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Cloning and Sequence Analysis of a Squalene Synthase Gene from *Ginkgo biloba*

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Abstract

To obtain the key genes for terpenoid saponins biosynthesis, a new squalene synthase gene was isolated by PCR from the leaves of *Ginkgo biloba*, named *GbSS*. And we also predicted the structure and physicochemical property of squalene synthase protein. The length of *GbSS* gene cDNA is 1998 bp, encoding 410 amino acids. The deduced protein sequence of GbSS has high homology with squalene synthase proteins from other plants, especially the gymnosperms. The putative GbSS protein is a hydrophilic protein and contains one putative transmembrane helices, it may be located in the plasma membrane and does not contain a signal peptide. Phylogenetic analysis revealed that the GbSS was closely related to TcSS, PmSS clustered into a single group, suggesting GbSS and squalene synthase from other Gymnosperms plants may be the same ancestor. These results provided a basis to further analyze the mechanism of terpenoid saponins biosynthesis of *Ginkgo biloba*.

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Keywords

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Introduction

The triterpenes in plants exhibit a wide range of structural diversity and biological activity, and their glycosides saponins are deemed to be natural medicines with economic importance (Connolly and Hill, 2005). Recent studies have shown that triterpenoids had antihyperglycemic, anti-cancer, and anti-inflammatory activities (Kikuchi et al., 2011; Yasukawa et al., 1998). Squalene synthase (SS) is an incipient and crucial branch point enzyme away from the main isoprenoids biosynthetic pathway and a potential regulatory point that control carbon flux into sterols and triterpenes biosynthesis (Huang et al., 2007). Squalene synthase catalyzes the reductive dimerization of farnesyl pyrophosphate (FPP), in which two identical molecules of FPP are converted into one molecule of squalene, and squalene is a committed precursor of the sterols and triterpenes biosynthetic pathways (Abe et al., 1993). Squalene synthase has been isolated and characterized from various species, such as *Taxus cuspidata* (Huang et al., 2007), *Arabidopsis thaliana* (Nakashima et al., 1995), tobacco (Devarenne et al., 1998), *Poria cocos* (Wang et al., 2014), *Dioscorea zingiberensis* (Ye et al., 2014), *Lotus japonicus* (Akamine et al., 2003), *Capsicum annuum*（Lee et al., 2002) and other plants, as well as mammals (Inoue et al., 1995) and yeast (Merkulov et al., 2000). The interest in the investigation of the squalene synthase regulation in plants increases steadily in recent years. Previous research has indicated that PgSS1 is a key regulatory enzyme not only for phytosterol but also for triterpene biosynthesis, *PgSS1* transcripts are expressed ubiquitously in the various plant tissues, but higher in shoot apex and root, overexpressing *PgSS1* is

able to produce high levels of phytosterol and ginsenosides in *Panax ginseng* (Lee et al., 2004). Zhang et al. (2016) also reported that birch *BpSS* and *BpSE* were indeed involved in the synthesis of triteropenoids. Moreover, overexpression of the *SS* gene in *Bupleurum falcatum* resulted in enhanced production of both phytosterol and saikosaponins and up-regulated mRNA expression of downstream genes including squalene epoxidase and cycloartenol synthase (Kim et al., 2011). Other studies have come to similar conclusions, overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* showed that the expression of the *35S-SS1* gene correlated positively with steroid and withanolide metabolism, confirming the pivotal role of the *SS1* gene in the regulation of triterpene metabolism (Mirjalili et al., 2011). Overexpression of the squalene synthase gene could enhance production of phytosterols and triterpenoids in *Eleutherococcus senticosus* (Seo et al., 2005).

Therefore, we believe that SS should play an important role in triterpenes biosynthesis in *Ginkgo biloba* species. For a better understanding of the role of SS in triterpenoid biosynthesis in *Ginkgo biloba*, we describe the isolation and analyze of the *SS* gene from *Ginkgo biloba* in the study, our results are an important foundation for further understanding function of SS in the control of triterpenoid biosynthesis in *Ginkgo biloba*.

