1] · Deok-Chun Yang[1,2]

Received: 20 July 2016 / Revised: 30 September 2016 / Accepted: 3 October 2016

Abstract Sesquiterpenes are an abundant group belonging to the terpenoid family, with a C15 structure comprise of three isoprene units. Many sesquiterpenes are volatile compounds and it act as chemical messenger in plant signalling, particularly in the defense mechanism against biotic and abiotic stresses. *Panax ginseng* Meyer is important medicinal herbs with various reported pharmacological efficacies in which its triterpenoid saponins, called ginsenosides, were mostly studied. However, there have been few studies on volatile sesquiterpenes compounds regulation on *P. ginseng*. As slow-growing perennial plant, *P. ginseng* received many kind of stresses during its cultivation. The pathogen attack is one of the most devastated perturbation for ginseng yield. Thus, we aimed to analyze *P. ginseng STS* gene (*PgSTS*) expressions in ginseng organs as well as mono-, sesquiterpenes contents from ginseng seedlings treated with elicitors. qRT-PCR and GC-MS analysis showed that two elicitors- salicylic acid (SA) and methyl jasmonate (MeJA) triggered *PgSTS* expression at different time points and significantly induced mono-, sesquiterpene yield. Overexpression of *PgSTS* in *Arabidopsis* also induced high terpene content and conferred tolerance against *Pseudomonas syringae* pv. *tomato* infection. These results suggested that *PgSTS* transcripts are involved in terpenoid biosynthesis in response to environmental stress mediated by MeJA and SA elicitors; thus, generate tolerance against pathogen attack.

Keywords Gene expression · Ginseng · Methyl jasmonate · Salicylic acid · Terpene content · Sesquiterpene synthase

Introduction

Terpenoids are the most abundant group with over 30,000 known compounds among secondary metabolites (Degenhardt et al. 2009). Sesquiterpenes (C15), the most diversified group of the terpenoid family, plays a variety of ecological roles such as defense signalling (Schnee et al. 2006) and also as medicines (i.e. artemisinin for antimalaria) (Kuhn and Wang 2008). All sesquiterpenes are produced either through mevalonic acid pathway (MVA) pathway or 1-deoxy-d-xylulose/2-C-methyl-d-erythritol-4-phosphate (DOXP/MEP) pathway (Hampel et al. 2005). Until now, at least 300 types of sesquiterpenes have been discovered and numerous sesquiterpenes are volatile compounds which usually are released from the leaves and flowers as a signal to attract pollinators or dispel predatory and parasitic insects. The synthesis and accumulation of volatile sesquiterpenes have also been demonstrated in rhizomes and roots (Arimura et al. 2008).

The synthesis of sesquiterpenes is catalyzed by sesquiterpene synthase (STS). STS frequently was revealed as rate-defining enzymes in the pathways they take part

Sung-Joo Yoon and Johan Sukweenadhi have contributed equally to this work.

✉ Yu-Jin Kim
yujinkim@khu.ac.kr

✉ Deok-Chun Yang
dcyang@khu.ac.kr

[1] Department of Oriental Medicinal Biotechnology, College of Life Science, Kyung Hee University, Yongin 449-701, Korea

[2] Graduate School of Biotechnology, College of Life Science, Kyung Hee University, Yongin 446- 701, Korea

[3] Woongjin Foods Co., Ltd., JEI-PLATZ, 186, Gasan Digital 1-ro, Room 201, Gemcheon-gu, Seoul 153-792, Korea

Published online: 14 October 2016

(Picaud et al. 2005). *In planta* transformation is one way to improve our understanding of *STSs* in plants. Gene transformation of several *STS*s from different sources of plant had been reported, including amorpha-4,11-diene synthase from *Artemisia annua* (Wallaart et al. 2001), (+)-δ-cadinene synthase (*CAD1*) from cotton (Xu et al. 2004), and *trans*-β-caryophellene synthase from both rice (*OsTPS3*) (Cheng et al. 2007) and *Arabidopsis* (*TPS21*) (Huang et al. 2012). Interestingly, transgenic plants overexpressing *STS* have a high level of sesquiterpene production, which acts as a defense mechanism against various abiotic and biotic stresses.

Panax ginseng Meyer is one of the most worthful medicinal herbaceous plant. Its roots are mainly used since the main constituents, triterpene saponins, also called as ginsenosides, are mainly found there. The pharmacological actions of ginseng include anti-tumor, anti-aging, anti-diabetes, anti-stress, and also improvement of immune function (Keum et al. 2000; Cheng et al. 2005; Lee et al. 2006; Yuan et al. 2012; Kang and Min 2012). There are many efforts to increase the content of ginsenosides using various elicitors and fungicides such as SA, MeJA, and lime-bordeaux mixture (Ali et al. 2006; Rahimi et al. 2015). Despite of that, there have been few studies on volatile compounds produced from ginseng. Some studies demonstrated sesquiterpene hydrocarbons production in roots and rootlets of ginseng (Yoshihara and Hirose 1975; Richer et al. 2005). Here, we investigated such volatiles in detail and isolated several interesting sesquiterpenes from ginseng seedlings. We previously isolated *PgSTS* gene (KF294501) from *P. ginseng* and its expression against various environmental stress conditions were analyzed (Khorolragchaa et al. 2010). Furthermore, we performed functional characterization of *PgSTS* overexpressing lines in *Arabidopsis* in response to exogenous elicitors against *Pseudomonas syringae* pv. *tomato* infection.

Materials and methods

Plant materials and growth conditions

From 4-year-old *P. ginseng*, different organs were sampled for gene expression pattern analysis. Meanwhile, ginseng seeds were sown and after it reached 4 weeks, the seedlings were used as terpene samples and gene expression analysis. All ginseng seeds and ginseng plants were obtained from the Ginseng Resource Bank, South Korea. Surface sterilization of ginseng seeds was done with 70 % ethanol for 30 s and subsequently placed in a 2 % solution of sodium hypochlorite for 15 min. The seeds then rinsed three times with double distilled water and sown on solidified Murashige and Skoog (MS) from Duchefa Biocheme, Netherland. Medium supplemented with 10 mg mL^{-1} gibberellic acid, 30 g L^{-1} sucrose with pH 5.7 adjustment. Cultures were kept under a 16 h photoperiod (30 la mol m^{-2} s^{-1}, Osram 36 W/ 30 warm white) at 25 °C for 4 weeks. On this study, *Arabidopsis thaliana* (Columbia ecotype) was used as a model plant. *Arabidopsis* seeds were sown on solidified ½ MS medium comprising 0.5 g L^{-1} 2-(N-morpholino) ethane sulfonic acid (MES), 10 g L^{-1} sucrose and 8 g L^{-1} agar.

