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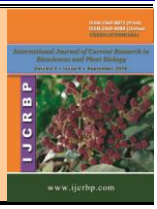

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Molecular Cloning and Sequence Analysis of a Novel Geranylgeranyl Diphosphate Synthase Gene (*GbGGPPS2*) from *Ginkgo biloba*

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Abstract

Geranylgeranyl diphosphate synthase (GGPPS), a key enzyme in the biosynthesis of ginkgolide, catalyzes the formation of geranylgeranyl diphosphate (GGPP) which is a biosynthetic precursor for ginkgolide. In this study, a novel full-length cDNA encoding GGPPS was isolated, designated as *GbGGPPS2* (GenBank accession no. KX756450), from *Ginkgo biloba*. The full-length cDNA of *GbGGPPS2* was 1568 bp and contained a 1149 bp open reading frame (ORF). The molecular weight and isoelectric point of the *GbGGPPS2* protein was 129.9 kDa and 5.01, respectively. Multiple alignments analysis revealed that *GbGGPPS2* showed extensive homology with GGPPSs from other species and possessed five conservative domains, indicating that the *GbGGPPS2* belonged to *GGPPS* gene family. Phylogenetic tree analysis showed that *GbGGPPS2* shared the same ancestor in evolution with other GGPPSs and had a further relationship with other gymnosperm species.

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Keywords

GbGGPPS2
Ginkgo biloba
Ginkgolide
Molecular cloning

Introduction

Ginkgo biloba is one of the most popular medicinal plants. It has been existing over 200 million years on the earth (He et al., 2009). The ginkgo leaf contains many active ingredients including ginkgolides, flavonol and flavone glycosides, diterpene lactones, sesquiterpenes, iron-based superoxide dismutase, p-hydroxybenzoic acid, ascorbic acid, and catechin (Jacobs and Browner, 2000). Ginkgolides, belonging to diterpenes, were first isolated by Furukawa from the root bark (Sabater-Jara et al., 2013). It has beneficial effect on cardiovascular and neurological systems (Lin et al., 1994). Further, the efficacy of reducing blood pressure and attack of migraine frequency has been proved (Usai and Bussone, 2011). Therefore, improving the quality and quantity of ginkgolide can bring tremendous medical effects and commercial value.

Terpenoids, a class of compound, are composited with several isoprene (Tholl, 2006). Isopentenyl diphosphate (IPP) is a universal biosynthetic precursor of isoprenoids. In plants, there are two pathway to synthesise IPP: mevalonate (MVA) pathway in cytoplasm and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids (Yu and Utsumi, 2009). In MVA pathway, acetyl-CoA finally synthesize IPP through a series of enzymatic reaction (Newman and Chappell, 1999); In MEP pathway, the reactant are both pyruvate and glyceraldehyde-3-phosphate (GA3P) and the final resultant is IPP (Cordoba et al., 2009). Geranylgeranyl diphosphate (GGPP), a key common diterpene precursor, is biosynthesized by geranylgeranyl diphosphate synthase (GGPPS) in the plastids (Fig 1; Dewick, 2002; Liao et al., 2004). GGPPS is a prenyltransferase that catalyzes the electrophilic coupling of farnesyl diphosphate (FPP) and IPP (Walker and Croteau, 2001). GGPPS protein contains five conservative domains:

domain I, II, III, IV and V. Meanwhile, two of these conservative domains are the first aspartate-rich motifs (FARM) and the second aspartate-rich motif (SARM) respectively (Engprasert et al., 2004).

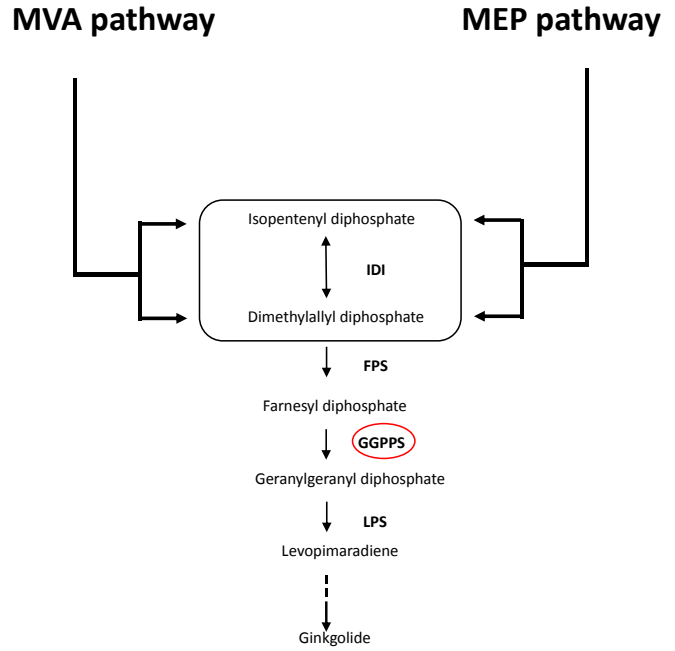

Fig. 1: Ginkgolides biosynthesis pathways in *Ginkgo biloba.*

In our previous report, transcriptome analysis of *G. biloba* was performed by us through using Illumina HiSeq™2500 sequencing platform. Based on the transcriptome data, we cloned a novel *GGPPS* gene from *G. biloba* and analyzed the structure of the sequence, aiming to provide the gene resource for increase the content of ginkgolide in *G. biloba* using genetic engineering.

