



Research article

Isolation and functional analysis of squalene synthase gene in tea plant *Camellia sinensis*



Jiangu Fu^{a,b,c,*}, Guanhua Liu^{a,b,c}, Mei Yang^{a,b,c}, Xinchao Wang^{a,d}, Xinlu Chen^e, Feng Chen^e, Yajun Yang^{a,d,**}

^a Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, 310008, China

^b Key Laboratory of Tea Quality and Safety Control, Ministry of Agriculture and Rural Affairs, Hangzhou, 310008, China

^c Graduate School of Chinese Academy of Agricultural Sciences, Beijing, 100081, China

^d National Center for Tea Plant Improvement, Hangzhou, 310008, China

^e Department of Plant Sciences, University of Tennessee, Knoxville, TN, 37996, USA

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ABSTRACT

Tea contains high quantities and diverse types of triterpenoids, particularly in the form of saponins. However, little is yet known about the molecular basis of triterpenoid biosynthesis in tea plant. Here we report on isolation and functional analysis of squalene synthase (SQS) gene from tea plant (*Camellia sinensis* var. *sinensis*), which controls the biosynthesis of triterpenoids precursor. First, a full-length cDNA of squalene synthase, designated CsSQS, was isolated from tea plant. The protein is highly homologous to SQSs from other plants. Using CsSQS-reporter assays, CsSQS was demonstrated to be endoplasmic reticulum membrane-bound. The coding region of CsSQS excluding transmembrane sequence was expressed in *Escherichia coli*. Recombinant CsSQS catalyzed the formation of squalene using farnesyl-pyrophosphate (FPP) as substrate with NADPH and Mg²⁺. In tea plant leaves, CsSQS expression was significantly induced by both herbivore and mechanical damages. Consistent with the stronger induction of CsSQS expression by mechanical damage than herbivory, tea plants injured mechanically released squalene as a volatile compound, which however was not detected from herbivore-damaged tea plants. Furthermore, it was found that the flowers of another tea plant cultivar *Camellia sinensis* var. *assamica* contain higher concentrations of squalene than the cultivar *sinensis*, indicating variations among tea plant varieties. With the identification and molecular characterization of squalene synthase in tea plant, next, we can ask the questions about the roles of squalene as a volatile product as well as a precursor for triterpenoids, which may promote product development from diverse tea materials and mining of excellent tea germplasm resources.

1. Introduction

Terpenoids are the largest class of natural products and numerous plant-derived terpenoids play important roles in both plant physiology and ecosystem functioning (Gershenson and Dudareva, 2007). In plants, two highly conserved pathways, the mevalonate pathway (MVA) in the cytosol and the methylerythritol phosphate pathway (MEP) in the plastids, are involved in terpenoid biosynthesis (Chappell et al., 1995; Külheim et al., 2015; Vranova et al., 2012). Although these two independent pathways are located in different compartments of plant cells, they can communicate through the common precursor isopentenyl diphosphate (IPP) (Vranova et al., 2012). Triterpenoids are ubiquitous in both plant and animal kingdoms. In plants they are

mainly synthesized via the MVA pathway (Thimmappa et al., 2014; Xu et al., 2004). As a large and structurally diverse group of terpenoids, triterpenoids are important for many processes of plant biology, especially in plant defense against biotic stresses (Xu et al., 2004). In addition, triterpenoids have a wide range of commercial applications in the food, cosmetics, pharmaceutical, and industrial biotechnology sectors (Rong et al., 2016; Thimmappa et al., 2014).

Squalene is the common precursor of triterpenoids. It also serves as a precursor for phytosterols (Busquets et al., 2008). As such, the biosynthesis of squalene is important for both plant secondary and primary metabolisms. Squalene is synthesized by squalene synthase (SQS), which converts two molecules of farnesyl diphosphate (FPP) into one molecule of squalene via a two-step reaction with the stable

* Corresponding author. Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, 310008, China.

** Corresponding author. Tea Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, 310008, China.

E-mail addresses: mybatigoal@tricaas.com (J. Fu), yjyang@tricaas.com (Y. Yang).

