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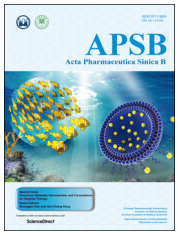

ORIGINAL ARTICLE

Cloning and characterization of squalene synthase and cycloartenol synthase from *Siraitia grosvenorii*

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KEY WORDS

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Subcellular localization

Abstract Mogrosides and steroid saponins are tetracyclic triterpenoids found in *Siraitia grosvenorii*. Squalene synthase (SQS) and cycloartenol synthase (CAS) are key enzymes in triterpenoid and steroid biosynthesis. In this study, full-length cDNAs of *SgSQS* and *SgCAS* were cloned by a rapid amplification of cDNA-ends with polymerase chain reaction (RACE-PCR) approach. The *SgSQS* cDNA has a 1254 bp open reading frame (ORF) encoding 417 amino acids, and the *SgCAS* cDNA contains a 2298 bp ORF encoding 765 amino acids. Bioinformatic analysis showed that the deduced SgSQS protein has two transmembrane regions in the C-terminal. Both *SgSQS* and *SgCAS* have significantly higher levels in fruits than in other tissues, suggesting that steroids and mogrosides are competitors for the same precursors in fruits. Combined *in silico* prediction and subcellular localization, experiments in tobacco indicated that SgSQS was probably in the cytoplasm or on the cytoskeleton, and SgCAS was likely located in the nucleus or cytosol. These results will provide a foundation for further study of *SgSQS* and *SgCAS* gene functions in *S. grosvenorii*, and may facilitate improvements in mogroside content in fruit by regulating gene expression.

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1. Introduction

Siraitia grosvenorii, a traditional medicinal plant native to Guangxi Zhuang Autonomous Region, belongs to the Cucurbitaceae family. Its fruits have been used as medicine for the treatment of cough and lung congestion, and they are extremely sweet substances with low energy content and no toxicity[1]. The active components for sweetness are known as mogrosides, a kind of triterpenoid, and are extracted from the fruit. They exhibit anti-tumor, anti-diabetic, anti-oxidation, and hypoglycemic activities[2–5]. Our previous research proposed a mogroside biosynthetic pathway[6], with triterpenoids synthesized *via* mevalonate (MVA) and plastidial 2-*C* methyl-D-erthritol-4-phosphate (MEP) in the upstream pathway and involves farnesyl diphosphate (FPP) conversion into squalene and then to 2,3-oxidosqualene followed by sequential cyclization, oxidation and other modifications. Squalene synthase (SQS) catalyzes the conversion of two FPPs to squalene, the first committed entry step of sterol, triterpene and brassinosteroid (BRs) biosynthesis[7]. This enzymatic reaction occurs in the membrane of endoplasmic reticulum (ER), and sterols and BRs can play important roles in membrane fluidity and permeability, and also serve as signaling molecules in plant growth and development[8]. Cycloartenol synthase (*CAS*) and cucurbitadienol synthase (*CS*) are members of the oxidosqualene cyclase (*OSC*) gene family, and catalyze the cyclization of 2,3-oxidosqualene to cycloartenol or cucurbitadienol[9]. This step catalyzed by OSCs is the key branch-point leading to sterol or triterpenoid synthesis. Further modification by cytochrome P450-dependent monooxygenases (CYP450s) and glycosyltransferases (GTs) ultimately yields steroids and triterpenoids.

As there is no sequence or structural information on SQS and CAS in *S. grosvenorii*, we focused on these two genes (*SgSQS* and *SgCAS*). The full-length sequences of both genes were obtained and expression patterns were investigated. The subcellular locations were determined by experiment and expression in *Escherichia coli* was investigated. The results could provide foundation for further exploration of gene function in yeast or in *S. grosvneorii*, and help reveal the regulation of mogroside and sterol biosynthesis in *S. grosvenorii*.

2. Materials and methods

2.1. Plant materials

The Nongyuan B6 variety of *S. grosvenorii* tissue culture seedlings are maintained in our laboratory. Fresh root, stem, leaf and fruits of *S. grosvenorii* from 5 to 50 days were harvested in Guangxi Botanical Garden of Medicinal Plant, Guangxi Zhuang Autonomous Region. All samples were cut into small pieces, frozen immediately with liquid nitrogen and stored at −80 °C for further use.

2.2. RNA extraction, cDNA synthesis and cloning of full-length SgSQS and SgCAS gene

Total RNA was extracted from the fruits of *S. grosvenorii* using Trizol (Invitrogen, USA) as described by Tang et al.[6]. First-strand cDNA was reverse-transcribed using 1 μg of total RNA and SMARTer™ RACE DNA Amplification Kit (Clonetech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's protocol. All the primers for rapid amplification of cDNA ends by PCR are shown in Table 1.

The first-strand cDNA for full-length cloning was synthesized using DNase I–treated RNA, Oligo dT primers and PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The specific primers for amplification of these two genes were designed by Primer Premier 5 (Table 1). PCRs were conducted in a total volume of 50 μL, containing 1 μL of cDNA, 10 μmol/L of forward and reverse primers, and 25 μL Taq Plus MasterMix (Tiangen, China). PCRs were carried out using the cyclic parameters as: initial denaturation at 94 °C for 5 min followed by 35 cycles of 20 s at 94 °C, 20 s at 56 °C and 1 min at 72 °C, and final extension of 10 min at 72 °C. The PCR products were purified and cloned into the pMD19-T (Takara, Dalian, China) vector for sequencing.

