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Molecular Cloning and Expression of SS Gene from *Panax japonicus*

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The squalene synthase (*SS*) gene from *Panax japonicus* is an important regulatory enzyme in the biosynthetic pathway of triterpenoid saponins (TS). Regulating the *SS* activity directly affects the synthesis of squalene (SQ), as well as the synthesis of TS, for which SQ acts as a precursor. By using the homologous cloning program RACE, the *SS* in the synthetic TS pathway of *P. japonicus* was successfully cloned , it was named for *PjSS*. Its fragment was 1353 bp, encoding 415 amino acids. The *PjSS* protein was an unstable and hydrophobic protein, with the molecular mass of 109559.7 kD. The secondary structure of SS was composed of α-helix, random coil, extended strand, and β-angle of 67.47%, 22.41%, 7.23%, and 2.89%, respectively. The *PjSS* protein exhibited folding properties to form the typical three-dimensional structure,which has eight functional motifs.RT-PCR indicated that the expressing quantity of *PjSS* is max in the hypogaeic tuber, the root is second, and the leaf is min.

Key words: Squalene synthase gene, *Panax japonicus*, Cloning, Expression.

Panax japonicus (C.A. Meyer.) is a perennial herb belonging to the Araliaceae ginseng genus. It is mainly distributed in China, Japan, and Korea, while southwest China shows the highest distribution of this species[1]. *P. japonicus* is a treasure in Chinese folk medicine and is known as the "king of herbs." It is one of the weven categories of rare and endangered Chinese medicinal herbs. In 2000, it was included in the "Pharmacopoeia of the People's Republic of China." *P. japonicus* has pharmacologically effects of promoting blood flow of *P. notoginseng* and strengthening tonics of *P.ginseng*[2]. The saponin substances in *P. japonicus* are mostly triterpenoid saponins (TS). More than 20 ingredients have been reported with important pharmacological activity[3-6].

Cloning, expression, and regulation of squalene synthase (*SS*) gene have recently become one of the cutting-edge research areas in the field of molecular biology[7-9]. A number of studies indicate that SS plays a key role in the TS biosynthetic pathway[10-12]. Squalene (SQ) is a common precursor of all TS and is synthesized by squalene synthase (SS). This response is the first critical step in the biosynthesis of TS. SS is an important regulatory enzyme in the synthetic TS pathway. Regulating the activity of SS direct affects the synthesis of SQ, as well as the synthesis of TS for which SQ acts as a precursor. Its content and activity determine the expression and production of TS downstream products[13-14]. Recently, studies of the *SS* have shown new methods for the metabolic control and breed improvement of *P. japonicus* TS.

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In this study, *P. japonicus* was used as the test materials to clone *PjSS* by RACE technology, and the *PjSS* was expressed analysis in the different organs. The aim to further study the functions of *PjSS*, explore the molecular mechanisms of *P. japonicus* TS and provide an experimental basis for future genetic engineering to breed improvement.

MATERIALS AND METHODS

Total RNA extraction and cDNA synthesis

The test material was leaves of *P. japonicus* sterile seedlings from Plant Biotechnology Laboratory of Anshun University.RNA was extracted by using RNA plant (mini) Kit (Shanghai Huashun Biotechnology Company) based on the operating instructions.The quality of RNA were determined by agarose gel electrophoresis (DU-640, Beckman, USA). The extracted RNA was stored at -86 °C in a refrigerator. The total RNA of *P. japonicus* was reverse transcribed to synthesize the first strand cDNA based on the instructions of the TAKARA RNA PCR Kit (AMV) Ver. 3.0 (Takara Dalian). Reaction reagents and volume: 2 µL $MgCl_2$, 1 µL 10× RT Buffer, 3.75 µL RNase Free dd H_2O, 1 µL dNTP Mixture, 0.25 µL RNase Inhibitor, 0.5 µL Oligo dT-Adaptor Primer, 0.5 µL AMV Reverse Transcriptase, and 1 µL RNA sample. All the reagents were mixed and incubated in the PCR at 42 °C for 60 min, reacted at 99 °C for 5 min, and maintained at 5 °C for 5 min. The reaction product was stored at -20 °C.