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presqualene diphosphate (PSP) as reaction intermediate (Busquets et al., 2008; Pandit et al., 2000). Currently, SQS genes have been isolated from a number of seed plants including *Arabidopsis* (Busquets et al., 2008), *Ornithogalum caudatum* (Liu et al., 2017), *Euphorbia tirucalli* (Uchida et al., 2009), *Centella asiatica* (Kim et al., 2005), *Bacopa monniera* (Vishwakarma et al., 2015), *Withania somnifera* (Gupta et al., 2012), *Dioscorea zingiberensis* (Ye et al., 2014), *Euphorbia pekinensis* (Zheng et al., 2013) and *Panax notoginseng* (Dan et al., 2017). In non-seed plants, SQS gene has been isolated from the lycophyte *Selaginella moellendorffii* (Jiang et al., 2015). Plant SQSs are highly homologous. Having entirely α -helical structure, SQSs contain two active sites that bind two farnesyl groups located in a central channel lined by 19 conserved aspartate and arginine residues. These residues, along with cofactor Mg^{2+} , bind to the diphosphate part of FPP (Pandit et al., 2000).

Tea is a popular beverage worldwide. Both tea and tea plants were found to contain high quantities of triterpenoids, especially in the form of saponins (Yu and He, 2018). Tea plant *C. sinensis* is classified into diverse varieties with *C. sinensis var. sinensis* and *C. sinensis var. assamica* being the two major varieties, which show much differences in secondary metabolism (Yang, 2005). Totally, 87 theasaponins had been identified from *C. sinensis var. sinensis*, while 24 theasaponins was identified from *C. sinensis var. assamica* (Cui et al., 2018). It does not mean that *C. sinensis var. sinensis* contains more theasaponins, just because which was much used for saponin isolation and identification at an earlier time. Theasaponins have been used as a hemolytic agent in shrimp aquaculture and a botanical pesticide in organic agriculture (Liu et al., 1996). Triterpenoid saponins have been implicated as a chemical defense of tea plants, which can reduce the damage of tea geometrid (*Ectropis obliqua*), a serious leaf-feeding pest (Zeng et al., 2018). The tea saponin mixture is also a potential candidate as a new plant-derived pesticide to control *Rhizoctonia damping-off* of vegetable seedlings (Kuo et al., 2010). Despite the importance of triterpenoids from tea plants, little is known about their biosynthesis. In this study, we aim at isolation and functional characterization of squalene synthase gene from tea plant that controls a critical step of the triterpenoid biosynthetic pathway.

2. Materials and methods

2.1. Tea plant materials and treatments

One-year-old clonally propagated tea plant seedlings (*C. sinensis var. sinensis* cultivar Longjing43) were grown in light incubators under controlled conditions: 25 °C, 10/14 h light/dark photoperiods, and 85% relative air humidity. Roots, stems, buds, leaves, styles and seeds were collected to investigate the tissue-specific expression of CsSQS. For mechanical damage leaves were injured along the vein using a sterilized blade. Larvae of tea geometrids (*Ectropis obliqua* Prout) were used for feeding damage, and the infested leaves were harvested after 6 h. All the samples were frozen in liquid N₂, and stored at –80 °C until use. The chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Volatile collection and GC-MS analysis

Volatiles emitted from leaves and flowers of tea plants were collected through a dynamic headspace collection method (Jiang et al., 2015; Li et al., 2012). The volatiles were carried out from a glass chamber by clean air (0.8 L/min), collected by SuperQ collection trap for 12 h (flowers) or 48 h (damaged leaves), and eluted using 200 μ L of methylene chloride. Analytes were separated by an Agilent DB-5ms column: internal length 30 m, diameter 0.25 mm and thickness 0.25 μ m (Haleyur et al., 2016). A splitless mode (injector temperature 240 °C) with an injection volume of 1 μ L was used, and a temperature gradient of 5 °C/min from 40 °C (3min hold) to 240 °C was applied (Yuan et al.,

2008). High purity helium was used as carrier gas with a flow rate of 1.0 mL/min in the column. Mass (MS) parameters were optimized by setting the source temperature of MS Quad at 230 °C, with positive electron ionization (+EI) energy mode of 70 eV. All MS data was collected from 40 to 400 m/z. The volatiles collected and squalene products from CsSQS enzymatic reaction were analyzed on an Agilent 7890B–7000C GC-MS system (Santa Clara, CA, USA) with three biological and technical replicates, respectively. Agilent MassHunter Workstation (version B.07.01/Build 7.1.524.0) was used for quantitative analysis. Squalene was validated by comparison of retention time and mass spectra with authentic standard diluted 3×10^8 folds using methylene chloride (Jiang et al., 2015; Yuan et al., 2008).