2.3. Bioinformatic analysis

Open reading frames (ORFs) were determined using NCBI online tools (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The physical and chemical parameters, such as molecular mass (MW), theoretical p*I* and stability of the deduced amino acids were predicted by ProtParam software online (http://web.expasy.org/protparam/), while conserved domains of both SgSQS and SgCAS were identified by ScanProsite (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The signal peptide, subcellular localization and transmembrane regions were identified using SignalP4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), PSORT (http://wolfpsort.org/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Table 1 List of primers used in this study.

Gene	Primer name	Sequence (5′–3′)
SgSQS 5′-RACE primer	*SgSQS*-5′GSP1	TCGCCATAATCTGAGGAATTGCAC
SgSQS 3′-RACE primer	*SgSQS*-3′GSP1	CCGTGCAATGCCTCAACGATTTGGTC
SgSQS ORF cloning	*SgSQS*-ORF1	ATGGGCAGCTTGGGGGCGAT
	SgSQS-ORF2	TCATACAGGTTGGTTAGCCGGT
SgSQS qRT-PCR primer	*SqSQS*-qPCR1	CTGAGACACCCAGATGACT
	SqSQS-qPCR2	GAGGGCTCGCAGAACAAGA
SgCAS 5′-RACE primer	5′RACE-*CAS*1	AGAACCGAACATTGTGCTTGGGCC
SgCAS 3′-RACE primer	3′RACE-*CAS*2	GAGGCAGTAACTGGCACTCTAAGAAGGG
SgCAS qRT-PCR primer	*SgCAS*-ORF1	ATGTGGCATCTCAAGATTGG
	SgCAS-ORF2	TTAAGGGGCTCGCAGTACC
SgCAS qRT-PCR primer	*SgCAS*-qPCR1	CAAATACAACATGCTCACC
	SgCAS-qPCR2	TAGCCCTTCTTAGAGTGCC
Reference gene primer	*SgUBQ*-qF	ATAAAAGACCCAGCACCACATTC
	SgUBQ-qR	CCCTTGCCGACTACAACATCC

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2.4. Phylogenetic trees analysis and multiple sequence alignment

Multiple alignment of proteins was conducted to visualize the conserved motifs by the BioEdit software. The phylogenetic trees were constructed by MEGA 6 software[10], and trees were generated using the neighbor-joining (NJ) method with one thousand bootstrap replicates.

2.5. Gene expression analysis of SgSQS and SgCAS in different tissues and at different developmental stages

RNA was extracted from different tissues (fruits at different stages of maturation, leaf, stem, and root), and the first strand cDNA was synthesized using PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). Quantitative Real-time PCR (qRT-PCR) was performed with SYBR Premix Ex Taq™ (Takara, Japan) on CFX96 real-time PCR platform (Bio-Rad, USA) using *GAPDH* as reference gene. Each sample had three replicates and the amplification specificity of primers was evaluated by melting curves (Table 1).

The relative gene expression analysis was done using the comparative C_t ($\Delta\Delta C_t$) method[11].

2.6. Subcellular localization of SgSQS-YFP and SgCAS-YFP

A vector pc-YFP containing enhanced yellow fluorescent protein (EYFP) was utilized in this study. The complete coding sequences of *SgSQS* and *SgCAS* were amplified and ligated into pc-YFP to generate fusion constructs. Transient expression in *Nicotiana benthsmiana* lower epidermal cells was done as described in the previous study by Zhang et al.[12], and tobacco plants were cultivated under short-day condition (8 h light/16 h dark). When the agrobacterium culture with fusion constructs reached stationary phase, the cells were centrifuged and resuspended in infiltration buffer (100 μmol/L acetosyringone in 10 mmol $MgCl_2$). After two days of incubation in the dark, YFP fluorescence was monitored under a confocal microscope (Zeiss, Germany).

2.7. Construction of expression vectors and prokaryotic expression

The *SgSQS* and *SgCAS* ORFs were cloned into pET28 vector, and recombinant vectors *SgSQS-pET28a* and *SgCAS-pET28a* were introduced into *E. coli* BL (DE3). The empty vector pET28a was transfected as a control. Recombinant proteins were expressed by induction with 1 mmol/L isopropy-*β*-D-thiogalactoside (IPTG) or lactose (Lac) at different temperatures for different times. The samples were centrifuged to obtain lysate and pellet. The pellets were resuspended with PBS (pH 7.4) and disrupted by sonication. Finally, the lysate and supernatant were loaded onto 10% SDS-PAGE gel after denaturation by boiling. The gel was stained with Coomassie Brilliant Blue G-250 to detect the protein distribution.

Table 3 Parameters computed by the online ExPASy Proteomics Server.

Protein characteristic	SgSQS	SgCAS
Number of the deduced amino acids	417	765
Molecular weight (kDa)	47.5	87.5
Theoretical p*I*	7.15	6.14
Instability index	42.40	43.03
Grand average of hydropathicity (GRAVY)	–0.037	–0.308

Table 2 Informations of *SgSQS* and *SgCAS* unigenes from *Siraitia grosvneoii* transcriptome and RACE-PCR fragments.

Gene name	Unigene length (bp)	TPM (3/50/70 days)	5′-RACE fragment (bp)	3′-RACE fragment (bp)	Full length of ORF (bp)
SgSQS	471	0.01/0.55/0.01	1235	888	1254
SgCAS	696	1.18/0.01/0.01	576	2157	2298

RACE-PCR, rapid amplification of cDNA-ends with polymerase chain reaction; TPM, transcript per million; ORF, open reading frame.