Materials and methods

Plant material

The 18-year-old *G. biloba* was used for cloning of the *GbSS* gene in this study. *G. biloba* was grown in the Botanical Garden of Yangtze University, in China. The leaves were collected, immediately frozen in liquid nitrogen and kept at -80°C prior to RNA extraction.

Cloning the full-length sequence of *GbSS* cDNA

Total RNA was extracted from fresh *G. biloba* leaves using the CTAB method (Cai et al., 2007). The purity and concentration of total RNA were quantified by spectrophotometer and agarose gel electrophoresis. The extracted RNA was reverse transcribed into cDNA using the PrimeScript™1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The obtained first-strand cDNA was used as template to amplify GbSS fragment. According to transcriptome sequence of G. *biloba*, a pair of specific primers GbSSF (5'-TGTTGAGGAGGATTTGGCCTGGAG-3') and GbSSR (5'-GCAAAATAATTTACATTATACTAGTGAAATG-3') were designed, and synthesized by Shanghai Sangon Biotechnology Company (In China). PCR reaction was performed under the following conditions: denaturation at 94°C for 3 min; 32 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 90s; and a final extension at 72°C for 10 min. The amplified product was purified with DNA Purification Kit (Dalian TaKaRa, China) and ligated into pMD18-T vector (Dalian TaKaRa, China) according to the manufacturer's instructions. The ligated products were transformed into *Escherichia coli* TOP10 competent cells, coated plates, and select monoclonal colony PCR was performed with M13 universal primers. The positive clones were sequenced by Shanghai Sangon Biotechnology Company.

Bioinformatics analysis

Vector NTI Suite V 11.5 and DNA man 8.0 were used to analyze cDNA sequences of *GbSS* gene. Physical and chemical parameters of GbSS protein were analysed using ExPASy (http://www.expasy.org/). Hydrophobic and hydrophilic features were analyzed using Protscale. SignalP 4.1 Server was used for the signal peptide prediction. TMHMM Server 2.0 was used for transmembrane structure prediction. PSORT WWW Server was employed to predict the protein localization. The secondary structure of GbSS protein was analyzed by SOPMA tool. The conserved domains and motifs were searched with the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ScanProsite tool (http://prosite.expasy.org/). Sequence similarity search was completed with Blastn and Blastp on NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

Multiple sequence alignment was performed by Align X (Vector NTI Suite V 11.5). Phylogenetic and genetic distance analyses of squalene synthase proteins were constructed with Clustal X 2.0 and MEGA 6.0 software based on the neighbor-joining (NJ) method (Tamura et al., 2013).

Results

Cloning of the cDNA sequence of GbSS

On the basis of sequence information of *G.biloba* transcriptome, a full-length cDNA designated as GbSS (GenBank accession number KX066188) was cloned from the cDNA template reversely transcribed from the *G.biloba* total RNA by using PCR. Sequence analysis noted that the cDNA was 1998 bp long, with

a 5′-untranslated region of 252 bp, an ORF from ATG codon to TAA stop codon of 1233 bp, and a 3′-untranslated region of 513 bp (Fig. 1).

The ORF region consists of bases A (28.1%), C (18.2%), G (23.9%) and T (29.8%) and encoding 410 amino acids.