Amplification of the core fragments of the *SS* gene

The gene sequences of *P. ginseng, P. notoginseng, P. quinquefolius, Glycyrrhiza uralensis, Centella asiatica, Nicotiana tabacum* were searched on the NCBI. The sequence numbers were EU502717, EU131090, ACS66750.1, AAV58897.1, and AAB02945.1. By using Oligo 7.0 primer design software, the following primers were designed:f*SS*, 52 -GGCCTCGCCAGATTTGGA GTAAA-32 ;r*SS*,52 -GCAATCAGGGCTGAA TTGTGTCC-32 .

The primers were synthesized by Nanjing GenScript Co. Ltd.

1 µL *P. japonicus* cDNA, 5 µL 10× EX Taqase PCR buffer, 3 µL 25 mmol·L^{-1} $MgCl_2$, 1 µL 10 mmol·L^{-1} dNTP, 1 µL 10 µmol·L^{-1} fSS, 1 µL 10 µmol·L^{-1} rSS, 0.5 µL (2.5 U) Taq DNA polymerase (TaKaRa EX Taq), and 37.5 µL dd H_2O was added into a 200 µL Eppendorf tube. The total reaction volume was 50 µL. After slight mixing and brief centrifuging, The PCR reaction was run using the following protocol: initial denaturing at 94 °C for 4 min, followed by 35 cycles of denaturing for 45 s at 94 °C, annealing for 45 s at 66°C and extension for 2 min at 72 °C, with a final extension for 10 min at 72 °C. After agarose/1× TAE gel electrophoresis and UV detection (120 V, 100 mA, 30 min) of the 10 µL PCR product, the sample was observed and analyzed using a gel imaging system. The target band was purified using Biospin Gel Extraction Kit (BioFlux), and detected by electrophoresis. A mixture of 4 µL purified SS DNA, 5 µL Solution I, and 1 µ plasmid pCXSN was transfected into *E. coli* DH5 a at 16 °C overnight for subcloning and sequencing.

Amplification of SS 32 and SS 52 gene fragments

On the basis of the known core fragment sequence of the *PjSS*, the Oligo 6.0 and DNAStar softwares were used to design nested RACE primers SS 32 -1 and SS 32 -2, as well as SS 52 -1 and SS 52 -2 for the amplification of the *SS* 32 and 52 terminal fragments, which were synthesized in Nanjing GenScript Co. Ltd. The sequences of primers SS 32 -1 and SS 32 -2, 32 -RACE Outer Primer and 32 -RACE InnerPrimer (TaKaRa 32 -Full RACE Core Set 2.0), primers SS 52 -1 and SS 52 -2, and 52 RACE Outer Primer and 52 RACE InnerPrimer (TaKaRa 52 -Full RACE Kit, TaKaRa Code: D315) were as follows:

SS 32 -1' 52 -TGCT GAAGTCCAAGGTTGACAA-32 , *SS* 32 -2ÿ52 -GAGTCAGGACACAA TTCAGCCC-32 ; 32 -RACE Outer Primer: 52 -TACCGTCGTTCCACTAGTGATTT-32 , 32 -RACE InnerPrimer: 52 -CGCGGATCCTCCA CTAGTG ATTTCACTATAGG-32 ; SS 52 -1ÿ52 -CCGGAAGATAGCAG GATCTCGC-32 , SS 52 -2ÿ52 -GTCATTCAGGCACTGCACTGCC-32 ; 52 -RACE Outer Primerÿ52 -CATGGCTACATGC TGACAGCCTA-32 , 52 -RACE Inner Primerÿ52 -CGCGGATCCACAGCCTACTGATGATCAGTCGATG-32 .

In accordance with TaKaRa 32 -Full RACE Core Set 2.0 and TaKaRa 52 -Full RACE Kit **,** the *SS* 32 and *SS* 52 gene fragments were synthesized.

Amplification of the full-length SS gene

The core fragment, 3-terminal and 5-

terminal fragments of *PjSS* were spliced by the Vector NTI Suite eight to obtain the full-length *PjSS* sequences. Primers Full-F-SS and Full-R-SS were designed based on the full-length *PjSS* sequences and synthesized by Nanjing GenScript Co. Ltd. The sequences of primers were as follows:
Full-F-SS: 52 -TAGAGAGAAAATGGG AAGTTTGGGG-32;
Full-R-SS: 52 -GAACTGGGGTTCTCACTGT TTGTTC-32.