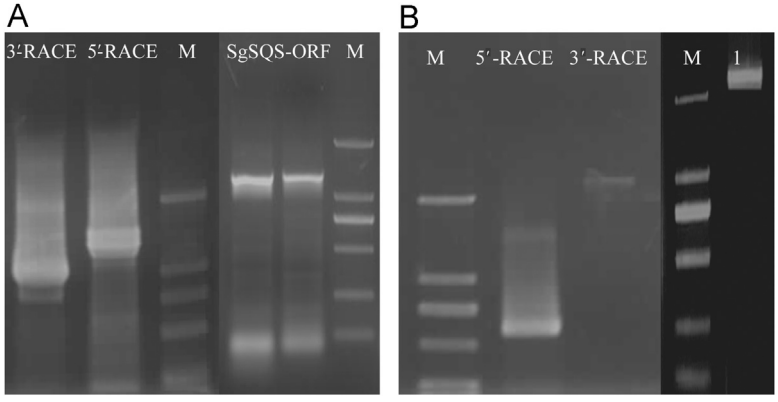

Figure 1 Amplification of full-length *SgSQS* and *SgCAS* gene by rapid amplification of cDNA end PCR. (A) 5′-RACE, 3′-RACE and open reading frame (ORF) of *SgSQS* gene. (B) 5′-RACE, 3′-RACE and ORF of *SgCAS* gene. The fragments in DL2000 marker were 2000, 1000, 750, 500, 250 and 100 bp, respectively.

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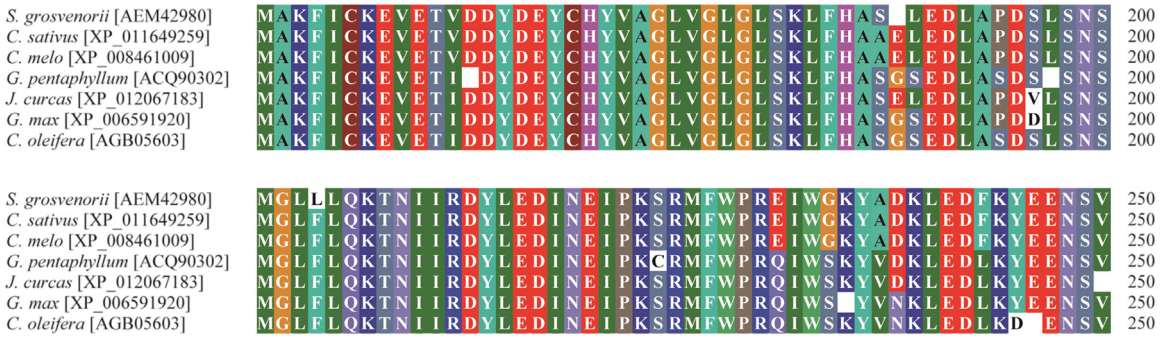

Figure 2 Multiple alignment of the deduced SgSQS protein with other plant SQS proteins using BioEdit software. SQS motif is shown in red box.