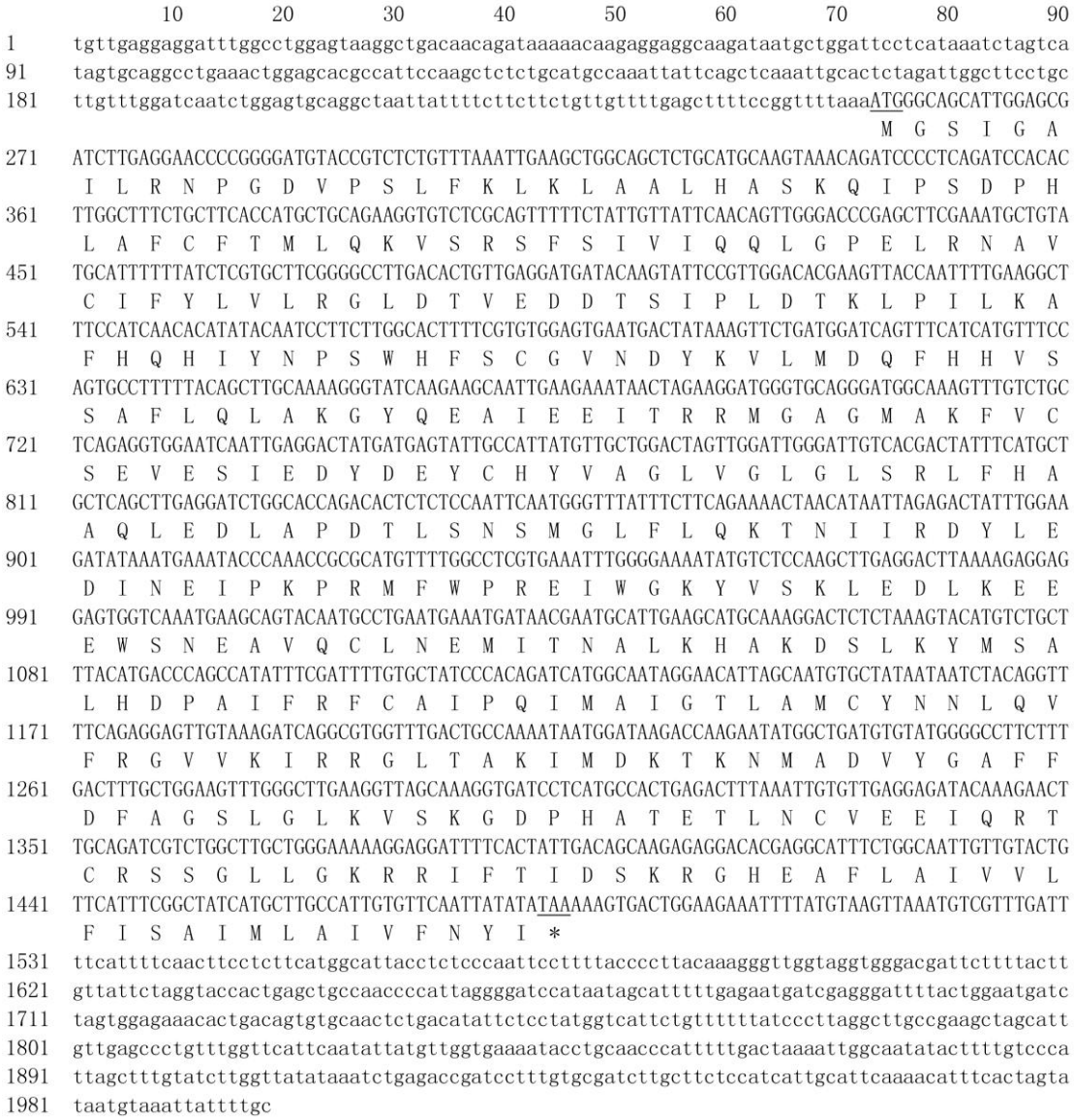

Fig. 1: The nucleotide acid sequence and deduced amino acid sequence of *GbSS*. The start condon (ATG) and the stop (TAA) are underlined, 5′-untranslated region and 3′-untranslated region are indicated in lowercase.

Characterization of the deduced GbSS protein

Putative protein of the ORF contains 410 amino acids with a calculated molecular mass of 46.23 kDa and a theoretical isoelectric point (pI) of 6.89. The percentages of Alpha helix, Extended strand, Beta turn and Random coil in the secondary structure were predicted to be 69.27, 8.05, 4.39 and 18.29%, respectively. According to Protscale application analysis, the squalene synthase amino acid sequence had the lowest hydrophilic score of -2.367, the highest hydrophobic score of 3.6. The hydrophilic amino acids were distributed throughout the whole peptide chain, and the number was larger than the number of hydrophobic amino acids, meaning the putative GbSS protein is a hydrophilic protein. The result of signal peptide prediction showed that the GbSS

protein does not contain a signal peptide. Amino acid sequences deduced from GbSS were predicted by the TMHMM program, GbSS contain one putative transmembrane helices between the protein position 387 and 409. With PSORT Prediction the subcellular localization of GbSS protein, we found that the GbSS may be located in the plasma membrane (0.730, Affirmative).