Sequence analysis, alignment and homology modeling of PgSTS protein

The *PgSTS* gene from our previous report (Khorolragchaa et al. 2010) was analyzed using BioEdit, ClustalX, MEGA5.1 Beta3 and NCBI BlastX (Alschul et al. 1997; Thompson et al. 1997; Tamura et al. 2011). GENSCAN server (http://genes.mit.edu/GENSCAN.html) was used to generate STS protein sequence, then, the obtained sequence was subjected to NCBI–BLAST. The information about appropriate structural templates and secondary structure elements was collected from Protein Structure Prediction Server/ PSIPRED (McGuffin et al. 2000). The PgSTS and (+)-δ-cadinene synthase of *Gossypium arboreum* (GaDCS) protein sequences (Gennadios et al. 2009) were aligned by using EBI-Align server (Lombard et al. 2002). Modeller9v2 software (Sali and Blundell 1993) was employed to construct a three-dimensional theoretical model of STS. Following the information collected from EBI-DaliLite, a monomeric structure was generated on Silicon Graphics Workstation by using InsightII (Accelrys, San Diego, CA, USA) (Holm and Park 2000). The theoretical monomer model was imposed to the SYBYL 7.1 (Tripos Inc., St. Louis, MO, USA) package in order to correct stereochemistry and bumps of the model at the interface regions. The geometric inaccuracies of the hypothetical model were validated by applying the model to PROCHECK (Laskowski et al. 1993). In addition, the theoretical model of PgSTS in a pentameric form was structurally associated with the test structure of GaDCS (Gennadios et al. 2009) and rendered using PyMOL (DeLano 2002).

Elicitor treatment of ginseng seedlings

Four-week-old ginseng seedlings elicited with MeJA and SA were used to investigate the *PgSTS* gene response and its terpene content. *P. ginseng* seedlings were placed in MS media supplemented with 100 µM of SA or 50 µM of MeJA. Then, samples were collected after 3, 6, 12, 24 and 48 h post-treatment (Cheng et al. 2005). All treated plants including Mock were cultured at 25 °C with 16 h photoperiod condition. After completing all treatments, the

plant materials were sampled, immediately frozen in liquid nitrogen and kept at −70 °C until needed. By using quantitative real-time PCR (qRT-PCR), the expression pattern of *PgSTS* against elicitor treatments was investigated. A total of 10 seedlings of each treatment (including Mock) were sampled from each time point for terpene content measurements and qRT-PCR analysis.

qRT-PCR analysis

Total RNA was extracted from 4-years old *P. ginseng* (flower bud, leaf, stem, rhizome, main roots, and lateral roots) for checking organ-specific expression profile of *PgSTS*. Also, total RNA from Mock and elicitor-treated leaves of 4-weeks old *P. ginseng* was used to check *PgSTS* temporal expression during elicitor treatment (SA and MeJA). RNeasy mini kit (Qiagen, Valencia, CA, USA) was used for RNA isolation. Reverse transcription-PCR was done using 2 μg of total RNA as template and 0.2 mM Oligo (dT) 15 primer (INTRON Biotechnology, Inc., South Korea) which mixed, heated at 75 °C for 5 min then incubated for 1 h with AMV Reverse Transcriptase (10 U μL^{-1}, INTRON Biotechology, Inc.) at 42 °C, followed by 5 min inactivation at 94 °C. 3 μL of cDNA was used for qRT-PCR analyisis in total of 10 μl reaction volume with SYBR® Green Sensimix Plus Master Mix (Quantace, Watford, England). Specific primers for *PgSTS* were used (forward 5′-CTGGCCCGAAGATTAATGACAAA-3′ and reverse 5′-GATGTCTATACTGAAATGGAG GAAGAAATG -3′) and *P. ginseng* actin gene primers were used as control (forward, 5′- CGTGATCTTACAG ATAGCTTGATG-3′ and reverse, 5′- AGAGAAGCTAAGATTGATCCTCC-3′). The thermal cycler conditions were used as follows: 95 °C for 10 min, followed 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C 20 s, as manufacturer recommendation. At the last step of each cycle, fluorescent products were detected. Rotor-GeneTM 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia) was used to carry out amplification, detection, and data analysis. Threshold cycle (Ct) data were collected, representing the number of cycles at which the fluorescence intensity was significantly higher than the background fluorescence at the initial exponential phase of PCR amplification. The Ct values for *PgSTS* were normalized with Ct value for β-actin to determine the relative fold change in template of each sample, which was used as a relative calibrator according to the formula $2^{-\Delta\Delta Ct}$. All experiments were replicated 3 times independently.

Vector constructions for in planta transformation

A complete sequence of *PgSTS* was amplified from *P. ginseng* cDNA using specific primers incorporating a *Sal*I (5-TCGTCGACATGGATGTGAATATCCTT-3) or *Eco*RI (5-TGGAATTCTATGGGAACAGGATCTAT-3) restriction site. To overexpress *PgSTS* into *Arabidopsis*, purified *PgSTS* was digested with *Sal*I–*Eco*RI restriction enzymes and inserted into pCAMBIA1390 (Pro35S: CFP, a gift from Elison Blancaflor), where CFP was integrated into *Sal*I and *Eco*RI sites. The generated construct designated as Pro35S: *PgSTS*-CFP were confirmed by nucleotide sequencing and used for *in planta* transformation of *Arabidopsis thaliana* C60000 using *Agrobacterium tumefaciens* C58C1 (pMP90) mediating transformation (Bechtold and Pelletier 1998). Seeds from five independent transgenic lines were randomly selected and the copy numbers of *PgSTS* cDNA in these lines were estimated on the basis of their segregation on hygromycin (50 mg L^{-1}) plates. PCR analysis was performed to investigate the stable integration of *PgSTS* transgenic plants. The transgenic lines *PgSTS* expression was then verified by qRT-PCR, which showed increased expression compared with wild-type (WT) plants.

Terpene extraction

Mono- and sesquiterpenes were extracted from 4-week-old ginseng seedlings following Lee and Chapell (2008). Briefly, to extract terpene compounds, approximately 0.5 g of ginseng seedlings were ground using mortar and pestle with liquid nitrogen, and extracted twice by dissolving on 1.5 mL of ethyl acetate: hexane (15:85). The extracts were consolidated and purified by using two rounds of silica column chromatography. The subsequent extract was applied to a silica column (7 mm × 146 mm) followed by eluting with 3 ml of fresh ethyl acetate: hexane (15:85). The gathered eluent was applied to a second silica column (7 mm × 146 mm), trailed by eluting oxygenated compounds and hydrocarbons with 2 mL of ethyl acetate: hexane (15:85). For GC-MS sample preparation, approximately 8 mL of final effluent was concentrated to 0.1 mL using nitrogen. Thermo Finnigan DSQ GC/MS (Thermo Fisher Scientific) system equipped with a Restek Rtx-5 capillary column (30 m x 0.32 mm, 0.25-μm phase thickness) was used to analyze terpene constituents from a different sample aliquot. The injector port was kept up at 220 °C in splitless mode. For monoterpene investigation, the oven temperature was set to 40 °C for 1 min followed by a 4 °C min^{-1}, 21 gradients to 150 °C and afterward 20 °C min^{-1}, 21 gradients to 300 °C. Meanwhile, for sesquiterpene investigation, the starting oven temperature of 70 °C (0.5 min) was expanded in a 4 °C min^{-1}, 21 gradients to 180 °C followed by a 20 °C min^{-1}, 21 gradients to 300 °C. Mass spectra were recorded at 70 eV and the scanning started from 35 to 300 atomic mass units (m z^{-1}). All identified compounds from the enzyme assays or leaf extracts were confirmed by comparing the mass

spectra and retention time with authentic standards and/or reported mass spectra in the MassFinder 2.3 and NIST mass spectra library version 2.0.