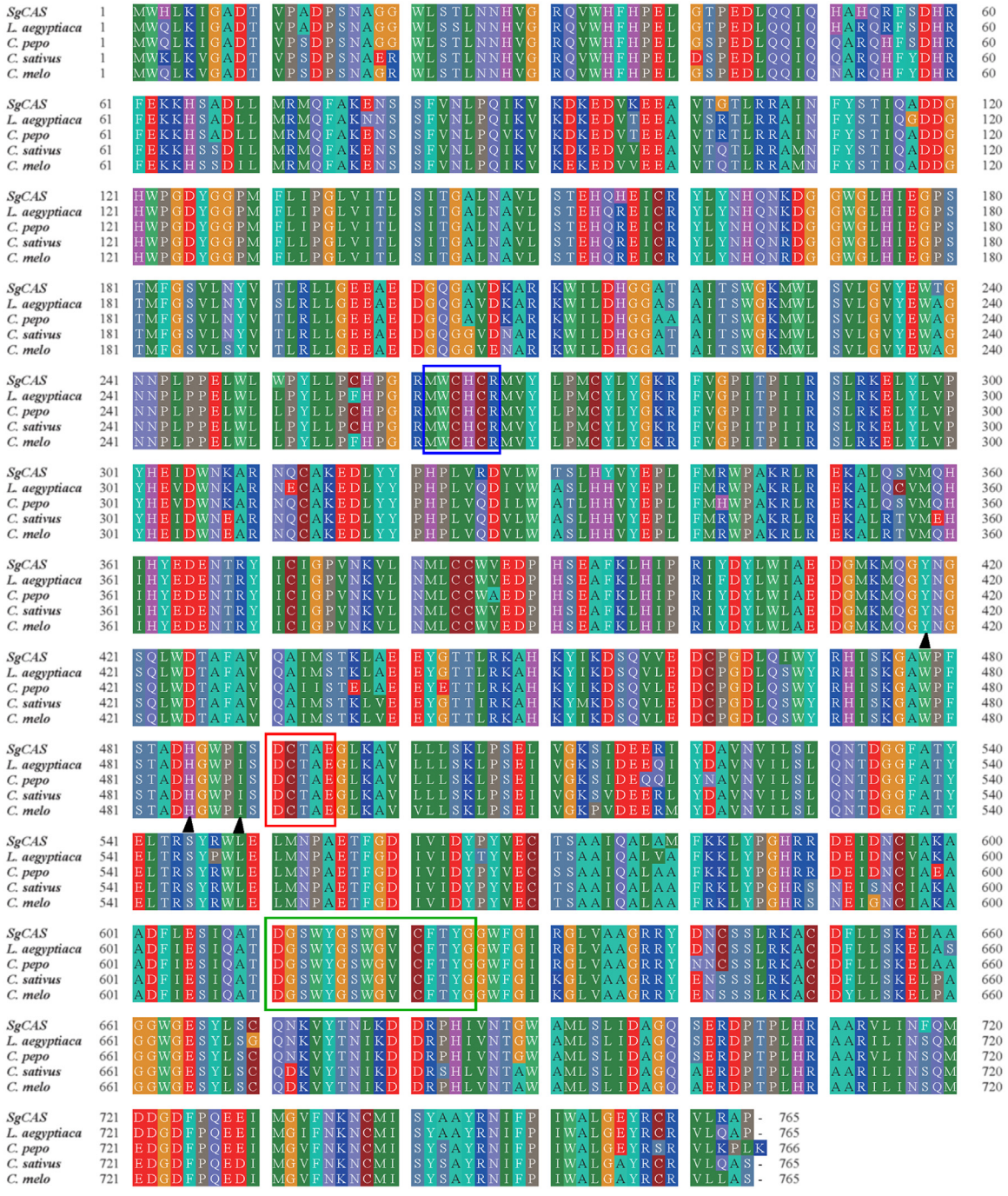

Figure 3 Multiple sequence alignment of the putative SgCAS protein and four other cycloartenol synthases. The highly conserved DCTAE motif is boxed in red. A terpene synthase feature of DGSWyGsWAVcFtYG is boxed in green. MWCHCR motif is shown in the blue box, in which Trp (W) plays key role in triterpene backbone synthesis. Important catalytic residues are marked with arrowheads (Tyr 418, His 485, and Ile 489) which are necessary for 2,3-oxidosqualene cyclization into cycloartenol.

3. Results

3.1. Full-length cloning of SgSQS and SgCAS from S. grosvenorii

After searching the *S. grosvenorii* transcriptome database (SRX064894), two unigenes annotated as *SQS* and *CAS* were selected for full-length cloning. The transcript per million (TPM) clean reads and lengths of these unigenes are listed in Table 2. Gene-specific primers were designed from these two unigene sequences and 5′ and 3′ RACE-PCR was conducted to obtain full-length cDNAs. A 1235 bp 5′ end and 888 bp 3′ end were generated for *SgSQS*, and 576 bp 5′ end and 2157 bp 3′ end were obtained for *SgCAS* (Fig. 1). After confirmation as SQS and CAS by BLAST analysis, the two genes contained 1254 bp and 2298 bp ORFs encoding 417 amino acid (AA) and 765 AA protein, respectively. These two full-length cDNAs of *SgSQS* and *SgCAS* have been deposited in Genebank with accession numbers of AEM42980 and AEM42981.

3.2. Bioinformatic analysis

The deduced protein parameters computed by the online Pxpasy's ProtParam tool are listed in Table 3. The predicted results classified SgSQS and SgCAS into unstable proteins. No signal peptides were predicted for either protein. The predicted localization results showed that SgSQS was likely to be located in cytoskeleton or cytosol, and that SgCAS was located in the chloroplast or nucleus. Although both putative proteins had negative GRAVY values indicating they were hydrophilic, the GRAVY value of SgSQS was close to zero which means SgSQS may have both hydrophilicity and lipotropy. Furthermore, two transmembrane domains (282–304 AAs and 386–408 AA, respectively) were found in the C-terminal region of SgSQS, while no transmembrane domain was predicted in SgCAS.

3.3. Homology analysis and sequence alignment

The putative SgSQS protein showed high similarity with other SQS from Cucurbitaceae, exhibiting 95% identity to *Cucumis sativus* (XP_011649259.1) and *Cucumis melo* (XP_008461009.1). The conserved motif of squalene synthase is boxed in Fig. 2. The deduced SgCAS protein had 95%, 94%, 92% and 92% identity to CAS in *Luffa aegyptiaca* (Q9SLP9.1), *Cucurbita pepo* (Q6BE25.1), *C. sativus* (XP_004141754.1) and *C. melo* (XP_008462186.1). Fig. 3 shows the DCTAE motif which was highly conserved in the OSC family for substrate binding and protonation.

To determine the evolutionary relationship of SQSs or CASs from different plants, phylogenetic trees were constructed based on their alignments. As showed in Fig. 4, SgSQS was clustered with squalene synthases from other Cucurbitaceae plants. Fig. 4 indicated that SgCAS belonged to cycloartenol synthase cluster, with over 90% sequence identity to other CASs in Cucurbitaceae.

