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ORIGINAL ARTICLE

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



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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 toxicity¹. 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 pathway⁶, with triterpenoids synthesized *via* mevalonate (MVA) and plastidial 2-C methyl-D-erythritol-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) biosynthesis⁷. 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 development⁸. 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 cucurbitadienol⁹. 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. grosvenorii*, 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 (Clontech 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 $\mu\text{mol/L}$ 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 *pI* 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/ipscan/>). The signal peptide, subcellular localization and transmembrane regions were identified using SignalP4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>), PSORT (<http://wolfsort.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')
<i>SgSQS</i> 5'-RACE primer	<i>SgSQS</i> -5'GSP1	TCGCCATAAATCTGAGGAATTGCAC
<i>SgSQS</i> 3'-RACE primer	<i>SgSQS</i> -3'GSP1	CCGTGCAATGCCTCAACGATTTGGTC
<i>SgSQS</i> ORF cloning	<i>SgSQS</i> -ORF1	ATGGGCAGCTTGGGGGCGAT
	<i>SgSQS</i> -ORF2	TCATACAGGTTGGTTAGCCGGT
<i>SgSQS</i> qRT-PCR primer	<i>SgSQS</i> -qPCR1	CTGAGACACCCAGATGACT
	<i>SgSQS</i> -qPCR2	GAGGGCTCGCAGAACAAGA
<i>SgCAS</i> 5'-RACE primer	5'RACE-CAS1	AGAACCGAACATTGTGCTTGGGCC
<i>SgCAS</i> 3'-RACE primer	3'RACE-CAS2	GAGGCAGTAACTGGCACTCTAAGAAGGG
<i>SgCAS</i> qRT-PCR primer	<i>SgCAS</i> -ORF1	ATGTGGCATCTCAAGATTGG
	<i>SgCAS</i> -ORF2	TAAAGGGGCTCGCAGTACC
<i>SgCAS</i> qRT-PCR primer	<i>SgCAS</i> -qPCR1	CAAATACAACATGCTCACC
	<i>SgCAS</i> -qPCR2	TAGCCCTTCTTAGAGTGCC
Reference gene primer	<i>SgUBQ</i> -qF	ATAAAAGACCCAGACCACATTC
	<i>SgUBQ</i> -qR	CCCTTGCCGACTACAACATCC

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 software¹⁰, 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 ($\Delta\Delta C_t$) method¹¹.

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 benthamiana* 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 $\mu\text{mol/L}$ 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 isopropyl- 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	<i>SgSQS</i>	<i>SgCAS</i>
Number of the deduced amino acids	417	765
Molecular weight (kDa)	47.5	87.5
Theoretical pI	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 grosvenorii* 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)
<i>SgSQS</i>	471	0.01/0.55/0.01	1235	888	1254
<i>SgCAS</i>	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.

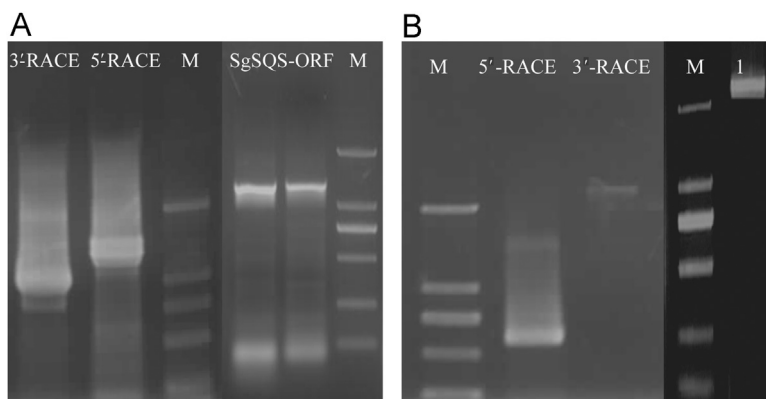


Figure 1 Amplification of full-length *SgSQS* and *SgCAS* gene by rapid amplification of cDNA end PCR. (A) 5'-RACE, 3'-RACE and open reading frame (ORF) of *SgSQS* gene. (B) 5'-RACE, 3'-RACE and ORF of *SgCAS* gene. The fragments in DL2000 marker were 2000, 1000, 750, 500, 250 and 100 bp, respectively.