Materials and methods

Plant material and reagents

The 15-year-old grafts of *G. biloba* were grown in the Botanical Garden of Yangtze University, China. The leaves of *G. biloba* were collected and preserved in a −80°C refrigerator immediately. Both of Primer synthesis and DNA sequencing were performed by Shanghai Sangon Biotechnology Company, in China.

Cloning the full-length cDNA of *GbGGPPS2*

Total RNA of *G. biloba* was isolated from young leaves using the CTAB method described by Cai et al. (2007). The first-strand cDNA was synthesized according to the instruction of PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Using the software of DNAMAN V6, the specific primers GbGGPPS-U (5′-TAGGAAGGAGGACAGAACAGTATC-3′) and GbGGPPS-D (5′-TTGATATCTAATTCTGTCTGTGAGC-3′) were designed based on the GGPPS unigene sequence of ginkgo transcriptome data. *GbGGPPS2* cDNA was amplified under the following conditions: per-denaturation at 94°C or 3 min; denaturation at 94°C for 30 sec; anneal at 51°C for 30sec; extension at 72°C for 90 sec. By 35 cycles, extend at 72°C for 10 min. The amplified product was purified, ligated into pMD18-T vector, and introduced into *Escherichia coli* strain DH5α followed by sequencing.

Bioinformatics and molecular evolution analysis

The sequence was analyzed by the bioinformatics software on websites (http://www.xpasy.org and http://www.ncbi.nlm.nih.gov), Plant GGPPS protein sequences were retrieved from NCBI GenBank. Sequence alignment was performed using ClustalX 2.0 and phylogenetic tree was constructed by neighbor-joining method using MEGA 6.0. A bootstrap statistical analysis was carried out with 1000 replicates.

Results and discussion

Cloning and sequence analysis of *GbGGPPS2*

The cDNA of *GbGGPPS2* (GenBank accession no. KX756450) was cloned by a pair of specific primers based on the *GbGGPPS2* unigene of transcriptome data. The full-length of GbGGPPS2 was 1568 bp and contained a 1149 bp opening read frame (ORF) which encoded a peptide of 383 amino acids (Fig. 2). In NCBI database, the nucleotide sequence of *GbGGPPS2* revealed high similarity with the entire coding region of *GGPPS* genes in *Populus euphratica* (81%), *Populus trichocarpa* (81%), *Lycium chinense* (79%), *Solanum pennellii* (76%). Thus, the *GbGGPPS2* might be a member of GGPPS2 gene family.

Characterization of GbGGPS2 protein

The calculated isoelectric point (pI) and molecular weight of *GbGGPPS2* were predicted to be 5.01 and 129.9kDa, respectively (http://cn.expasy.org/tools/protparam.html). Multi-alignment of *GbGGPPS2* with other plant GGPPSs showed high similarity (Fig. 3). The amino acid sequence of the *GbGGPPS2* was highly similar to previously reported *TcGGPPS* of *Taxus canadensis* (Hefner and Croteau, 1998).