3.4. Gene expression patterns of SgSQS and SgCAS in S. grosvenorii

To explore the expression patterns of the two genes involved in triterpene biosynthesis of *S. grosvenorii*, qRT-PCR was conducted with RNA from root, stem, leaf and fruits of different stages. As

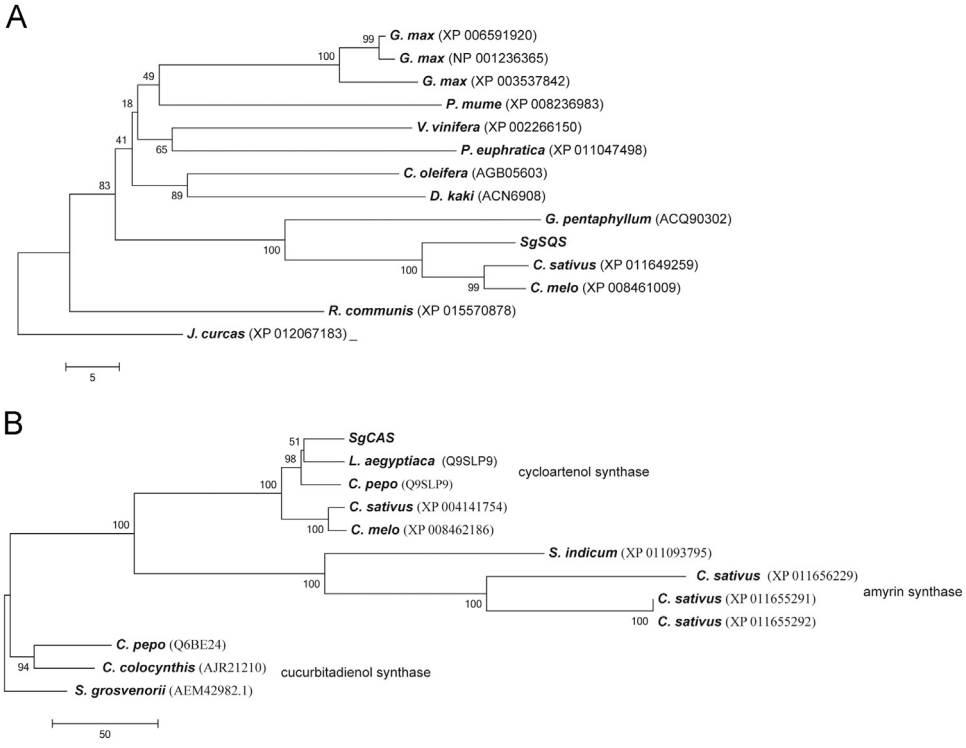

Figure 4 The phylogenetic trees obtained with MEGA 6.0 software. (A) A phylogenetic tree from the putative SgSQS protein and other plant SQSs; (B) A phylogenetic tree constructed from the deduced SgCAS and other plant OSCs. These OSCs included cycloartenol synthase from *Luffa aegyptiaca* (Q9SLP9), *Cucurbita pepo* (Q6BE25), *Cucumis sativus* (XP_004141754) and *Cucumis melo* (XP_008462186), cucurbitadienol synthase from *Siraitia grosvenorii* (AEM42982.1), *Cucurbita pepo* (Q6BE24) and *Citrullus colocynthis* (AJR21210), amyrin synthase from *C. sativus* (XP_011655291 and XP_011655292), *Sesamum indicum* (XP_011093795) and lupeol synthase from *C. sativus* (XP_011656229). OSC, oxidosqualene cyclase.

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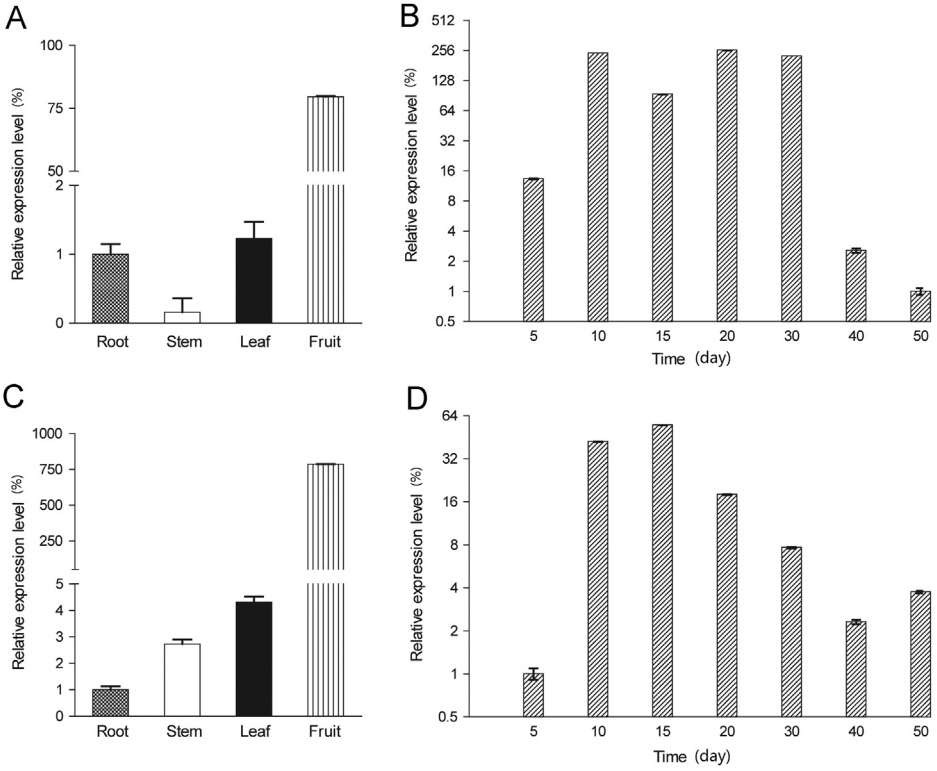

Figure 5 Quantitative RT-PCR analysis of the expression patterns of *SgSQS* and *SgCAS* genes in *Siraitia grosvenorii*. (A and C): Expression patterns of *SgSQS* and *SgCAS* genes in different tissues (root, stem, leaf and 5-day fruit). (B and D): Expression patterns of *SgSQS* and *SgCAS* genes in different stages of fruits from 5 to 50 days.

presented in Fig. 5, *SgSQS* and *SgCAS* were remarkably abundant in the fruits, indicating terpenoid and sterol were active in fruits.