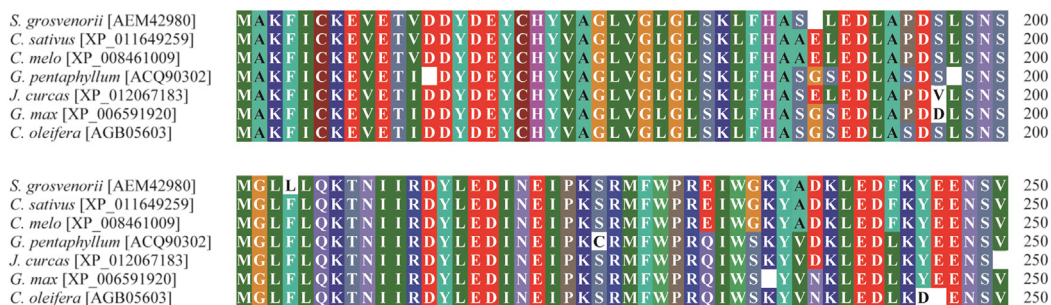


Figure 2 Multiple alignment of the deduced SgSQS protein with other plant SQS proteins using BioEdit software. SQS motif is shown in red box.

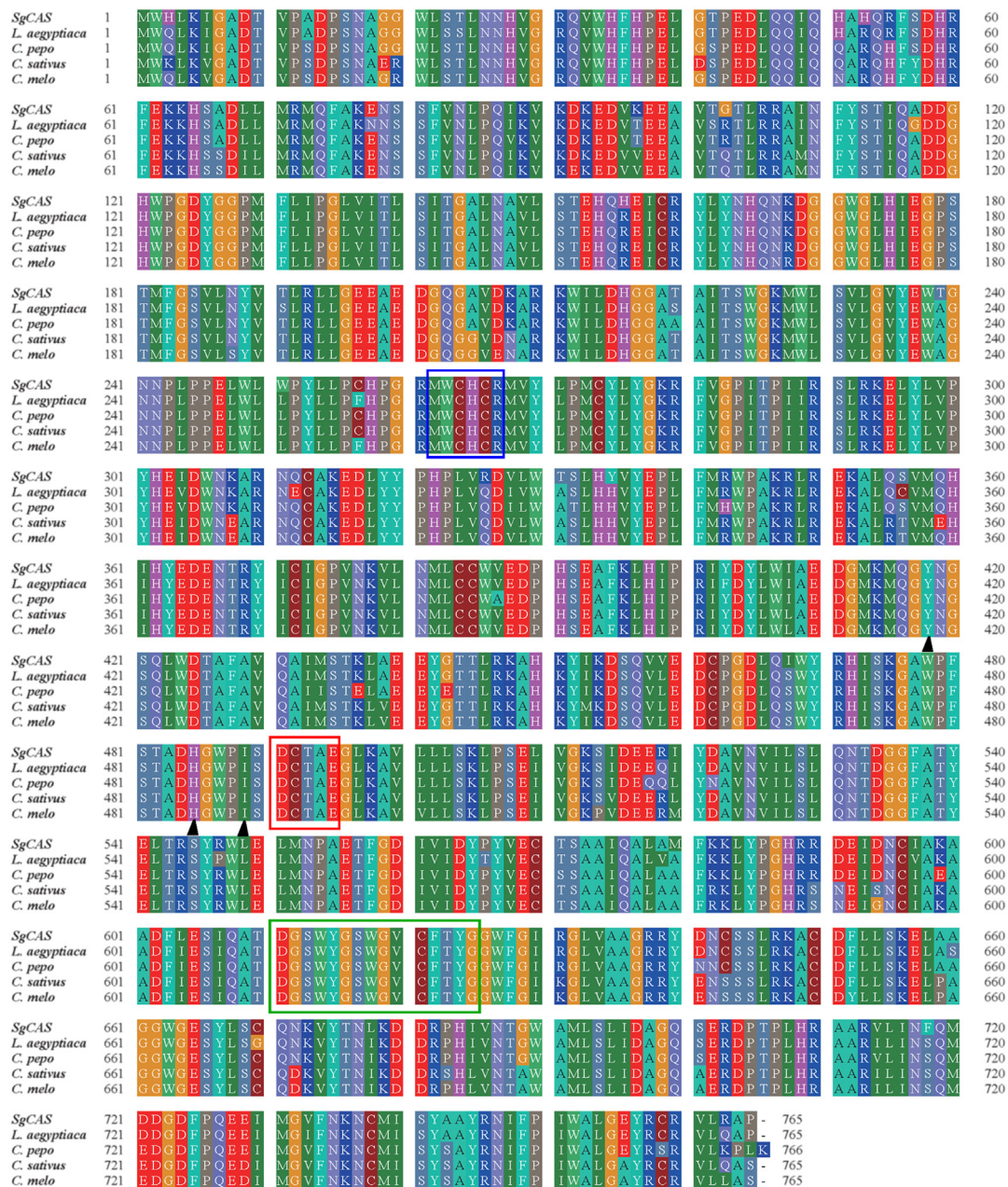


Figure 3 Multiple sequence alignment of the putative SgCAS protein and four other cycloartenol synthases. The highly conserved DCTAE motif is boxed in red. A terpene synthase feature of DGSWYGSWAVcFYG is boxed in green. MWCHCR motif is shown in the blue box, in which Trp (W) plays key role in triterpene 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 *SQS*s or *CAS*s 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 *CAS*s 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