2.3. Gene cloning and tissue expression of CsSQS in tea plant

The coding sequence of a tea plant squalene synthase gene was initially identified from a transcriptome of tea plant flowers (GenBank Accession No. MF774226). Total RNA of tea plant (*C. sinensis var. sinensis* cultivar Longjing43) was extracted from leaves by RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's instructions, and cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TAKARA, China) according to the manufacturer's protocol. The CsSQS gene was amplified by PCR, and the PCR products were confirmed and sequenced bi-directionally. According to the protein structure analysis, the full-length and two truncated fragments of CsSQS were cloned from tea plant cDNA using the same forward primer and different reverse primers (Table S1). The CsSQS transcript-level in roots, stems, leaves, buds, styles and seeds of tea plant was investigated by one step RT-PCR. The qRT-PCR reactions were performed with three biological replicates following the manual of SYBR Premix Ex Taq II Kit (TAKARA, China) on an ABI 7500 Real-Time PCR System (Applied Biosystems, USA), and *GAPDH* was used as the internal control (Sun et al., 2010) (Table S1). Relative gene expression data was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A phylogenetic tree of squalene synthases was constructed based on the Maximum Likelihood method with 1000 bootstrap using MEGA 6.0.

2.4. Subcellular location of CsSQS

The full-length CDS of SQS were cloned and ligated into the 35S::GFP vectors (modified from pCambia1300). The resultants of 35S::SQS::GFP and 35S::GFP::SQS plasmids, 35S::RFP vector, and the vector with ER marker were introduced into *Agrobacterium tumefaciens* strain GV3101, respectively (Ju et al., 2012; Nelson et al., 2007). Positive clones were then incubated in Luria-Bertani medium supplemented with 50 mg/L kanamycin at 28 °C until OD₆₀₀ at 0.8, and the cells were collected and resuspended with infiltration buffer (10 mM MgCl₂, 0.2 mM acetosyringone, and 10 mM MES, pH 5.6). The buffer was then injected into the epidermal cells of tobacco leaves (*Nicotiana benthamiana*) (Busquets et al., 2008). GFP and RFP fluorescence was observed via confocal laser scanning microscopy (LSM710: Karl Zeiss).

2.5. Protein structure analysis and in vitro enzymatic activity determination

The signal peptide and hydrophobic region of CsSQS were analyzed by SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) and TMHMM Server (<http://www.cbs.dtu.dk/services/TMHMM/>), respectively. The full-length and two truncated fragments of CsSQS were subcloned into pEXP5-CT/TOPO vector (Invitrogen) and confirmed by sequencing. The plasmids carrying CsSQS were transformed into *E. coli* BL21 (DE3) for heterologous expression. The cultures were grown at 37 °C to an OD₆₀₀ of 0.6, and gene expression was induced by adding 1 mM of isopropyl-1-thio-D-galactopyranoside, and then cultured at 18 °C for 20 h. The cells were collected by centrifugation at 8000g and sonicated for 6 \times 30 s in chilled 1 \times PBS buffer (pH = 7.2). Cell debris was removed by centrifugation at 12000g and supernatant was desalted

through Econo-Pac 10DG columns (Bio-Rad). The CsSQS activity was determined using the method as previously reported, and n-hexane with 0.003% (w/v) 1-Octanol as an internal standard was used to extract the reaction mixture (Jiang et al., 2015).

2.6. Expression level of CsSQS under biotic and abiotic stresses

The expression of CsSQS induced by mechanical and herbivore damages was measured by qRT-PCR. The RNA pool from ten leaves was set as one biological replicate and three biological replicates were analyzed. The cDNAs were synthesized and qRT-PCR was performed as above described for analysis for expression of CsSQS in different tissues.

3. Results

3.1. Gene cloning and expression of CsSQS in tea plant

A full length cDNA of CsSQS was successfully cloned from the leaves of tea plant cultivar *sinensis* and verified by sequencing. It is 1245 bps in length. The protein encoded by CsSQS is highly homologous to SQSs from other plants and even to human SQS. CsSQS contains two conserved domains of ‘DTVED’ and ‘DYLED’, which are typical of known SQSs (Fig. S1). By computing genetic distance with 23 SQSs retrieved from NCBI, the phylogenetic tree based on ML method indicated that CsSQS clustered with SQSs of other angiosperm plants (Fig. 1).

