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

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**KEY WORDS**

*Siraitia grosvenorii*; Triterpenoids; Steroids; Cloning; Expression; Squalene synthase; Cycloartenol synthase; 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. 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. Total RNA was extracted from the fruits of *S. grosvenorii* using Trizol (Invitrogen, USA) as described by Tang et al. 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 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. 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

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.ncbi.nlm.nih.gov/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://psort.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 | TGGCCATAATCTGAGGAATTCGAC        |
| SgSQS 3′-RACE primer | SgSQS-3′GSP1 | CCGTGCAATGGCTCAAAGATTTGTC       |
| SgSQS ORF cloning | SgSQS-ORF1 | ATGGCACTTGGGCGCTGAT             |
|            | SgSQS-ORF2 | TCATACAGGTTGGTTAGCCGGTG         |
| SgSQS qRT-PCR primer | SgSQS-qPCR1 | CTGAAACACCCGATACGT             |
|            | SgSQS-qPCR2 | TAGCCGTGGCAGAACATTG             |
| SgCAS 5′-RACE primer | 5′RACE-CAS1 | AGACCCTGAGGATTGCTTGGCC         |
| SgCAS 3′-RACE primer | 3′RACE-CAS2 | GGGCATGAACTGGCCACTCTAAGAGGG     |
| SgCAS qRT-PCR primer | SgCAS-ORF1 | ATGGGCCATCTCAGATTG             |
|            | SgCAS-ORF2 | TTAGGGGTCGCTGGCAGT             |
| SgCAS qRT-PCR primer | SgCAS-qPCR1 | CAAATACAACATGGCTCACACC         |
|            | SgCAS-qPCR2 | TAGGCCCTTCTTATAGTGGC           |
| Reference gene primer | SgUBQ-qF | ATAAAAAGGCCACGACCACATTC        |
|            | SgUBQ-qR | CCGCCGACTACAACATCC            |
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 (ΔΔC_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 μmol/L acetosyringone in 10 mmol MgCl2). 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 | SgSQS | SgCAS |
|------------------------|-------|-------|
| 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 |

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 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.

Figure 2  Multiple alignment of the deduced SgSQS protein with other plant SQS proteins using BioEdit software. SQS motif is shown in red box.
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
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 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. SgCAS genes have been identified in many organisms, including algae, yeast, animals and plants. SgSQS was remarkably highly expressed in fruit, especially in fruit after 30 days in *S. grosvenorii*. SQSs are a small gene family, with
one, two and three SQSs identified in *Medicago truncatula*, *Panax notoginseng* and *Panax ginseng*. 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. demonstrated that RNAi for *SgCAS* was a competitor for the substrate pattern similar to that of cucurbitadienol synthase (data not published), indicating *SgCAS* was also highly expressed in fruits with an expression content when *SgCAS* expression 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. 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, the expression in *E. coli* can regulate withanolide biosynthesis. Intro-duction of RO 48-8071 as a CAS inhibitor caused diminished phytosterol synthesis but did not affect triterpenoid accumulation.

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.

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