Bacterial treatment of PgSTS ox lines of Arabidopsis thaliana

King's B medium with additional rifampicin (50 mg L^{-1}) wa used to culture *Pseudomonas syringae* pv tomato DC3000 (ATCC No. BBA-871). Inoculum (OD_{600} = 0.4–0.5) was prepared from overnight bacteria cultures by centrifugation, followed by resuspension in 10 mM $MgCl_2$. Then, bacteria suspension (OD_{600} = 0.01 in 10 mM $MgCl_2$) was sprayed into rosette leaves of 4-week-old *Arabidopsis* plants. Total of 6 plants (each of Mock and infected) were used for quantitative analysis, from which 3 leaves were taken from each plant. Each leaf was gently inverted, exposing the abaxial (under) side, then 1 mL needless syringe containing the bacterial suspension (1×10^6 CFU mL^{-1}) was used to pressure-infiltrate leaf intracellular spaces. After that polythene bag was used to cover plant for maintaining its humidity. 2 and 4 days post-treatment, the leaves were sampled, surface-sterilized using 70 % ethanol then rinsed with distilled water. Excised leaf disks (1 cm^2) were put in 1.5 mL microfuge tubes with addition of 1 mL sterile distilled water and macerated using TissueLyser II (Qiagen). Serial dilutions of the resulting lysates were plated on King's B medium with additional rifampicin (50 mg L^{-1}) and the colony-forming units (CFU) of each sample were enumerated 2 days after.

Sesquiterpene analysis of PgSTS ox lines of Arabidopsis thaliana

Solid-phase microextraction (SPME)-trapped volatiles were performed by GC-MS to investigate sesquiterpene (Aharoni et al. 2003). Briefly, 0.3 g of 5-week-old *Arabidopsis* inflorescence was brought into a 20 mL vial (Gerstel, Mülheim, Germany). The fused silica fiber of the SPME device (coated with 100 µm of polydimethylsiloxane) was introduced into the vial through an aluminum cap. At that point, the volatiles were caught by presenting the fiber to the headspace for 30 min. A 65 um polydimethylsiloxane–divinylbenzene (PDMS-DVB) coated fiber was utilized next. The SPME fiber was exposed in the head-space at 45 °C for 20 min. Later, the fiber was pulled into the needle and moved to the injector of the GC-MS. Utilizing He (37 kPa) as the carrier gas, an HP-5 column (50 m × 0.32 mm, film thickness 1.05 pro) was used. The GC oven temperature was schemed as follows: Hold at 80 °C for 2 min, increment to 250 °C at a rate of 8 °C/min, and hold at 250 °C for 5 min. Mass spectra in the electron impact mode were produced at 70 eV. The compounds were identified by comparing GC retention records and mass spectra with those of authentic reference compounds.

Results and discussion

Sesquiterpenes along with monoterpenes are an crucial essential oils constituents from plants, as the most diverse group of isoprenoids. In addition, sesquiterpenes have been reported to act as defense agents against various environmental stresses, thus, it is important to analyze terpene content in elicitor-treated conditions. In the present study, we characterized *PgSTS* gene expression in various organs

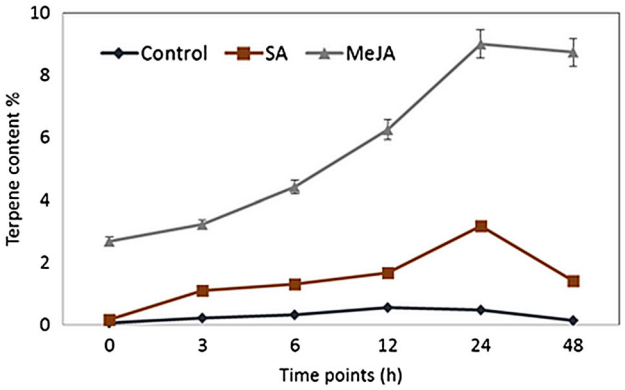

Fig. 1 Terpene contents of 4-week-old control and SA or MeJA treated ginseng seedlings. The data represent the mean ± SE of three independent replicates

Table 1 Mono- and sesquiterpenes present in 4-week-old ginseng seedlings (48 h post SA or MeJA treatments)

Compounds (%)	Control	SA (100 µM)	MeJA (50 µM)
Monoterpenes			
β -pinene	0.03	–	0.03
β -myrcene	–	0.22	0.66
$_L$-limonene	–	0.03	0.08
β -phellandrene	–	–	0.02
Sesquiterpenes			
β -panasinsene	–	0.11	0.34
β -elemene	0.05	0.41	1.19
(-)-α-neoclovene	–	0.13	0.03
Aromadendrene	–	–	0.05
Trans- β -farnesene	0.45	2	5.53
Trans- β -caryophyllene	0.01	0.13	0.37
Germacrene D	–	0.15	–
β -neoclovene	–	–	0.05
β -cubebene	–	–	0.44
(-)-isoledene	–	–	0.02
α-selinene	–	–	0.02
Trans-α-bisabolene	–	–	0.1

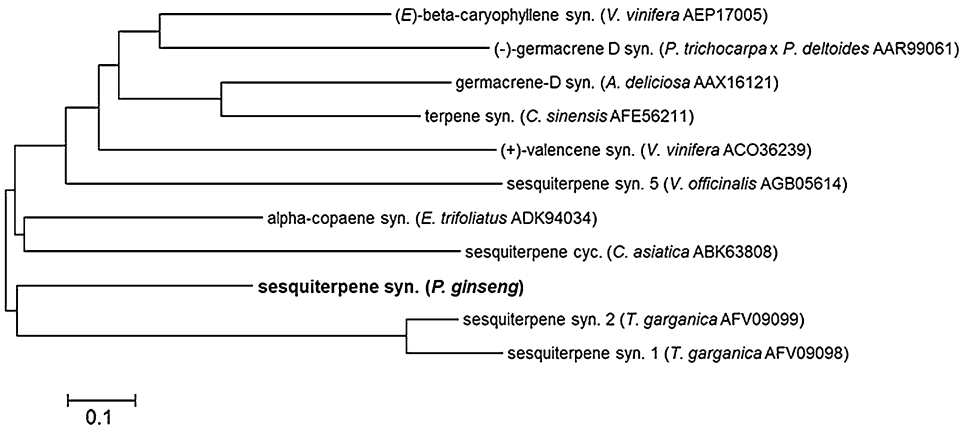

Fig. 2 Phylogenetic tree of PgSTS proteins and close homologs. The phylogenetic tree was constructed using the ClustalX program (neighbor-joining method). The *bar* represents 0.1 substitutions per amino acid position