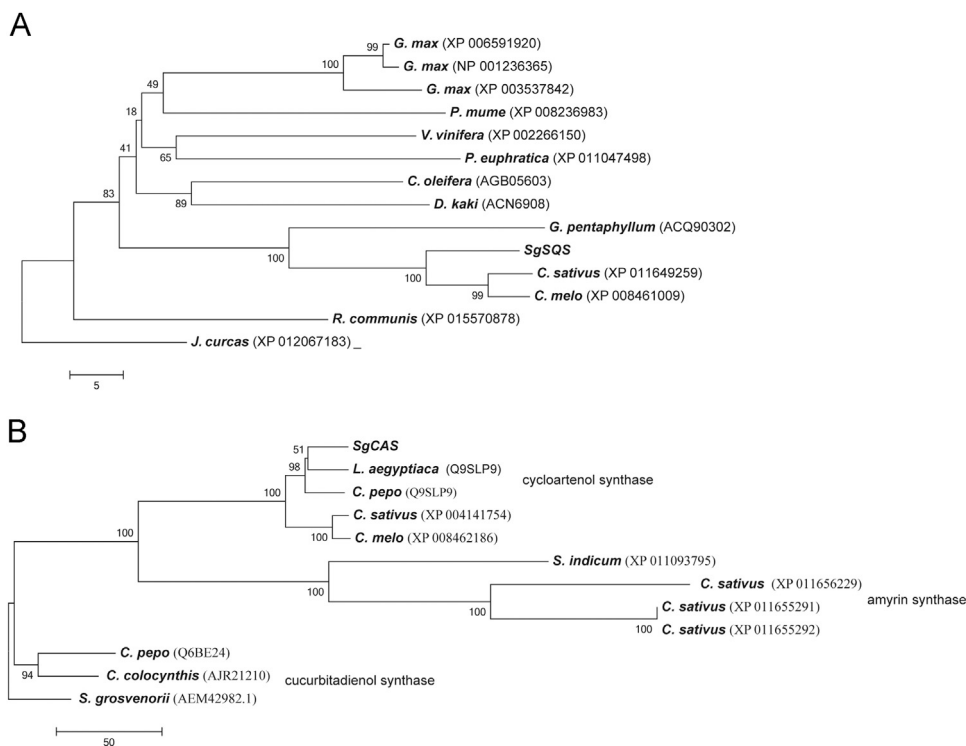


Figure 4 The phylogenetic trees obtained with MEGA 6.0 software. (A) A phylogenetic tree from the putative *SgSQS* protein and other plant *SQS*s; (B) A phylogenetic tree constructed from the deduced *SgCAS* and other plant OSCs. These OSCs included cycloartenol synthase from *Luffa aegyptiaca* (Q9SLP9), *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.