CsSQS expressed in all tissues including roots, stems, buds, leaves, styles and seeds. It showed the highest and second highest expression levels in roots and young leaves, respectively. While it showed the lowest expression level in old leaves. The expression levels of CsSQS in roots and young leaves were approximately 100 and 30-fold higher than that in the old leaves (Fig. 2). The gene expression pattern in tissues was consistent with the observations in *Euphorbia pekinensis* Rupr (Zheng et al., 2013), *Bacopa monniera* (Vishwakarma et al., 2015) and *Selaginella moellendorffii* (Jiang et al., 2015), their SQS genes expressed much higher in roots than other tissues.

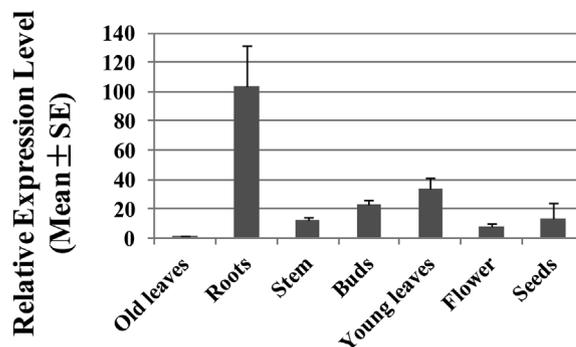


Fig. 2. The gene expression profiles of CsSQS in tea plant tissues. The transcripts of CsSQS in roots, stems, buds, leaves, styles and seeds were quantified by qRT-PCR. The *GAPDH* gene was used as the internal control.

3.2. Subcellular location of CsSQS

In order to investigate the subcellular localization of CsSQS, the SQS with C-terminal and N-terminal GFP fusion constructs, free ER and RFP markers, were transiently expressed in *N. benthamiana* leaves, respectively. The results showed that both SQS-GFP and GFP-SQS co-localized with ER-marker in endoplasmic reticulum, while the control vector with RFP protein could not co-localized with SQS-GFP (Fig. 3). It proved that CsSQS is an ER membrane-localization protein. A previous study have demonstrated that plant SQSs are targeted to ER membrane and the location exclusively depend on the presence of the SQS C-terminal hydrophobic transmembrane domain (Busquets et al., 2008). Our subcellular localization results for CsSQS were in line with the observations in plant.