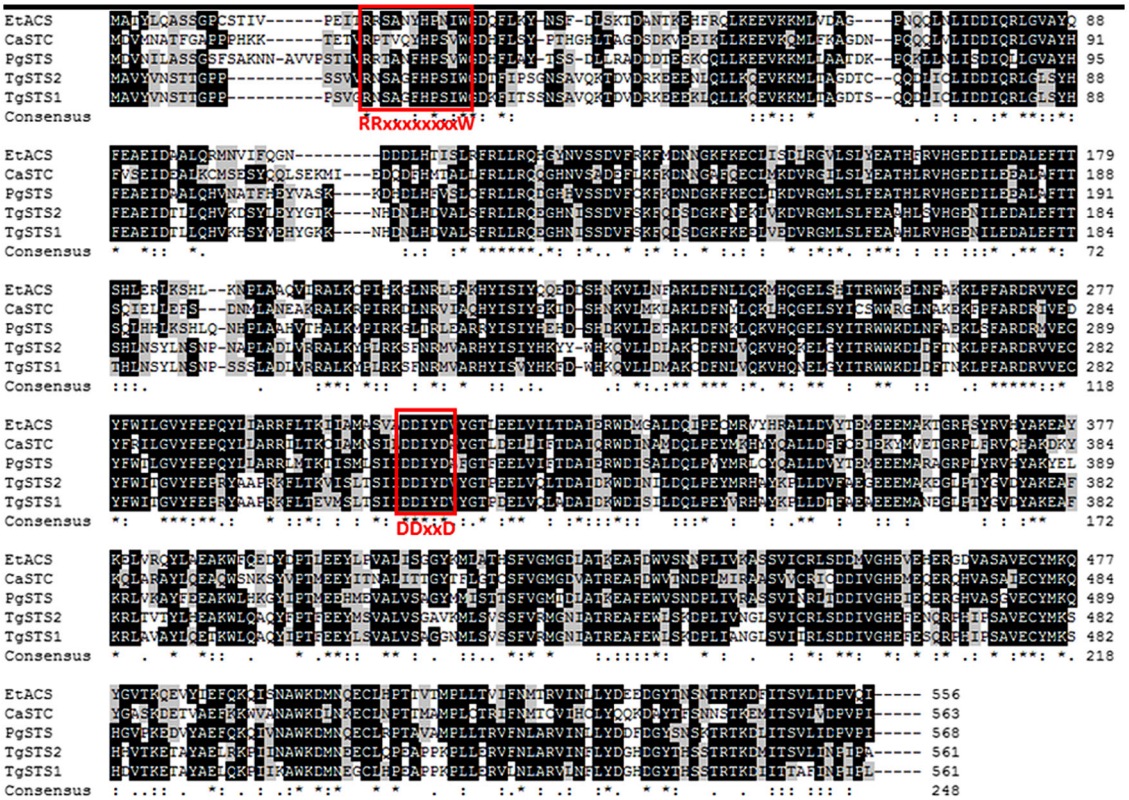

Fig. 3 Alignment of PgSTSs and close homologs. Boxed domains represent conserved motifs RRx_8W and DDxxD, which are known STS functional domains. Residues conserved in all sequences are marked with *asterisks*. *Colons* and *dots* indicate the positions of amino acid residues with strong and weak similarity, respectively. Residues that are identical in at least half of the sequences are printed with a *black* background, residues that are similar in at least half of the sequences are printed with a *gray* background, and residues with <50 % similarity are printed with a *white* background. EtACS (ADK94034), CaSTC (ABK63808), PgSTS (AGS16741), TgSTS2 (AFV09099), and TgSTS1 (AFV09098)

of ginseng as well as determined sesquiterpene content in *P. ginseng* seedlings and overexpressed *Arabidopsis* lines treated with different elicitors. Furthermore, we checked the *Arabidopsis* transgenic lines tolerance against *Pseudomonas syringe* infection.

The roots of ginseng have long been used for medicinal purposes. Many reports on ginsenosides have been published. However, there have been few studies of the volatile compounds produced by ginseng, except some reports that sesquiterpene hydrocarbons were thought to be present only in the roots and rootlets of ginseng (Yoshihara and Hirose 1975; Richer et al. 2005). Here, the detailed investigation of the volatiles of ginseng and isolated several sesquiterpenes from ginseng

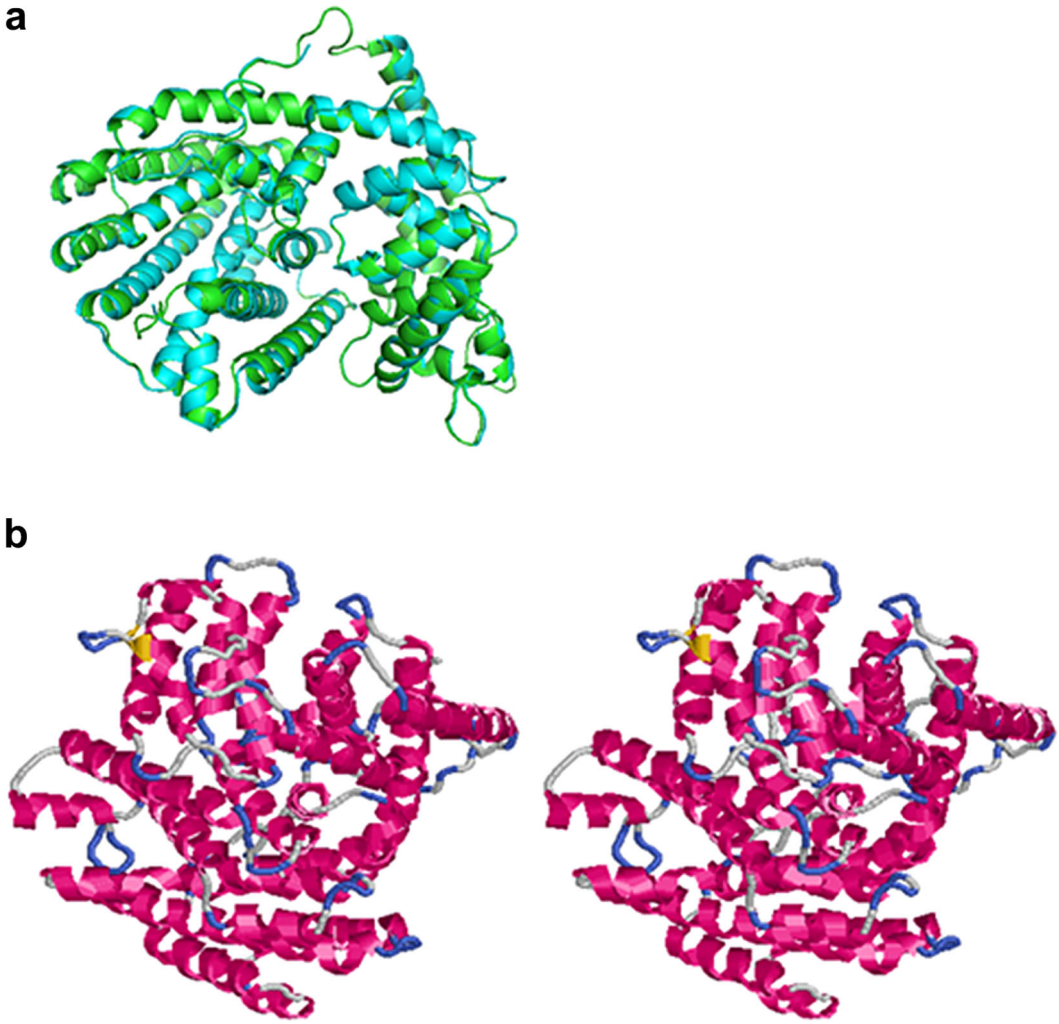

Fig. 4 3-D structural view of STS. **a** Stereo view of a cartoon diagram with alpha and beta helices. **b** PgSTS was superimposed with the X-RAY structure of (+)-δ-cadinene synthase from *Gossypium arboreum* (GaDCS) (Gennadios et al. 2009). Structurally conserved regions (SCRs) and structurally variable regions (SVRs) are shown in *green* and *blue*, respectively (color figure online)