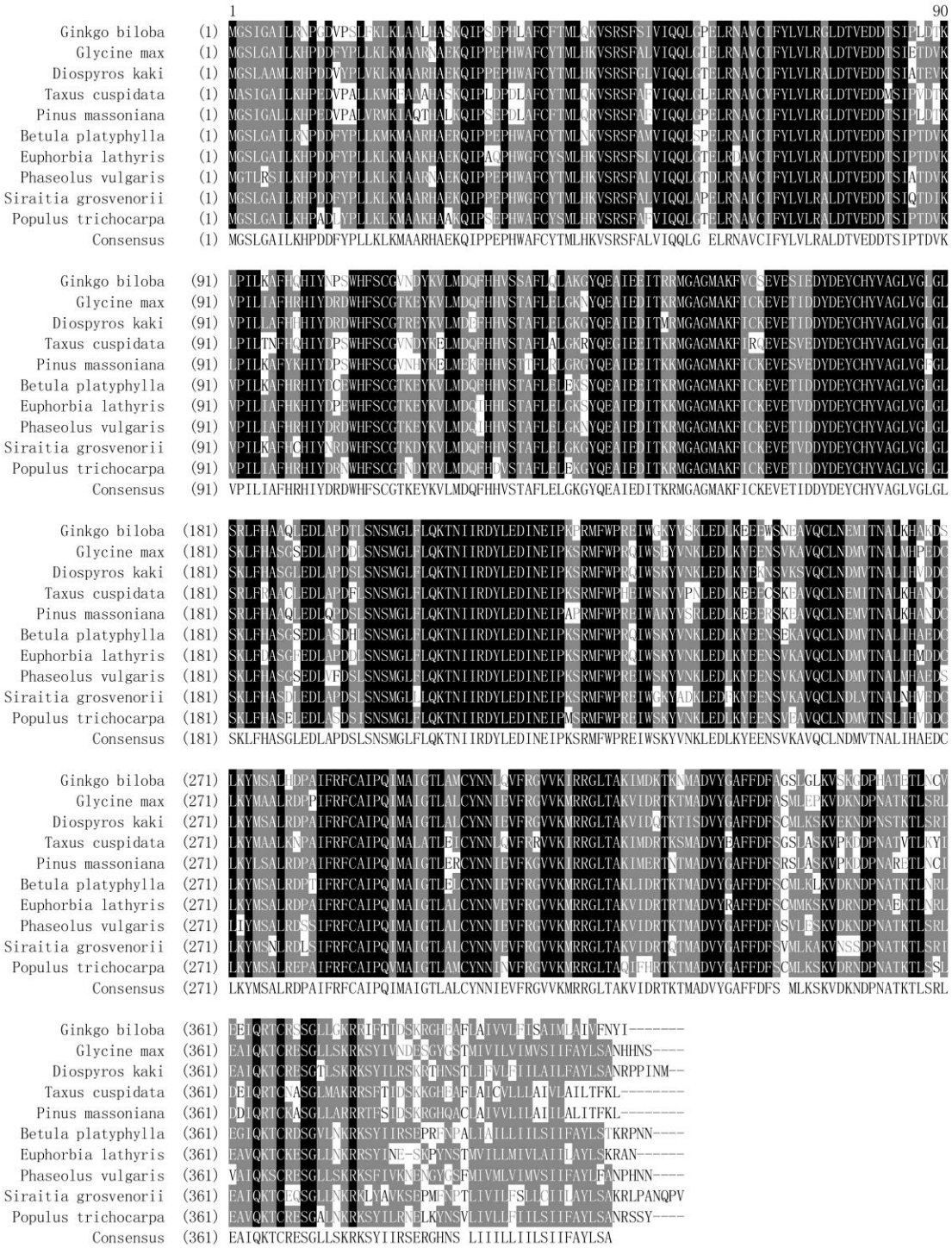

Fig. 2: Homology comparison of GbSS amino acid sequence with SS protein from other plants. The completely identical amino acids are indicated with white foreground and black background. The conserved aminoacids are indicated with white foreground and grey background. Non-similar amino acids are indicated with black foreground and white background.