3.5. *Subcellular localizations of SgSQS and SgCAS proteins in tobacco*

In silico analysis using WoLF PDORT software showed that SgSQS was predicted to be located in cytoskeleton or in cytosol with high reliability, while SgCAS was predicted to be located in the chloroplast and nucleus. To confirm the predicted localizations, these two proteins were transiently expressed in tobacco leaf epidermal cells as fusions with C-terminal of YFP. Two fluorescent protein fusions (SgSQS-GFP and SgCAS-GFP) were expressed. As shown in Fig. 6, the fluorescent signal of SgSQS-YFP was detected in cytosol, nucleus and cytoplasm, which was consistent with the prediction. Fig. 6 also shows that SgCAS was located both in the nucleus and cytoplasm. Dynamic monitoring of SgCAS-YFP protein in the cell was conducted, and SgCAS-YFP did not co-locate with autofluorescent chlorophyll, but was intensely mobile and connected with the nucleus in filiform, suggesting SgCAS protein may be associated with the cell cytoskeleton.

3.6. *Prokaryotic expression of pET28a-SgSQS and pET28a-SgCAS*

The recombinant vectors pET28a-SgSQS and pET28a-SgCAS were confirmed by sequencing. As shown in Fig. 7, the recombinant SgSQS (49.5 kDa) was found after induction with IPTG and lactose at 37 °C for 4 h, while the recombinant SgCAS protein (87 kDa) was expressed in the cell pellet after induction with IPTG

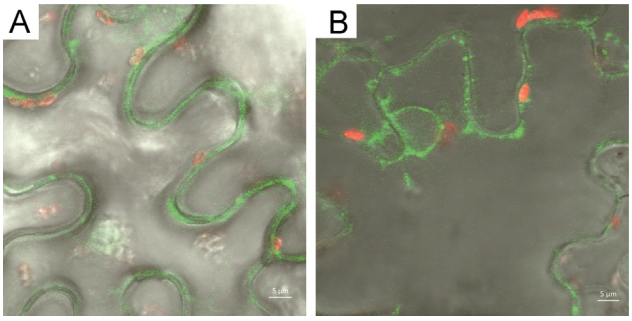

Figure 6 Subcellular localization of SgSQS and SgCAS proteins. (A) Confocal image of an epidermal leaf cell expressing SgSQS-YFP. (B) Confocal image of an epidermal leaf cell expressing SgCAS-YFP.

for 4 h at 37 °C or lactose for 16 h at 20 °C. Both recombinant proteins were expressed as inclusion bodies.

4. Discussion

Here we obtained the full-length *SgSQS* and *SgCAS* genes from *S. grosvnorii* fruit and performed bioinformatic analysis on their deduced proteins. SgSQS has two transmembrane domains in the C-terminal region, which is consistent with the reported presence of SQSs in the ER membrane[13,14]. *SQS* genes have been identified in many organisms, including algae[15], yeast, animals and plants[16]. *SgSQS* was remarkably highly expressed in fruit, especially in fruit after 30 days in *S. grosvenorii*. *SQSs* are a small gene family, with

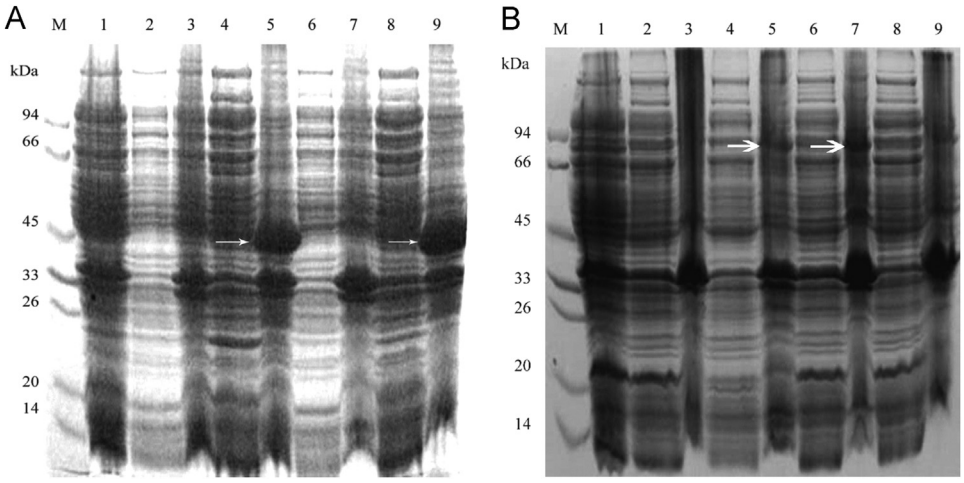

Figure 7 SDS-PAGE analysis of recombinant SgSQS-pET28a and SgSQS-pET28a proteins. (A) SDS-PAGE of SgSQS-pET28a, arrow marks the SgSQS protein. (B) SDS-PAGE of SgCAS-pET28a, arrow marks SgCAS protein. M: Protein molecular weight marker. (1) Control. (2) 20 °C for 16 h by IPTG (Supernatant); (3) 20 °C for 16 h by IPTG (Precipitant); (4) 37 °C for 4 h by IPTG (Supernatant); (5) 37 °C for 4 h by IPTG (Precipitant); (6) 20 °C for 16 h by lactose (Supernatant); (7) 20 °C for 16 h by lactose (Precipitant); (8) 37 °C for 4 h by lactose (Supernatant); (9) 37 °C for 4 h by lactose (Precipitant). IPTG, isopropy-β-D-thiogalactoside.