seedlings were carried out. The levels of terpenes under SA and MeJA treatments were quantified by using GC-MS. Levels of terpenes in ginseng seedlings increased within 24 h, reached 3.18 and 9 % of emission after the exposure to SA and MeJA, respectively (Fig. 1). Two (β-myrcene and l-limonene) and four types (β-pinene, β-myrcene, l-limonene and β-phellandrene) of monoterpenes were released at 48 h in response to SA and MeJA, respectively (Table 1). Likewise, SA and MeJA caused a significant increase in sesquiterpene yield in ginseng seedlings. The types and levels of sesquiterpene were increased after 48 h (Table 1) in which only 3 types of sesquiterpenes were found in control plants. However, upon treatment with SA and MeJA, the number of sesquiterpenes increased to 6 and 11 %, respectively. After MeJA treatment, four-week-old ginseng seedlings produced 11 different kinds of sesquiterpenes, while SA treated seedlings produced 6 kinds of sesquiterpenes. There were also increases in the sesquiterpenes, *trans*-β-farnesene, β-elemene, and *trans*-β-caryophyllene by 2, 0.41, and 0.13 % after SA treatment and 5.53, 1.19, and 0.37% after MeJA treatment, respectively (Table 1). Both of treated seedlings have *trans*-β-farnesene as the highest percentage of volatiles. *Trans*-β-farnesene is an acyclic sesquiterpene olefin found from both plant and animal taxa, reported as an important chemical messenger. Indeed, *trans*-β-farnesene is one of essential oil found in hundreds of angiosperms and gymnosperms species (Crock et al. 1997). *Trans*-β-farnesene was originally found in both fresh and dried roots of ginseng, but not at a high percentage compared with other sesquiterpenoid volatiles (Yoshihara and Hirose 1975). Thus, it convinced that elicitors treatment raised the production of sesquiterpenes.

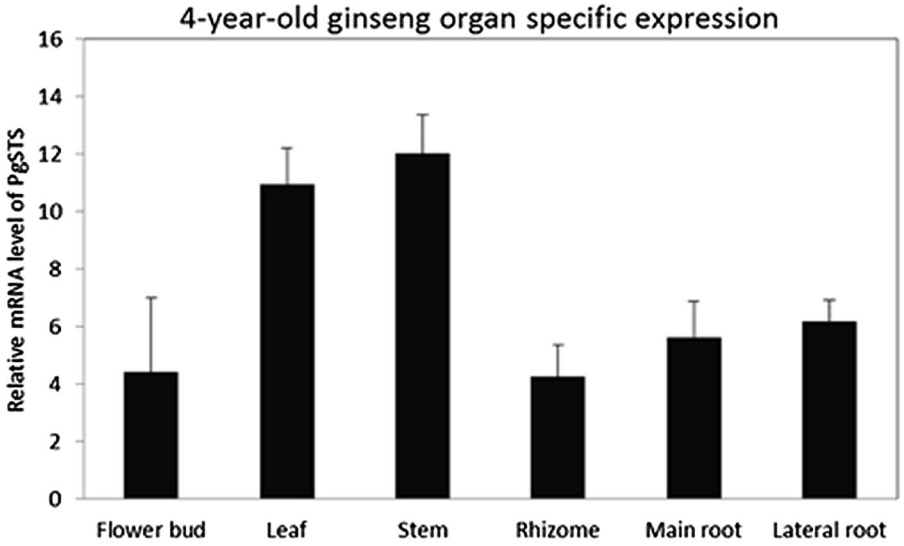

Fig. 5 Organ-specific expression of *PgSTS*. Four-year-old field samples were collected and used to quantify the expression pattern of *STS*. Relative gene expression was determined by qRT-PCR. The data represent the mean ± SE of three independent replicates

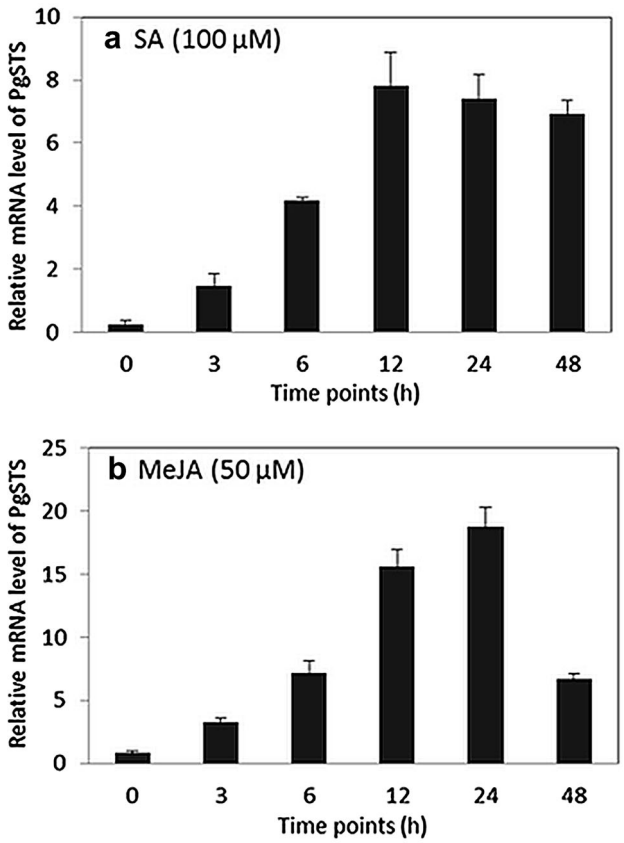

Fig. 6 Expression levels of *PgSTS* in SA- (**a**) and MeJA- (**b**) treated seedlings of *P. ginseng*. A quantitative expression profile of *STS* was determined at various time intervals. The Ct value of the actin gene was obtained and used as the relative calibrator according to the formula $2^{-\Delta\Delta Ct}$. The data represent the mean ± SE of three independent replicates. Average values of treated samples are significantly different compared to the control at $^{*}P < 0.001$ according to Student's *t* test

Previously, *PgSTS* is found to have 1883 bp nucleotide length and has an open reading frame (ORF) of 1704 bp encoding a precursor protein of 568 amino acids (Khorolragchaa et al. 2010). PgSTS had the highest sequence homology to STS 1 and 2 from *Thapsia garganica* (AFV09098 and AFV09099), with similarities of 59 and 62 %, respectively. Sequence analysis also revealed high similarity and identity with other species such as α-copaena synthase of *Eleutherococcus trifoliatus* (ADK94034) (identity 53 % and similarity 69 %), and sesquiterpene cyclase of *Centella asiatica* (ABK63808) (identity 51 % and similarity 61 %) based on phylogenetic tree made from the retrieved amino acid sequences of STSs (Fig. 2). Multiple alignment of PgSTS indicated that the amino-acid residues were highly conserved with all of the sequences (Fig. 3), including PgSTS containing an arginine-arginine/tryptophan conserved motif RRx_8W which is often found in the N-terminal portion (Bohlmann et al. 1998), at 26–36 positions from the N-terminal and also aspartate-rich DDxxD active-site motif, which is found in all terpene enzymes (Whittington et al. 2002), at 320–324 positions from the N-terminal (Fig. 3). These motifs and variations thereof are described to play an crucial role in enzyme function (Davis and Croteau 2000) and are commonly found in plant terpene synthases (Aubourg et al. 2002) and. The short N-terminal region upstream of the RRx_8W motif indicated the absence of a plastid transit peptide (Bohlmann et al. 1998). In addition, a three-dimensional model of *PgSTS* indicated a similar composition as other homologous plant terpene synthases (Fig. 4). By utilizing the crystal structure of previous structurally characterized plant STS, the GaDCS (Chain A) monomer as the model (Gennadios et al. 2009), a theoretical structure of PgSTS was