Sequence homology and conserved domains

Homology analysis with BLASTP and Align X indicated that the putative GbSS shared 82, 81, 76, 73, 72, 72, 72, 71 and 70% identity with other known SS proteins from *Taxus cuspidata* (ABI14439.1), *Pinus massoniana* (AHI96421.1), *Betula platyphylla* (AKR76253.1), *Diospyros kaki* (ACN69082.1), *Glycine max* (NP_001236365.1), *Populus trichocarpa* (XP_002313765.1), *Siraitia grosvenorii* (AEM42980.1), *Euphorbia lathyris* (AFZ93644.1) and *Phaseolus vulgaris* (AHA84150.1), respectively, indicating they are highly homologous (Fig. 2). Conserved domain prediction of NCBI Domain Database revealed that the GbSS belongs to Isoprenoid_Biosyn_C1 Superfamily and contained squal_synth (farnesyl-diphosphate farnesyltransferase), Trans_IPPS_HH (Trans-Isoprenyl Diphosphate Synthases), SQS_PSY (Squalene/phytoene synthase), PLN02632 (phytoene synthase) and ERG9 domain.

Conserved domain analysis by using ScanProsite showed that the GbSS had two functional motifs SQUALEN_ PHYTOEN_SYN_1 and SQUALEN_PHYTOEN_ SYN_2, which were same as SS from *Pinus massoniana* and *Betula platyphylla* (Fig. 3). Squalene synthase (farnesyl-diphosphate farnesyltransferase) catalyzes the conversion of two molecules of farnesyl diphosphate into squalene. Phytoene synthase catalyzes the conversion of two molecules of geranylgeranyl diphosphate into phytoene. All the above evidences substantiate that the putative GbSS protein is a squalene synthase.