one, two and three SQSs identified in *Medicago truncatula, Panax notoginseng* and *Panax ginseng*[17]. In our transcriptome data, we have found three unigenes of 288 bp, 194 bp and 471 bp, and the full-length *SgSQS* gene was obtained from the last and longest unigene. This data suggests that there may be three *SgSQSs* with different roles. Manavalan et al.[18] demonstrated that RNAi for *SQS* in rice could produce stomatal conductance and more grain yield. As reported in other plants, over-expression of *SQS* could increase phytosterols, triterpenoids and steroids in *Withania coagulans*[19], *P. ginseng*[20], *Eleutherococcus senticosus*[21], and *Bupleurum falcatum*[22]. Squalene accumulated by SgSQS is the precursor for triterpenoids and steroids, and it would likely increase end-product content when *SgSQS* was over-expressed in *S. grosvenorii*. As the bioinformatics analysis suggests the presence of a C-terminal transmembrane region in the SgSQS protein, it would likely inhibit the expression in *E. coli*. To obtain a more soluble SgSQS protein in the future, we can attempt to generate a recombinant protein by removing the C-terminal transmembrane region.

Cycloartenol synthase belongs to OSCs gene family, which include different types of enzymes, such as cucurbitadienol synthase from *C. sativus*, amyrin synthase from *Glycyrrhiza uralensis*, dammarenediol synthase from *P. ginseng*, and lanosterol synthase from yeast. The DCTAE motif was highly conserved in CASs, and moreover all CASs had a strict requirement for His (485 bp) and Ile (489 bp) near the DCTAE motif[23]. Phylogenetic analysis clustered SgCAS with other cycloartenol synthases from Cucurbitaceae, separating them from the cucurbitadienol synthase and amyrin synthase groups. The recombinant protein expressed from SgCAS-pET28a was mostly insoluble in the prokaryotic system; however, yeast can provide the substrate naturally. Thus, we can introduce SgCAS into yeast to identify its function like other OSCs[24–28]. SgCAS was also highly expressed in fruits with an expression pattern similar to that of cucurbitadienol synthase (data not published), indicating SgCAS was a competitor for the substrate 2,3-oxidosqualene with SgCAS. Mishra et al. [29] tried to explore the *WsCAS* gene function and discovered that RNAi and over-expression of *WsCAS* can regulate withanolide biosynthesis. Introduction of RO 48-8071 as a CAS inhibitor caused diminished phytosterol synthesis but did not affect triterpenoid accumulation[30]. Therefore, we hypothesize that mogrosides can accumulate significantly by reducing by-pass of SgCAS expression. *SgSQS* and *SgCAS* may be used to engineer the effective production of triterpenoid or steroids in *S. grosvenorii* or in heterotrophic systems in the future.

Acknowledgments

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References

1. Lee CH. Intense sweetener from Lo Han Kuo (*Momordica grosvenori*). *Experientia* 1975;**31**:533–4.
2. Takasaki M, Konoshima T, Murata Y, Sugiura M, Nishino H, Tokuda H, et al. Anticarcinogenic activity of natural sweeteners, cucurbitane glycosides, from *Momordica grosvenori*. *Cancer Lett* 2003;**198**:37–42.
3. Takeo E, Yoshida H, Tada N, Shingu T, Matsuura H, Murata Y, et al. Sweet elements of *Siraitia grosvenori* inhibit oxidative modification of low-density lipoprotein. *J Atheroscler Thromb* 2002;**9**:114–20.
4. Ukiya M, Akihisa T, Tokuda H, Toriumi M, Mukainaka T, Banno N, et al. Inhibitory effects of cucurbitane glycosides and other triterpenoids from the fruit of *Momordica grosvenori* on epstein-barr virus early antigen induced by tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. *J Agric Food Chem* 2002;**50**:6710–5.
5. Suzuki YA, Murata Y, Inui H, Sugiura M, Nakano Y. Triterpene glycosides of *Siraitia grosvenori* inhibit rat intestinal maltase and suppress the rise in blood glucose level after a single oral administration of maltose in rats. *J Agric Food Chem* 2005;**53**:2941–6.
6. Tang Q, Ma XJ, Mo CM, Wilson IW, Song C, Zhao H, et al. An efficient approach to finding *Siraitia grosvenorii* triterpene biosynthetic genes by RNA-seq and digital gene expression analysis. *BMC Genom* 2011;**12**:343.
7. Jarstfer MB, Zhang DL, Poulter CD. Recombinant squalene synthase. Synthesis of non-head-to-tail isoprenoids in the absence of NADPH. *J Am Chem Soc* 2002;**124**:8834–45.