3.3. Protein structure and enzymatic activity analyses for CsSQS

According to the analysis performed by SignalP, CsSQS does not

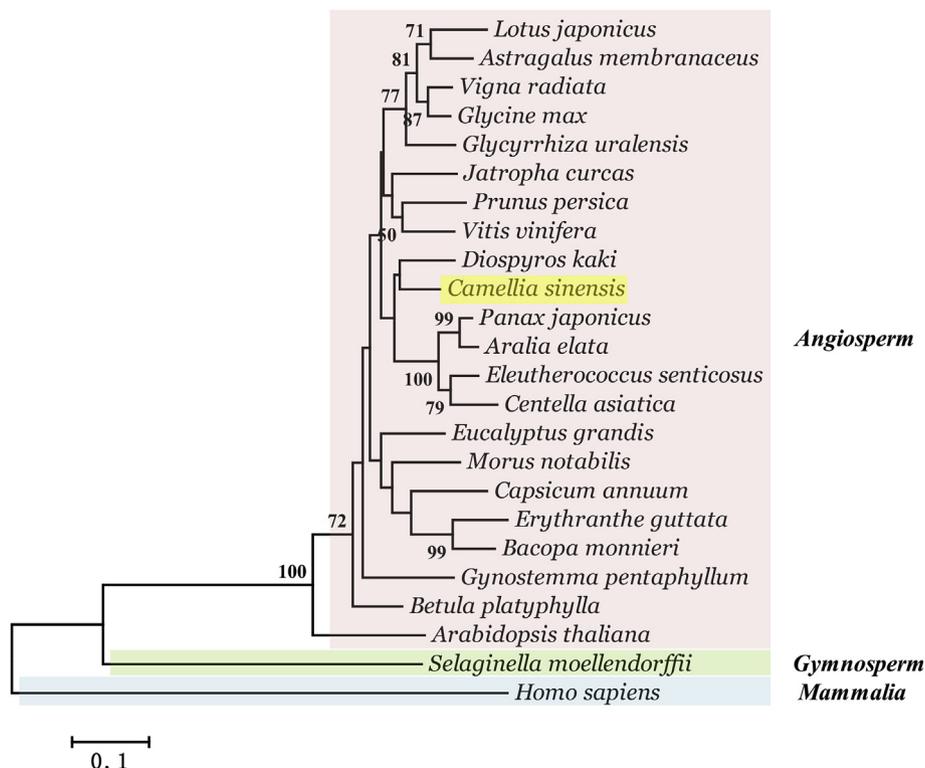


Fig. 1. The phylogenetic tree of CsSQS. The analysis included CsSQS, 21 SQSs from higher plants, one SQS from lower plant, and one SQS from human. Three clades of Angiosperm, Gymnosperm and Mammalia were highlighted on the right, with bootstrap values over 50% are shown on the branches.

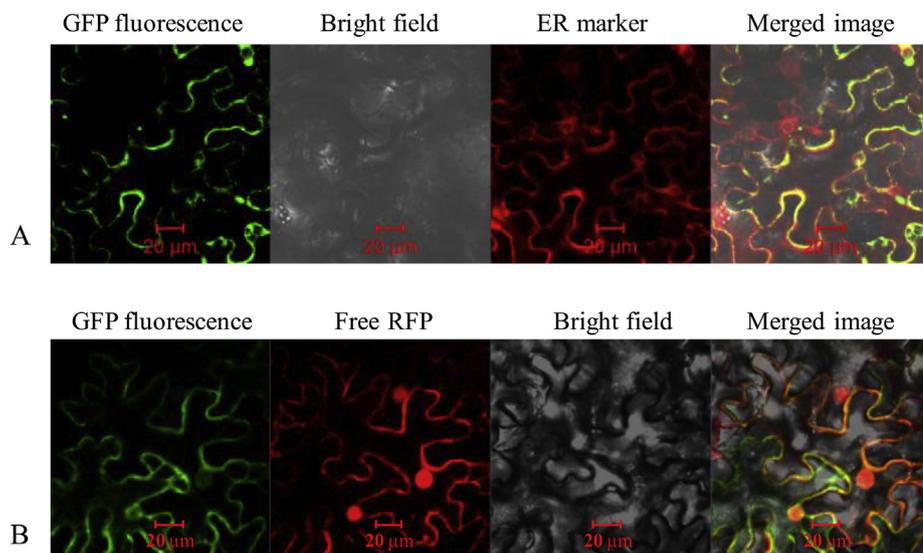


Fig. 3. The subcellular localization of GFP-tagged CsSQS. The results of *35S::SQS::GFP* and *35S::GFP::SQS* plasmids, *35S::RFP* vector, and the vector with ER marker were introduced into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into leaves of *Nicotiana benthamiana*. (A) The subcellular localization results of C-terminal GFP fusion; (B) The subcellular localization results of N-terminal GFP fusion proteins. GFP and RFP fluorescence were observed via confocal laser scanning microscopy (LSM710; Karl Zeiss). Bars = 20 µm.