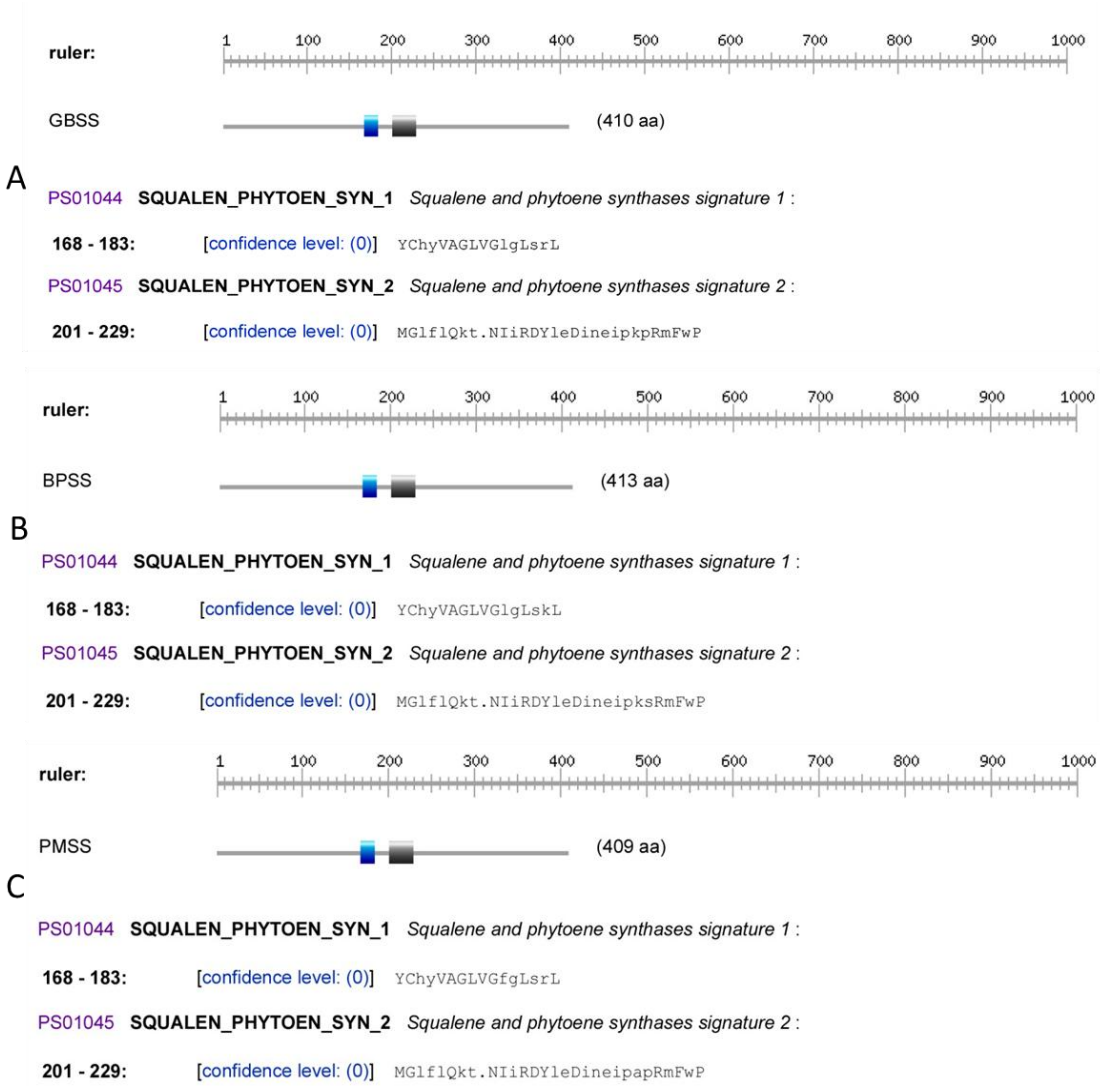

Fig. 3: Conserved domain analysis of squalene synthase from *Ginkgo biloba* (A), *Pinus massoniana* (B) and *Betula platyphylla* (C).

Phylogenetic analysis

To understand the relationship between the GbSS protein sequence and SS protein sequences from other plants, a phylogenetic tree was constructed by using the neighbor-joining method (Fig. 4). It was clearly observed that SS enzymes were clustered into two group Gymnosperm and Angiosperm, and the putative GbSS protein was clustered into the branch of Gymnosperm plants. GbSS had a closer relationship with SS from *Pinus massoniana* and *Taxus cuspidata* than with SS from other plants. SS proteins from *Phaseolus vulgaris*, *Glycine max*, *Glycyrrhiza uralensis* and *Astragalus membranaceus* shared the same subclades, this group comprised the Fabaceae family. In addition, SS proteins from poaceae such as *Oryza sativa* and *Zea mays* were grouped into the same cluster. These show that SS proteins from the same family having the closest relationship. The analysis indicates that GbSS protein had a high homology to other plant SS proteins, and shared a common evolutionary origin with the Gymnosperm species SS proteins.