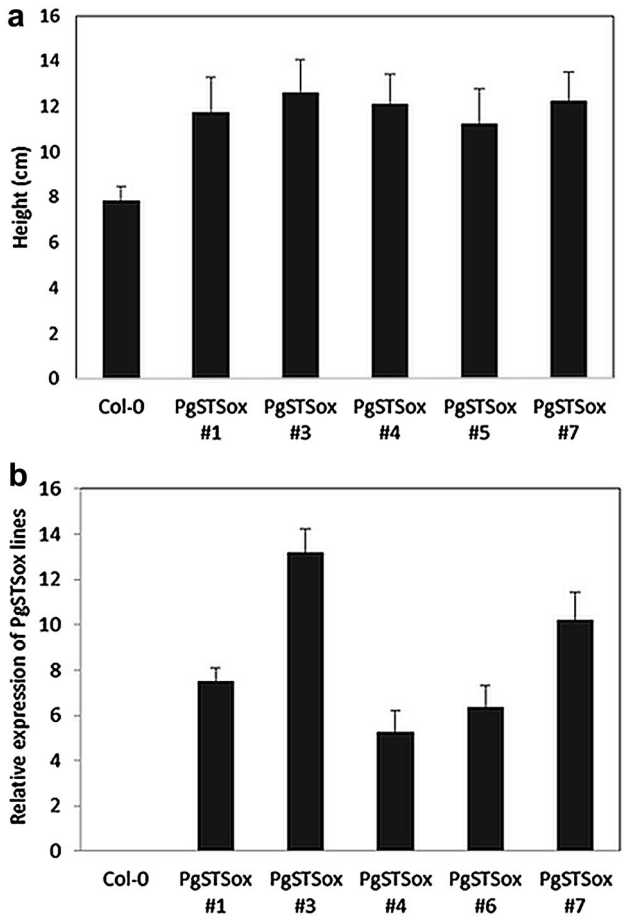

Fig. 7 Plant height (**a**) and relative mRNA expression levels (**b**) of *PgSTS*ox lines. The data represent the mean ± SE of three independent replicates

derived from the program MODELLER, based on the alignment generated by EBI-Align. For one subunit modeling, 20 different models were produced and the structure with the lower energy value was chosen. The structural superimposition of tentatively available monomer residue and hypothetically acquired structure was collected from EBI-DaliLite server (Fig. 4a). Then, by using InsightII, a theoretical structure of PgSTS was generated refer to the information provided by EBI-DaliLite. The superimposition results revealed that the structurally conserved regions (SCRs) and the structurally variable regions (SVRs) were quite similar compared to the target PgSTS and template GaDCS (Fig. 4b). The monomer was imposed to 100 iterations in order to strip steric clashes at the subunit interfaces. Procheck was used to examine the stereochemical quality which is apparently the most powerful determinant of theoretical protein coordinates quality. The Ramachandran plot quality was worthy good compared to that of the template structure, which justified the theoretical model reliability. The average pairwise RMS fit of the Cα coordinates of the experimental and theoretical structures was 0.163 Å, indicating a strong structural conservation among models and similarity in the structural folds. Likewise, the major segment of the PgSTS sequence was in good consensus with the GaDCS (Chain A) template. GaDCS is a sesquiterpene cyclase catalyzes biosynthesis of cadinane-type sesquiterpenes, such as gossypol. This compound belong to sesquiterpene phytoalexins which was found to be able providing constitutive and inducible defence protection against pests and diseases (Chen et al. 1995). Similarity with this protein indicating possible role of PgSTS on pathogen defense response.

An organ-specific expression pattern of *PgSTS* was determined using mRNA samples of 4-year-old *P. ginseng* by qRT-PCR. As shown in Fig. 5, *PgSTS* expressions were observed higher in the leaf (10.97-fold) and stem (12.03-fold) rather than in the flower bud (4.43-fold), rhizome (4.26-fold), main roots (5.65-fold) and lateral roots (6.20-

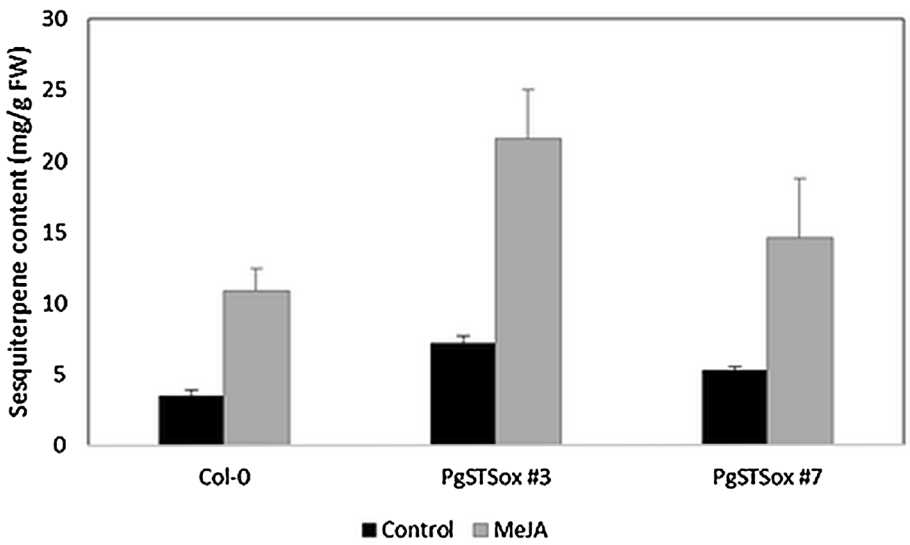

Fig. 8 Sesquiterpene content of *PgSTS*-overexpressing transgenic *Arabidopsis* plants. The data represent the mean ± SE of three independent replicates