1 µL *P. japonicus* cDNA, 5 µL 10× EX Taqase PCR buffer, 3 µL 25 mmol·L^{-1} $MgCl_2$, 1 µL 10 mmol·L^{-1} dNTP, 1 µL 10 µmol·L^{-1} fSS, 1 µL 10 µmol·L^{-1} rSS, 0.5 µL (2.5 U) Taq DNA polymerase (TaKaRa EX Taq), and 37.5 µL dd H_2O were added into a 200 µL Eppendorf tube. The total volume was 50 µL. After slightly mixing and briefly centrifuging, the mixture was placed in the PCR instrument. Reaction conditions were as follows: pre-denaturation at 94 °C for 4 min, 30 cycles of amplification (denaturation at 94 °C for 45 s, annealing at 66 °C for 45 s, extension at 72 °C for 2 min), and extension at 72 °C for 10 min. After agarose/1× TAE gel electrophoresis and UV detection (120 V, 100 mA, 30 min) of the 10 µL PCR product, the sample was observed and analyzed using a gel imaging system. The target band was purified using Biospin Gel Extraction Kit (BioFlux), and detected by electrophoresis. A mixture of 4 µL purified SS DNA , 5 µL Solution I, and 1 µ plasmid pCXSN were transfected into *E. coli* DH5 a at 16 °C overnight for subcloning and sequencing.

Bioinformatics analysis

The physicochemical properties of the protein encoded by the *SS* gene were predicted using the online tool Protparam (ExPASy Proteomics Server). The SS protein structure was predicted using the online tool ExPASy PROSITE. SWISS-MODEL was used to analyzing the protein secondary structure and establish three-dimensional (3D) modeling. SignalP4.0 Server was used to predict the secreted protein. WOLF PSORT was used to predict the protein localization signal. The transmembrane region was predicted using the online tool HMMTOP. MEGA 5.0 was used to establish the hylogenetic tree.

Expression of *PjSS*

The total RNA was extracted from the root , stem , leaf and under block stem separately , and inverted to cDNA accordingly. With ²-actin for internal standard reference, the expressing quantity of *PjSS* was analyzed in different organs by RT-PCR.

RESULTS AND DISCUSSIONS

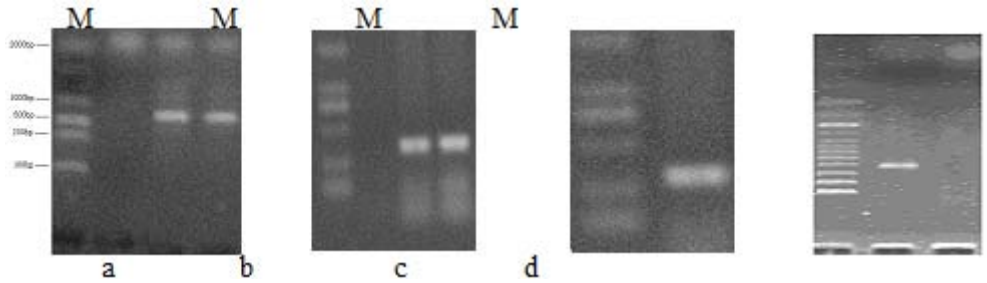
Coding sequence of *PjSS*

The primers F-SS and R-SS were employed to amplify the *P. japonicus* cDNA, which was obtained via reverse transcription. After 1% agarose gel electrophoresis, a specific fragment about 550 bp was detected , it was recovered , transfected and sequenced to obtain the nucleotide sequence. It was showed that the amplified PCR fragment was the core fragment of the *PjSS* (Fig.1-a). The 3-terminal and 5-terminal RACE nested primers (SS 32 -1, SS 32 -2; SS 52 -1, and SS 52 -2) designed based on the core fragment of the *PjSS*, were PCR amplified by TaKaRa 32 -Full RACE Core Set 2.0 and TaKaRa 52 -Full RACE Kit (TaKaRa Code: D315) at 55.81 °C and 59.54 °C, respectively. After electrophoresis, the 3-terminal and 5-terminal fragments were recycled, subjected to transfected, and sequenced to obtain approximately 400 bp and 350 bp, respectively (Figs. 1-b, 1-c). The core fragment, 3-terminal and 5-terminal fragments of the *PjSS* were spliced together by the Vector NTI Suite 8, and obtained full-length sequence of the *PjSS* was about 1352 bp. The full-length PCR primers (Full-F-SS and Full-R-SS) were designed based on spliced full-length SS gene. At 56.9 °C, after PCR amplification, electrophoresis, recycling, transformation, and sequencing, the sequence was consistent with that of the full-length cDNA (Fig. 1-d). Vector NTI 8.0 and DNAMAN softwares were used to analyze the full-length *P. japonicus* cDNA sequence. The length of *PjSS* was 1353 bp, including 45 bp 52 -UTR, 60 bp 32 -UTR, and 1248 bp ORF. The ORF included the start codon ATG and the stop codon TAA and encoded 415 amino acid. Molecular weight of *PjSS* protein was 109559.7kD, and the formula was $C_{4054}H_{6790}N_{1320}O_{1706}S_{257}$. Total number of atoms was 14127. Theoretical pI was 4.96. Instability index was 44.25; hence, the protein was considered unstable. Aliphatic index was 29.32. Grand average of hydropathicity was 0.722. A_{57} (G) was the most hydrophilic residue in SS protein (Minimum: -0.5). A_{1017} (T) and A_{1018} (C) were the most hydrophobic residues (Maximum: 2.033). The number of