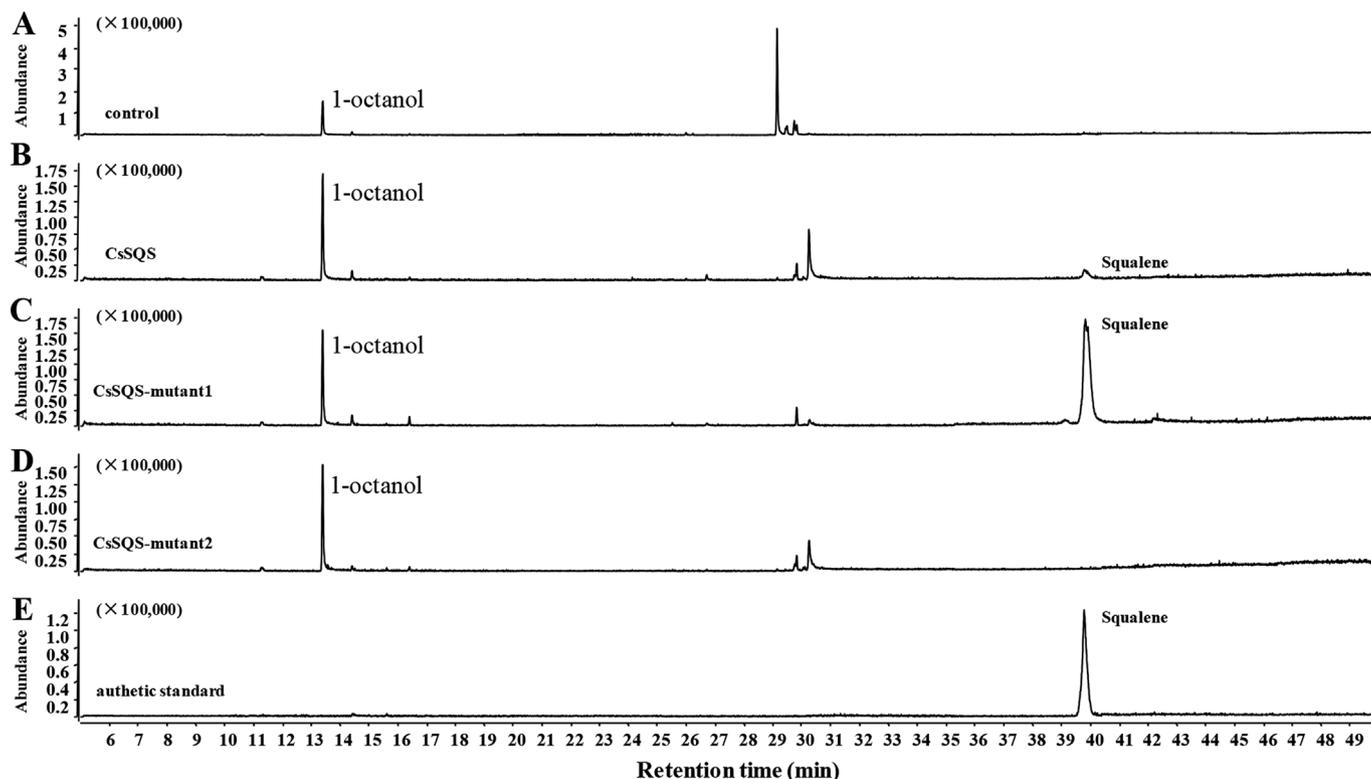


Fig. 4. The gas chromatogram of *in vitro* enzymatic products of CsSQS and two truncated proteins deleted one or both transmembrane regions. Proteins were expressed in *E. coli* and desalted, and the squalene synthesis activities were identified by GC-MS. (A) The catalyzed products of an empty expression vector that was used as a negative control; (B) The catalyzed products of full-length CsSQS; (C) The catalyzed products of CsSQS-mutant1 deleted one transmembrane region; (D) The catalyzed products of CsSQS-mutant2 deleted both transmembrane regions; (E) The authentic standard of squalene.

contain signal peptides at the N-terminus, consistent with subcellular localization assays (Fig. 3). In contrast, the analysis with TMHMM revealed two transmembrane domains, which are located at 282Ile-304Ile and 386 Arg-408 Ala of the C-terminus (Fig. S2). The transmembrane regions at C-terminus localized eukaryotic SQSs to the ER membrane (Busquets et al., 2008; Stamellos et al., 1993), which could produce insoluble protein when eukaryotic SQSs expressed in *E. coli*. Therefore, a CsSQS-mutant1 truncating both transmembrane regions and CsSQS-mutant2 truncating the second transmembrane region were created to detect the activities of SQS. The full-length and two fragments deleted membrane-anchoring peptides were all introduced into *E. coli* to test the

synthesis activities for squalene using FPP as substrate under the condition of NADPH and Mg^{2+} . Compared with empty vector controls, CsSQS-mutant1 produced a large quantity of squalene while CsSQS-mutant2 generated no product, and even full-length CsSQS produced trace squalene. It indicated that CsSQS was a functional gene of squalene-synthesizing and the soluble protein deleted two transmembrane regions had the strongest activity (Fig. 4). The recombinant proteins were checked by SDS-PAGE electrophoresis (Fig. S3). The catalyzed products of recombination of three CsSQSs were confirmed to be squalene using the authentic standard (Fig. S4).