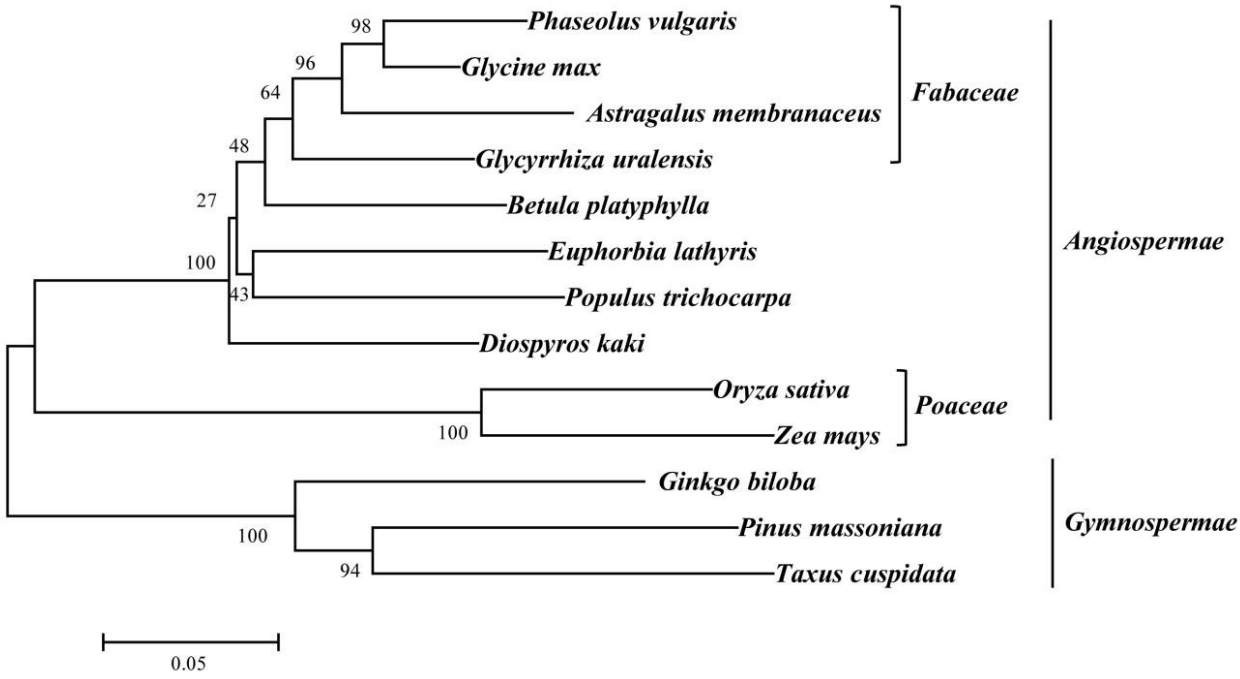

Fig. 4: Phylogenetic tree analysis of squalene synthase in different species. The numbers at each node represent the bootstrap values (with 1,000 replicates). GenBank accession numbers of SS sequences are as follows: *Phaseolus vulgaris* (AHA84150.1), *Glycine max* (NP_001236365.1), *Astragalus membranaceus* (ADV57358.1), *Glycyrrhiza uralensis* (ADG36702.1), *Betula platyphylla* (AKR76253.1), *Euphorbia lathyris* (AFZ93644.1), *Populus trichocarpa* (XP_002313765.1), *Diospyros kaki* (ACN69082.1), *Oryza sativa* (AAS07223.1), *Zea mays* (NP_001104839.1), *Pinus massoniana* (AHI96421.1), *Taxus cuspidata* (ABI14439.1).

Discussion

Squalene synthase plays an important role in the regulation of isoprenoid biosynthesis (Wentzinger et al., 2002) since it catalyzes the first committed step in sterol and triterpenoid biosynthesis. The levels of phytosterol and triterpene accumulation in plants such as *Panax ginseng* (Lee et al., 2004) and *Eleutherococcus senticosus* (Seo et al., 2005) can be increased by enhancing expression of SS genes.

In the present study, an SS gene was isolated from *Ginkgo biloba* by using the PCR method. Sequence analysis indicated that its coding region was 1233 bp, encoding 410 amino acids. Homology analysis found that the deduced GbSS was high homologous with SS proteins from other plants, and they all belong to Isoprenoid_Biosyn_C1 Superfamily. Ding et al. (2015) reported that SS amino acid sequences in 9 kinds of Ginseng species were hydrophilic protein and located in the plasma membrane, which was consistent with our results. GbSS amino acid contains one putative transmembrane helices. In contrast, SS from 9 kinds of Ginseng species were all have two transmembrane (Ding

et al., 2015). Phylogenetic analysis suggested that the GbSS and squalene synthase from other Gymnosperms plants may be the same ancestor. In general, the squalene synthase cDNA sequence structure and the encoding amino acid sequence in different species had high levels of similarity. The GbSS maybe also involve in regulation of triterpenoid biosynthesis.

Conclusion

In this study, a squalene synthase gene was successfully cloned and characterized for the first time from *Ginkgo biloba*, which is involved in the terpenoid saponins biosynthesis. Multiple sequence alignment showed that GbSS had a high identity with other plant SS proteins. The phylogenetic tree analysis also showed that the squalene synthase proteins are highly conserved during the molecular evolution. The cloning and sequence analysis of GbSS is useful for understanding the function of SS in triterpenoid biosynthesis in *Ginkgo biloba*.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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