hydrophobic residues was much higher than the number of hydrophilic residues. Therefore, *P. japonicus* SS protein was considered as hydrophobic.

Secondary structure and tertiary structure of SS protein

The prediction by the online tool SOPMA indicates that the secondary structure of *PjSS* protein was composed of α-helix, random coil, extended strand, and β-turn, at 67.47%, 22.41%, 7.23%, and 2.89%, respectively (Fig. 2). Thus, α-helix and random coils were the major structural elements of *PjSS* protein, while extended strands and β-turns are dispersed throughout the protein. The 3-structural model of *PjSS* protein was predicted by the Online Tool SWISS-MODEL, and



M. DNA marker; a. core PCR fragment; b. 3-terminal PCR fragment; c. 5-terminal PCR fragment; d. full-length cDNA

Fig. 1. Gel electrophoresis of the PCR fragment of *PjSS*

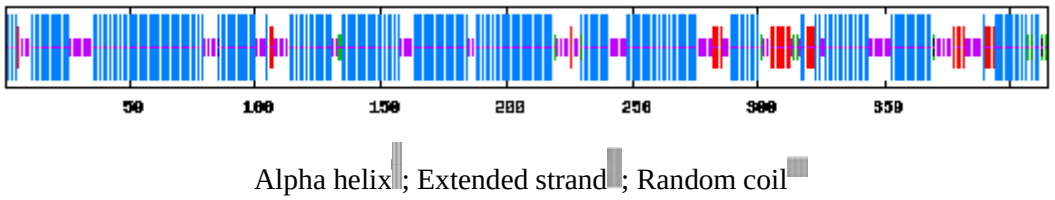


Fig. 2. Secondary structure of *PjSS* protein

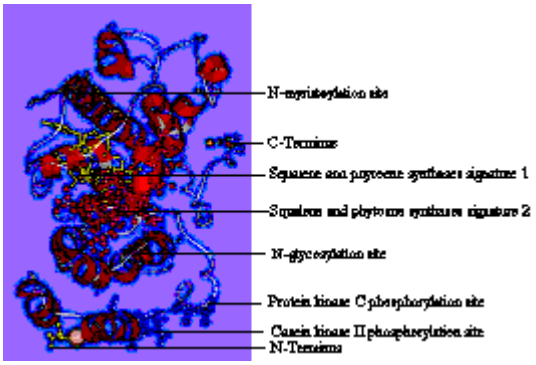


Fig. 3. Tertiary structure of *PjSS* protein

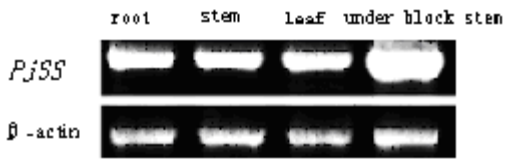


Fig. 4. Expression of *PjSS* in different organs

the structural sites were tagged by WebLab ViewerLite software to obtain the tertiary structure of *PjSS* protein (Fig. 3).

Expressing analysis of *PjSS*

The expressing quantity of *PjSS* was analysized in different organs(root , stem , leaf and under block stem) by RT-PCR, the result showed that the *PjSS* has a different expression in different organs.Among of them, the expressing amount was the max in the under block stem, the root was for the second, the stem and leaf were the min. This expressing result was in concordance with its synthesis. Therefore, the *P. japonicus* TS biosynthesis was controled by *PjSS*, its regulating level was different in different organs(Fig.4).

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