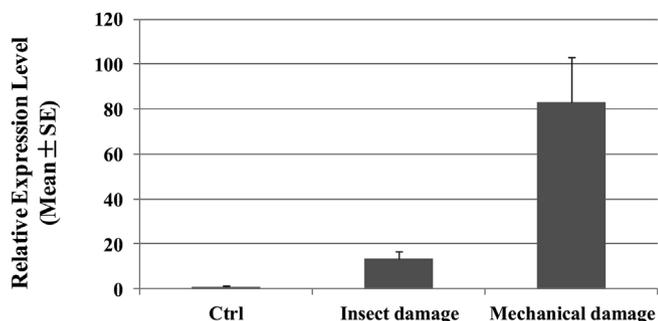


Fig. 5. Gene expression of CsSQS induced by mechanical and herbivore damages in tea plant. Gene transcript level was detected by qRT-PCR analysis, and the values were the mean (\pm SE) of three replicates and *GAPDH* gene was used as the internal control.

3.4. Gene induction and squalene volatile release in *Camellia sinensis* var. *sinensis*

Because squalene volatile was hardly detected in intact tissues, we tried to induce it using herbivore and mechanical damages. The leaves of *C. sinensis* var. *sinensis* were damaged with tea geometrid larvae or with a sterilized blade, and gene expression was investigated by qRT-PCR. Compared with intact leaves, CsSQS expression enhanced almost 12-fold and 83-fold under herbivore and mechanical damages, respectively (Fig. 5). This suggested that mechanical damage had much stronger induction effect for squalene synthase gene. To investigate the release of squalene in tea plants of *C. sinensis* var. *sinensis*, the volatiles of treated leaves were collected through the dynamic headspace method. Although insect and mechanical damages could both obviously induce CsSQS expression, the squalene volatile release from leaves of *C. sinensis* var. *sinensis* was only detected under mechanical damage (Fig. 6). This indicated that squalene metabolism in tea plants have a much stronger response to mechanical damage than herbivore damage.

3.5. Squalene contents in two major varieties of *Camellia sinensis*

Squalene volatile was not detected from intact tissues such as roots, stems, leaves, flowers and seeds using the dynamic headspace method. The departed flowers were ground to fine powder in liquid N_2 , and 0.1 g tissue powder were extracted overnight on a shaker by 1 mL ethyl acetate to collect the compounds. The extracts were analyzed by GC-MS, and it showed that the flowers of *C. sinensis* var. *assamica* contained more squalene while *C. sinensis* var. *sinensis* contained trace amounts of squalene (Fig. S5).

4. Discussion

Squalene synthase is a membrane-bound enzyme anchored at the endoplasmic reticulum (ER) through its C-terminal hydrophobic region (Lograsso et al., 1993; Ye et al., 2014). Most plant SQSs contain one or two transmembrane regions at the C-terminus. Consequently, truncated SQSs with their C-terminus removed exhibit higher solubility and catalytic activity than their respective full-length proteins (Liu et al., 2017; Uchida et al., 2009; Vishwakarma et al., 2015; Ye et al., 2014; Zheng et al., 2013). Tea plant SQS was found to also contain two C-terminal transmembrane regions (Fig. S2). We produced two versions of truncated CsSQS with either one or both transmembrane domains removed. Unlike some other plant SQSs (Dan et al., 2017), the solubility of CsSQS could not be rescued with the mere removal of its last C-terminal transmembrane region. Only when a large fragment of 132 amino acids covering both transmembrane domains were removed, the resulted truncated protein exhibited the largest solubility and activity. This indicated that the first transmembrane region located at 282Ile-304Ile also affected the solubility of CsSQS.

Squalene is accumulated in both animals and plants, and it can either be an end product or serve as a committed precursor for complex saponins and sterols. As such, characterizing the synthesis and release of squalene in tea plants is important for our understanding of the saponin metabolism in tea plant. Here, for the first time, we isolated the SQS gene in tea plants and characterized its activity. We also found that the two varieties of tea plants, although sharing a same SQS gene, showed a great difference in squalene content (Fig. S5). It will be interesting to determine whether such differences are related to the difference in triterpenoid saponin chemistry of the two varieties (Yoshikawa et al., 2008). Additionally, one tea germplasm belonging to the variety of *C. sinensis* var. *assamica* with a high content of squalene in flowers was identified, which could be a valuable material for utilization of tea flowers. Although the biofunctional effects of tea leaves have been studied extensively, much less attention was paid to those of tea flowers (Matsuda et al., 2016). Recently, a few reports described that tea flowers had radical scavenging, gastroprotective and hypoglycemic activities (Chen et al., 2014; Yoshikawa et al., 2008). As far, tea flowers represent an abundant yet non-utilized resource material from tea plants in China (Chen et al., 2014) and have a potential for the development of triterpenoid saponins-based pesticides, surfactants and bioactive materials (Matsuda et al., 2016; Yoshikawa et al., 2008).