8. Clouse SD. Plant development: a role for sterols in embryogenesis. *Curr Biol* 2000;**10**:R601–4.
9. Dai LH, Liu C, Zhu YM, Zhang JS, Men Y, Zeng Y, et al. Functional characterization of cucurbitadienol synthase and triterpene glycosyltransferase involved in biosynthesis of mogrosides from *Siraitia grosvenorii*. *Plant Cell Physiol* 2015;**56**:1172–82.
10. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;**30**:2725–9.
11. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc* 2008;**3**:1101–8.
12. Zheng HQ, Camacho L, Wee E, Batoko H, Legen J, Leaver CJ, et al. A Rab-E GTPase mutant acts downstream of the Rab-D subclass in biosynthetic membrane traffic to the plasma membrane in tobacco leaf epidermis. *Plant Cell* 2005;**17**:2020–36.
13. Zhou CH, Zhao DQ, Sheng YL, Liang GH, Tao J. Molecular cloning and expression of squalene synthase and 2,3-oxidosqualene cyclase genes in persimmon (*Diospyros kaki* L.) fruits. *Mol Biol Rep* 2012;**39**:1125–32.
14. Stamellos KD, Shackelford JE, Shechter I, Jiang G, Conrad D, Keller GA, et al. Subcellular localization of squalene synthase in rat hepatic cells. Biochemical and immunochemical evidence. *J Biol Chem* 1993;**268**:12825–36.
15. Kajikawa M, Kinohira S, Ando A, Shimoyama M, Kato M, Fukuzawa H. Accumulation of squalene in a microalga *Chlamydomonas reinhardtii* by genetic modification of squalene synthase and squalene epoxidase genes. *PLoS One* 2015;**10**:e0120446.
16. Spanova M, Daum G. Squalene – biochemistry, molecular biology, process biotechnology, and applications. *Eur J Lipid Sci Technol* 2011;**113**:1299–320.
17. Niu YY, Luo HM, Sun C, Yang TJ, Dong LL, Huang LF, et al. Expression profiling of the triterpene saponin biosynthesis genes *FPS*, *SS*, *SE*, and *DS* in the medicinal plant *Panax notoginseng*. *Gene* 2014;**533**:295–303.
18. Manavalan LP, Chen X, Clarke J, Salmeron J, Nguyen HT. RNAi-mediated disruption of squalene synthase improves drought tolerance and yield in rice. *J Exp Bot* 2012;**63**:163–75.
19. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J. Overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* hairy root cultures. *Biol Plant* 2011;**55**:357–60.
20. Zhang J, Dai L, Yang J, Liu C, Men Y, Zeng Y, et al. Oxidation of cucurbitadienol catalyzed by CYP87D18 in the biosynthesis of mogrosides from *Siraitia grosvenorii*. *Plant Cell Physiol* 2016;**57**:1000–7.
21. Seo JW, Jeong JH, Shin CG, Lo SC, Han SS, Yu KW, et al. Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. *Phytochemistry* 2005;**66**:869–77.
22. Kim YS, Cho JH, Park S, Han JY, Back K, Choi YE. Gene regulation patterns in triterpene biosynthetic pathway driven by overexpression of squalene synthase and methyl jasmonate elicitation in *Bupleurum falcatum*. *Planta* 2011;**233**:343–55.
23. Dhar N, Rana S, Razdan S, Bhat WW, Hussain A, Dhar RS, et al. Cloning and functional characterization of three branch point oxidosqualene cyclases from *Withania somnifera* (L.) dunal. *J Biol Chem* 2014;**289**:17249–67.
24. Lin YL, Lee YR, Tsao NW, Wang SY, Shaw JF, Chu FH. Characterization of the 2,3-oxidosqualene cyclase gene from *Antrodia cinnamomea* and enhancement of cytotoxic triterpenoid compound production. *J Nat Prod* 2015;**78**:1556–62.
25. Zheng XS, Luo XX, Ye GB, Chen Y, Ji XY, Wen LL, et al. Characterisation of two oxidosqualene cyclases responsible for triterpenoid biosynthesis in *Ilex asprella*. *Int J Mol Sci* 2015;**16**:3564–78.
26. Jin ML, Lee DY, Um Y, Lee JH, Park CG, Jetter R, et al. Isolation and characterization of an oxidosqualene cyclase gene encoding a β-amyrin synthase involved in *Polygala tenuifolia* Willd. saponin biosynthesis. *Plant Cell Rep* 2014;**33**:511–9.
27. Huang LL, Li J, Ye HC, Li CF, Wang H, Liu BY, et al. Molecular characterization of the pentacyclic triterpenoid biosynthetic pathway in *Catharanthus roseus*. *Planta* 2012;**236**:1571–81.
28. Wang ZH, Guhling O, Yao RN, Li FL, Yeats TH, Rose JKC, et al. Two oxidosqualene cyclases responsible for biosynthesis of tomato fruit cuticular triterpenoids. *Plant Physiol* 2011;**155**:540–52.
29. Mishra S, Bansal S, Mishra B, Sangwan RS, Asha, Jadaun JS, et al. RNAi and homologous over-expression based functional approaches reveal triterpenoid synthase gene-cycloartenol synthase is involved in downstream withanolide biosynthesis in *Withania somnifera*. *PLoS One* 2016;**11**:e0149691.
30. Gas-Pascual E, Simonovik B, Schaller H, Bach TJ. Inhibition of cycloartenol synthase (CAS) function in tobacco BY-2 cells. *Lipids* 2015;**50**:761–72.

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