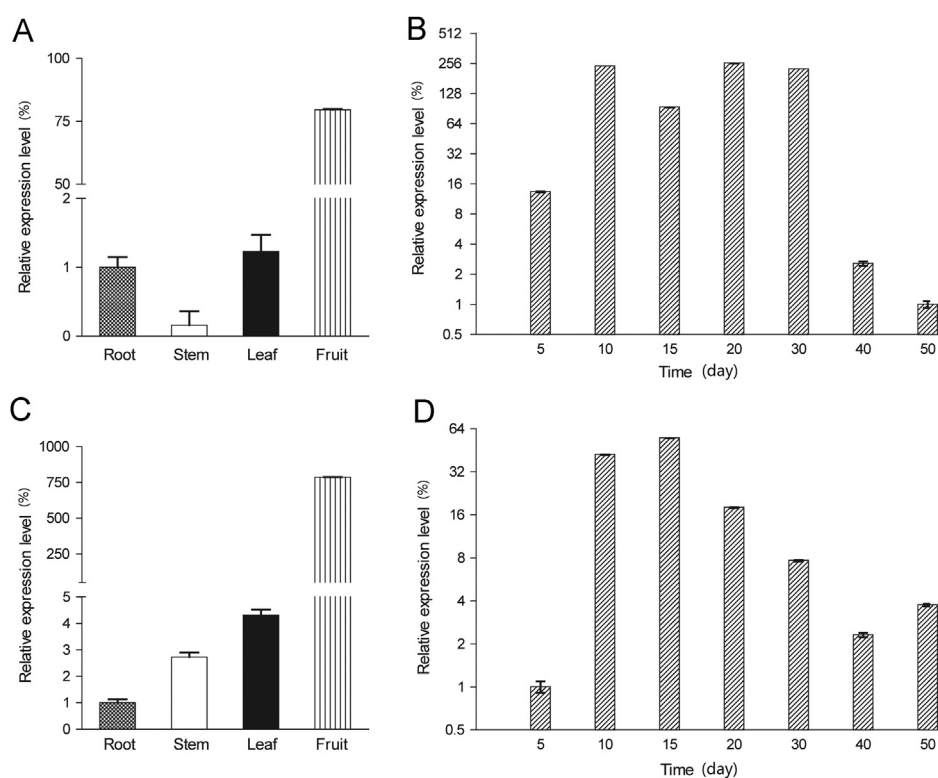


Figure 5 Quantitative RT-PCR analysis of the expression patterns of *SgSQS* and *SgCAS* genes in *Siraitia grosvenorii*. (A and C): Expression patterns of *SgSQS* and *SgCAS* genes in different tissues (root, stem, leaf and 5-day fruit). (B and D): Expression patterns of *SgSQS* and *SgCAS* genes in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

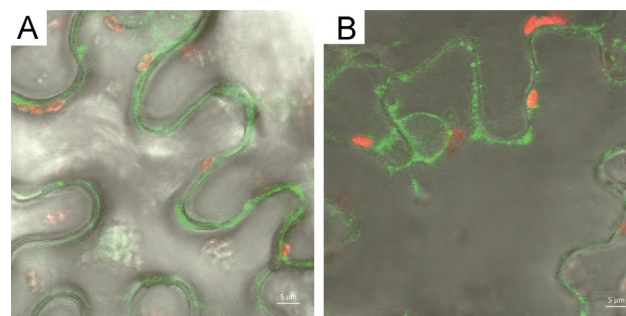


Figure 6 Subcellular localization of *SgSQS* and *SgCAS* proteins. (A) Confocal image of an epidermal leaf cell expressing *SgSQS*-YFP. (B) Confocal image of an epidermal leaf cell expressing *SgCAS*-YFP.

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. grosvenorii* 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 algae¹⁵, yeast, animals and plants¹⁶. *SgSQS* was remarkably highly expressed in fruit, especially in fruit after 30 days in *S. grosvenorii*. *SQS*s are a small gene family, with

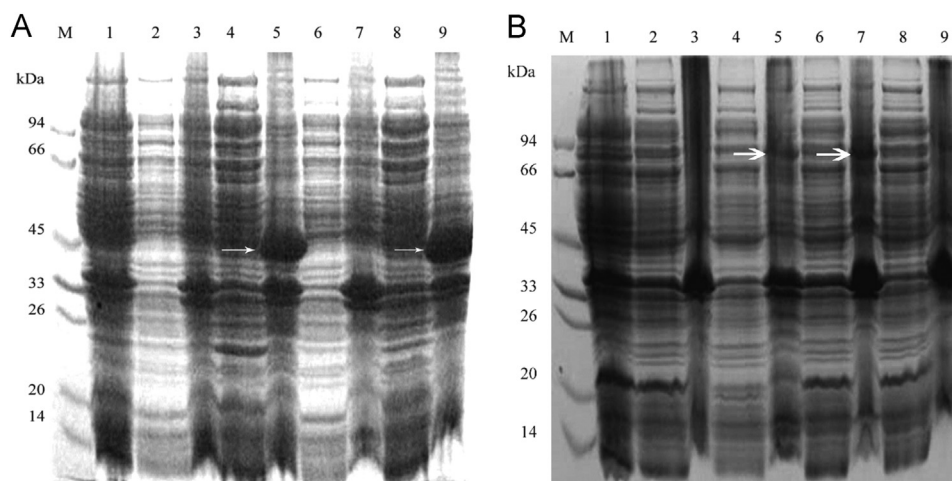


Figure 7 SDS-PAGE analysis of recombinant SgSQS-pET28a and SgCAS-pET28a proteins. (A) SDS-PAGE of SgSQS-pET28a, arrow marks the SgSQS protein. (B) SDS-PAGE of SgCAS-pET28a, arrow marks SgCAS protein. M: Protein molecular weight marker. (1) Control. (2) 20 °C for 16 h by IPTG (Supernatant); (3) 20 °C 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, isopropyl- 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*, amyirin 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 motif²³. Phylogenetic analysis clustered SgCAS with other cycloartenol synthases from Cucurbitaceae, separating them from the cucurbitadienol synthase and amyirin 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 accumulation³⁰.

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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