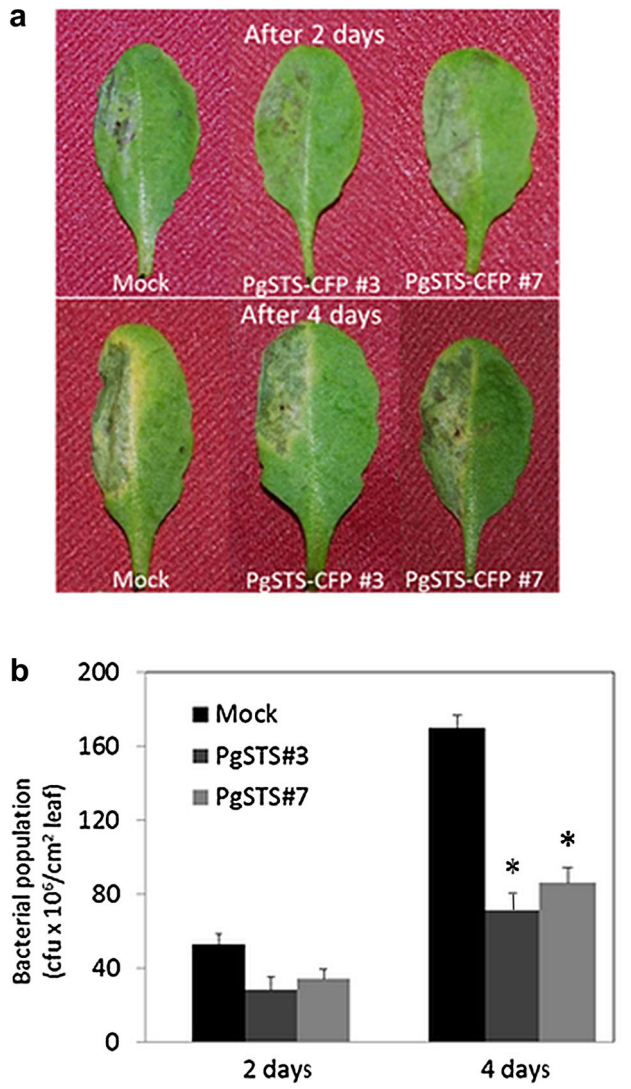

Fig. 9 In vitro assay of *PgSTS*ox lines against bacterial pathogens. Healthy leaves of wild-type and transgenic plants were infected with *Pseudomonas syringe* **a** photographs were taken after 2 and 4 days of treatment, **b** bacterial populations per leaf disc (1 cm^2) were counted after 2 and 4 days of syringe infiltration containing *P. syringe*, and the corresponding leaves were compared with the empty vector control (Pro35S: YFP) and two independent transgenic lines. The data represent the mean ± SE of three independent replicates. Averages of treated samples were significantly different compared to the control, $^*P < 0.05$

fold). It was corresponded with *Oryza sativa* terpene synthase (*OsTPS3*) which is strongly expressed in leaf, spikelet and sheath tissues (Cheng et al. 2007). In contrast, *Zingiber* STS (*ZSS1*) expressed strongly in rhizomes and very weakly in stems (Yu et al. 2008a, b). qRT-PCR analysis was performed using four-week-old seedlings of *P. ginseng* to check whether *PgSTS* transcription can be influenced by different elicitors such as SA and MeJA. Specifically, *PgSTS* was expressed differentially when ginseng roots were exposed to these elicitors as a function of exposure time (Fig. 6). SA induces gene expression which related to biosynthesis of several classes of plant secondary metabolites, regarded as a general inducer of plant defensive metabolite production. *PgSTS* expression was increased 0.24-fold at 3 h and continued to increase until 12 h post treatment in ginseng seedlings upon treatment with 100 μM SA (Fig. 6a). *PgSTS* transcript levels were increased to 7.82-fold (highest) at 12 h and slightly reduced to 6.91-fold at 48 h. *PgSTS* exhibits a similar transcription profile in ginseng hairy roots (Khorolragchaa et al. 2010). Sometimes SA cannot induce terpene expression in plants, such as *trans*-β-ocimene synthase from *A. thaliana* (Faldt et al. 2003). Jasmonates including MeJA have been reported to be signal transducers for plant secondary metabolites production (Gundlach et al. 1992) and stimulate accumulation of metabolites consist of different structural classes, such as alkaloids, terpenoids, phenolic compounds and others. RT-PCR results showed that the *AtTPS03* expression 16 h post treatment was greater than that of a 2 h post treatment with JA (Faldt et al. 2003). Similar with that, MeJA treatment also up-regulated the *PgSTS* expression on ginseng seedlings after 1 h (0.82-fold), then increased thereafter, with a maximum accumulation of 18.73-fold observed at 24 h (Fig. 6b). On our previous report (Khorolragchaa et al. 2010), abiotic stress such as chilling (4 °C) was induced *PgSTS* transcript level to 2.95 fold at 1 h and reached its peak at 3.57 fold at 4 h. Meanwhile, abscisic acid (ABA) treatment stimulated *PgSTS* expression level to 3.56 fold at 2 h and increased gradually until 3.56 fold at 48 h post treatment. ABA, defined as a stress hormone, plays a central role in responses to biotic and abiotic stresses (Smet et al. 2006).

In order to elucidate the functional roles of *PgSTS*, a key gene involved in the biosynthesis of terpenes, we overexpressed *PgSTS* in the well-defined model plant *Arabidopsis*. Transgenic plants were confirmed using genomic DNA as the template and *PgSTS* were amplified in all five of the selected lines but not Col-0 (as WT). Under the same growth conditions, the phenotypes of transgenic plants did not differ greatly both in shape and size of leaves compared to WT. However, the shoots heights of transgenic lines were higher than WT (Fig. 7a). As shown in Fig. 7b, the transcript levels of *PgSTS*ox#3 and *PgSTS*ox#7 were higher than WT plants by 13.15 and 10.17-fold. Based on the phenotype and expression levels, these two lines were chosen for further analysis. Terpene content was considerably modified by expression of *PgSTS* in the transgenic lines. In addition, the transgenic lines increased sesquiterpene contents compared with WT plants (Fig. 8). Plant height, expression level, and sesquiterpene content were higher in *PgSTS*ox lines compared to WT (Figs. 7, 8). A similar pattern existed in transgenic tomato (Davidovich-Rikanati et al. 2008) and spearmint (Kang et al. 2012).

Thus, increased terpene levels as a result of overexpression of *PgSTS* in *Arabidopsis* resulted in decreased susceptibility to biotic stress, suggesting a role as a plant defense mechanism.

P. syringae is one of the pathogens which infects a broad variety of plants including *Arabidopsis*. This established pathosystem model has contributed enormously to the molecular understanding of plant-pathogen interactions. With black spots on the surface of veins, the leaves of *PgSTS*ox plants exhibited less severe symptoms compared to mock-treated WT plants which showed discoloration and severe necrosis (Fig. 9a). The bacterial populations size of mock and transgenic lines was quantified by titration of living cells in leaves 2 and 4 days post-inoculation. Bacterial growth increased continuously after inoculation treatment. However, bacterial growth was significantly less in both transgenic lines compared with WT plants at 4 days post-inoculation (Fig. 9b), indicating that sesquiterpenoid emission either increased the plant tolerance or inhibited the pathogen growth.

In summary, qRT-PCR and GC-MS analysis showed MeJA and SA triggered *PgSTS* expression at different time points resulting on higher terpene yield. Upon MeJA and SA treatment, the sesquiterpenes content in ginseng seedlings was significantly increased. Moreover, overexpression of *PgSTS* in *Arabidopsis* increased level of terpene content and induced tolerance against *Pseudomonas* infection. Taken together, these results suggested that *PgSTS* transcripts are involved in terpenoid biosynthesis of ginseng and it can be enhanced by giving MeJA and SA as elicitors; also, high terpene content can generate tolerance against pathogenic attack.

Acknowledgments This research was supported by Basic Science Research Program, National Research Foundation (NRF), Ministry of Education, Republic of Korea (2013R1A1A2064430) to YJ Kim, and Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea (iPET 112142-05-4-SB010) to DC Yang.