Plant volatiles play key roles in interactions between plants, herbivores and their parasitoids as chemical communication signals. Most monoterpenes and sesquiterpenes, some diterpenes and the triterpene squalene can function as such important chemical signals. Despite the isolation and functional characterization of SQS genes from a variety of

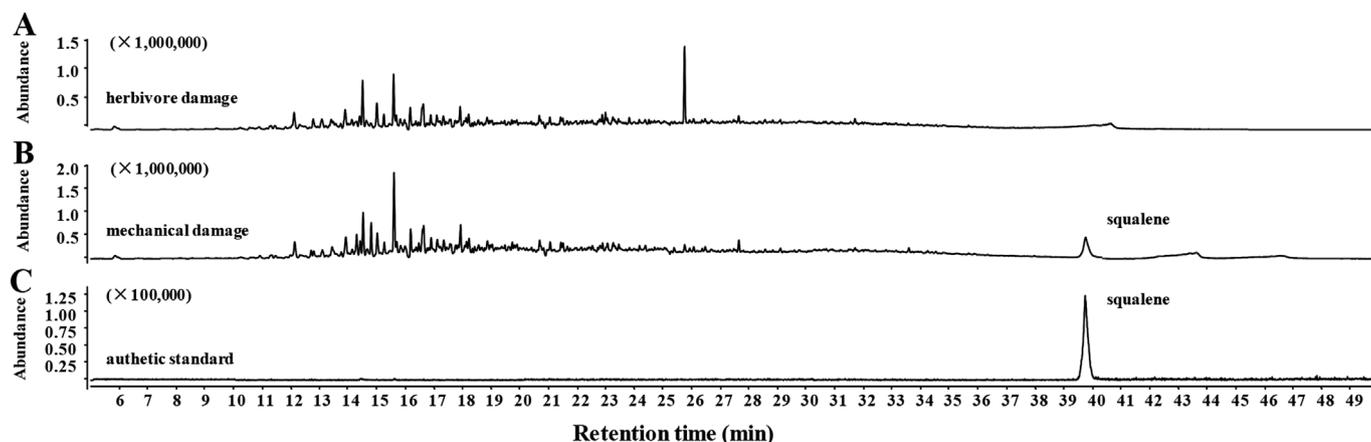


Fig. 6. The gas chromatogram of volatile squalene induced by mechanical and herbivore damages in tea plant. (A) The volatile squalene release induced by herbivore damage; (B) The volatile squalene release induced by mechanical damage; (C) The authentic standard of squalene.

plants (Vishwakarma et al., 2015; Ye et al., 2014; Zhang et al., 2015; Zheng et al., 2013), only a few studies reported the detection of squalene as a volatile compound released by plants (Jiang et al., 2015). In this paper, herbivore damage was performed to induce squalene release. Although the expression of CsSQS gene was upregulated, squalene was not detected from herbivore-damaged tea leaf. Interestingly, mechanical damage could significantly induce both CsSQS expression and volatile squalene emission in the tea plant (Figs. 5 and 6). In a previous study, a fungal elicitor alamethicin induced the emission of volatile squalene from *S. moellendorffii* plants (Jiang et al., 2015). This suggests that the response mechanisms to stress factors are diverse among plants. The detection of volatile squalene from physically injured leaves indicates that squalene could be stored as a final product in intact tissues. This also indicated that tea plucking and pruning could, just like mechanical damages, induce squalene release, which may help protect tea plants from insect and pathogen invasions. The elucidation of squalene biosynthesis in tea plants paves the way to understanding its biological function and its role in triterpenoid metabolism, which, in turn, may help promote product development using diverse tea materials and the mining of excellent germplasm resources.

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Notes

The authors declare no competing financial interest.

Author contribution

J.F. Fu: conceived the project. G.H. Liu, M. Yang, X.C. Wang and X.L. Chen: performed the experiments and analyzed the data. J.F. Fu, F. Chen and Y.J. Yang: wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.06.030>.

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