References

Aharoni A, Giri AP, Deuerlein S, Griepink F, Kogel WJ, Verstappen FWA, Verhoeven HA, Jongsma MA, Schwab W, Bouwmeester HJ (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. Plant Cell 15:2866–2884

Ali MB, Yu KW, Hahn EJ, Paek KY (2006) Methyl jasmonate and salicylic acid elicitation induces ginsenosides accumulation, an enzymatic and non-enzymatic antioxidant in suspension culture *Panax ginseng* roots in bioreactors. Plant Cell Rep 25:613–620

Alschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Arimura GI, Garms S, Maffei M, Bossi S, Schulze B, Leither M, Mithofer A, Boland W (2008) Herbivore-induced terpenoid emission in *Medicago truncatula*: concerted action of jasmonate, ethylene and calcium signaling. Planta 227:453–464

Aubourg S, Lecharny A, Bohlmann J (2002) Genomic analysis of the terpenoid synthase (AtTPS) gene family of *Arabidopsis thaliana*. Mol Genet Genom 267:730–745

Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. *Arabidopsis* protocols. Method Mol Biol 82:259–266

Bohlmann J, Meyer-Gauen G, Croteau R (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. PNAS 98:4126–4133

Chen XY, Chen Y, Heinstein P, Davisson VJ (1995) Cloning, expression, and characterization of (+)-δ-cadinene synthase: a catalyst for cotton phytoalexin biosynthesis. Arch Biochem Biophys 324(2):255–266

Cheng Y, Shen LH, Zhang JT (2005) Anti-amnestic and anti-aging effects of ginsenoside Rg1 and Rb1 and its mechanism of action. Acta Pharmacol Sin 26:143–149

Cheng AX, Xiang CY, Li JX, Yang CQ, Hu WL, Wang L, Lou YG, Chen XY (2007) The rice (*E*)-β-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes. Phytochemistry 68:1632–1641

Crock J, Wildung M, Croteau R (1997) Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha* × *piperita*, L.) that produces the aphid alarm pheromone (E)-β-farnesene. PNAS 94:12833–12838

Davidovich-Rikanati R, Lewinsohn E, Bar E, Iijima Y, Pichersky E, Sitrit Y (2008) Overexpression of the lemon basil a-zingiberene synthase gene increases both mono- and sesquiterpene contents in tomato fruit. Plant J 56:228–238

Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Biosynthesis 209:53–95

Degenhardt J, Köllner TG, Gershenzon J (2009) Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry 70:1621–1637

DeLano WL (2002) The PyMOL molecular graphics system. DeLano Scientific, San Carlos

Faldt J, Arimura G, Gershenzon J, Takabayashi J, Bohlmann J (2003) Functional identification of AtTPS03 as (*E*)-β-ocimene synthase: a monoterpene synthase catalyzing jasmonate and wound-induced volatile formation in *Arabidopsis thaliana*. Planta 216:745–751

Gennadios HA, Gonzalez V, Costanzo LD, Li A, Yu F, Miller DJ, Allemann RK, Christianson DW (2009) Crystal structure of (+)-δ-cadinene synthase from *Gossypium arboreum* and evolutionary divergence of metal binding motifs for catalysis. Biochemistry 48(26):6175–6183

Gundlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. PNAS 89:2389–2393

Hampel D, Mosandl A, Wüst M (2005) Biosynthesis of mono-and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry 66(3):305–311

Holm L, Park J (2000) DaliLite workbench for protein structure comparison. Bioinformatics 16:566–567

Huang M, Sanchez-Moreiras AM, Abel C, Sohrabi R, Lee S, Gershenzon J, Tholl D (2012) The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (*E*)-b-caryophyllene, is a defense against a bacterial pathogen. New Phytol 193:997–1008

Kang S, Min H (2012) Ginseng, the 'immunity boost': the effects of *Panax ginseng* on immune system. J Ginseng Res 36:354–368

Kang YM, Park DJ, Song HJ, Ma HS, Karigar C, Choi MS (2012) Comparative analysis of terpenoids in in vitro culture media of metabolically engineered transgenic and wild-type spearmint (*Mentha spicata* L.) Korean. J Med Crop Sci 20:301–307

Keum YS, Park KK, Lee JM, Chun KS, Park JH, Lee SK, Kwon HJ, Surh YJ (2000) Antioxidant and anti-tumor promoting activities of the methanol extract of heat-processed ginseng. Cancer Lett 150:41–48

Khorolragchaa A, Parvin S, Shim JS, Kim YJ, Lee OR, In JG, Kim YJ, Kim SY, Yang DC (2010) Isolation of sesquiterpene synthase homolog from *Panax ginseng* C.A. Meyer. J Ginseng Res 34:17–22

Kuhn T, Wang Y (2008) Artemisinin-an innovative cornerstone for anti-malaria therapy. Prog Drug Res 66(383):385–422

Laskowski RA, Macarthur MW, Moss DS, Thornton JM (1993) PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 26:283–291

Lee S, Chapell J (2008) Biochemical and genomic characterization of terpene synthases in *Magnolia grandiflora*. Plant Physiol 147:1017–1033

Lee SH, Jung BH, Kim SY, Lee EH, Chung BC (2006) The antistress effect of ginseng total saponin and ginsenoside Rg3 and Rb1 evaluated by brain polyamine level under immobilization stress. Pharmacol Res 54:46–49

Lombard V, Camon EB, Parkinson HE, Hingamp P, Stoesser G, Redaschi N (2002) EMBL-Align: a new public nucleotide and amino acid multiple sequence alignment database. Bioinformatics 18:763–764

McGuffin LJ, Bryson K, Jones DT (2000) The PSIPRED protein structure prediction server. Bioinformatics 16:404–405

Picaud S, Olofsson L, Brodelius M, Brodelius PE (2005) Expression, purification, and characterization of recombinant amorpha-4,11-diene synthase from *Artemisia annua* L. Arch Biochem Biophys 436:215–226

Rahimi S, Kim YJ, Yang DC (2015) Production of ginseng saponins: elicitation strategy and signal transductions. Appl Microbiol Biotechnol 99:6987–6996

Richer R, Basar S, Koch A, Konig WA (2005) Three sesquiterpene hydrocarbons from the roots of *Panax ginseng* C.A. Meyer (Araliaceae). Phytochemistry 66:2708–2713

Sali A, Blundell TL (1993) Comparative protein modeling by satisfaction of spatial restraints. J Mol Biol 234:779–815

Schnee C, Köllner TG, Held M, Turlings TCJ, Gershenzon J, Degenhardt J (2006) The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. PNAS 103:1129–1134

Smet ID, Zhang H, Inz D, Beeckman T (2006) A novel role for abscisic acid emerges from underground. Trends Plant Sci 11:434–439

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Maijers NCA (2001) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. Planta 212:460–465

Whittington DA, Wise ML, Urbansky M, Coates RM, Croteau RB, Christianson DW (2002) Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. PNAS 99:15375–15380

Xu R, Fazio GC, Matsuda SPT (2004) On the origins of triterpenoid skeletal diversity. Phytochemistry 65:261–291

Yoshihara K, Hirose Y (1975) The sesquiterpenes of ginseng. Bull Chem Soc Jpn 48:2078–2080

Yu F, Harada H, Yamasaki K, Okamoto S, Hirase S, Tanaka Y, Misawa N, Utsumi R (2008a) Isolation and functional characterization of a β-eudesmol synthase, a new sesquiterpene synthase from *Zingiber zerumbet* Smith. FEBS Lett 582:565–572

Yu F, Okamto S, Nakasone K, Adachi K, Matsuda S, Harada H, Misawa N, Utsumi R (2008b) Molecular cloning and functional characterization of α-humulene synthase, a possible key enzyme of zerumbone biosynthesis in shampoo ginger (*Zingiber zerumbet* Smith). Planta 227:1291–1299

Yuan HD, Kim JT, Kim SH, Chung SH (2012) Ginseng and diabetes: the evidences from in vitro, animal, and human studies. J Ginseng Res 36:27–39