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1       CAACGATTAAAACAGCAACAAGGAGGAGAAGAAATGGACCCAATAAAATGCATGTATGAC
61      ACATGCATGATGATCGCTTCGAAACTAAGTAAACCAGGAAAGGAAGCTAGTCGGGTAATT
121     TCCTGGAATTGATGTCCGCGAGTTACGACATATAATAGGAAGGAGGACAGAACAGTATCT
181     TTGAACTTGGCCAGATGAGTACTGCATTACTGAATTCGGGTTTCCATCAGTTCCTTGGCA
1                       M  S  T  A  L  L  N  S  G  F  H  Q  F  L  G
241     TCTGCTCCAACAAGACAACTTCAATGTCTCCAGGTAGCTGCAGCCTCAGAAGTTCCATAG
16      I  C  S  N  K  T  T  S  M  S  P  G  S  C  S  L  R  S  S  I
301     TTCACATTAGCAATCAGGACAAAAGCACAGGTTCTATCTATAAACCAAACCCTGTATCCT
36      V  H  I  S  N  Q  D  K  S  T  G  S  I  Y  K  P  N  P  V  S
361     CTCTTACTGCATCTTGGCTAACAAGGACAAGACCCAGTTGTTCCATCACAACATCACAGC
56      S  L  T  A  S  W  L  T  R  T  R  P  S  C  S  I  T  T  S  Q
421     TTCTCAATGAAGTTGAGGCAAAGGAAGAAAATTCTTTTTCATTTGATTTCAAGAAATACA
76      L  L  N  E  V  E  A  K  E  E  N  S  F  S  F  D  F  K  K  Y
481     TGGTTTTGAAGGCCAATGCCATAAATGAGGCCCTAGACAAATCTGTAGCTCTTAGATATC
96      M  V  L  K  A  N  A  I  N  E  A  L  D  K  S  V  A  L  R  Y
541     CTGAAAAGATACATGAAGCAATGAGATATTCACTCCTTGCTGGAGGAAAACGTGTTAGAC
116     P  E  K  I  H  E  A  M  R  Y  S  L  L  A  G  G  K  R  V  R
601     CCATTCTCTGCATTGCTGCATGTGAGCTTGTTGGTGGGTCTGAGGAATCTGCCACGCCAG
136     P  I  L  C  I  A  A  C  E  L  V  G  G  S  E  E  S  A  T  P
661     CAGCCTGTGCTATTGAGATGATTCATACAATGTCTCTTATTCATGATGATTTGCCTTGTA
156     A  A  C  A  I  E  M  I  H  T  M  S  L  I  H  D  D  L  P  C
721     TGGATAATGATGATCTGAGAAGAGGAAAACCCACCAACCATAAGGTCTTTGGTGAAGATA
176     M  D  N  D  D  L  R  R  G  K  P  T  N  H  K  V  F  G  E  D
781     TTGCTGTTCTGGCAGGAGATGCTCTGTTAGCATTTGCATTTGAACATATTGCGACATCCA
196     I  A  V  L  A  G  D  A  L  L  A  F  A  F  E  H  I  A  T  S
841     CAAAGGGTATTTTGGCAGAAAGAGTTTTGAGGGTTATTTCTGAATTGGGCAAGGCAATTG
216     T  K  G  I  L  A  E  R  V  L  R  V  I  S  E  L  G  K  A  I
901     GGTCGGAAGGGCTCGTTGCAGGCCAGGTCGTGGATATTTCTAGTGGGGGTGTCTCTGATG
236     G  S  E  G  L  V  A  G  Q  V  V  D  I  S  S  G  G  V  S  D
961     TGGCTTTGGATCTGCTTGAGTACATTCATGTGCACAAAACAGCTTCTCTTTTAGAGGGAT
256     V  A  L  D  L  L  E  Y  I  H  V  H  K  T  A  S  L  L  E  G
1021    CTGTAGTGATTGGTGCAATTATAGGGGGTGGATCTGATGATGAGGTCGAGAGATTGAGGA
276     S  V  V  I  G  A  I  I  G  G  G  S  D  D  E  V  E  R  L  R
1081    GATTTGCGCGTTGTATAGGTTTGCTTTTCCAGGTGGTTGATGACATTCTTGATGTCACTA
296     R  F  A  R  C  I  G  L  L  F  Q  V  V  D  D  I  L  D  V  T
1141    AATCATCTCAGGAGCTGGGAAAGACTGCTGGCAAGGACCTCCTGGCTGATAAGGTCACAT
316     K  S  S  Q  E  L  G  K  T  A  G  K  D  L  L  A  D  K  V  T
1201    ACCCTAAATTGCTGGGGCTAGAGGAATCAAGAGAATTTGCTGAAGAGTTGAACAGGCAAG
336     Y  P  K  L  L  G  L  E  E  S  R  E  F  A  E  E  L  N  R  Q
1261    CCAAGGATCAGCTTTCTGTTTTTGATATGGAAAAAGCAGCACCTTTGCTTTGTTTAGCTG
356     A  K  D  Q  L  S  V  F  D  M  E  K  A  A  P  L  L  C  L  A
1321    ATTACATTGCTCACAGACAGAATTAGATATCAAAGGACTTGGCAGTTTACACTTTCAATT
376     D  Y  I  A  H  R  Q  N  *
1381    TTCTGCTACCATGTTATGCCAAATTGGAAGCCCATTATCAAGTGTATAAAGCGCTTCTCA
1441    AATTTATTAGTTCTCTGATTAATATTCCTTTTCAGTAAGTTACTAGTATTCCAACTTTCT
1501    TGTTTGCCATCTACCTGCTTGCTTTTTGTTTCTAGAACACTATTTTCTTGTATTAGGGCA
1561    TTTTATAA
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Fig. 2: Nucleotide sequence and deduced amino acid sequence of *GbGGPP2*.

Amino acid sequence alignment of *GbGGPPS2* and other plant GGPPS showed that *GbGGPPS2* exhibited high similarity to GGPPS proteins from other plants. The deduced *GbGGPPS2* protein sequence showed 76%, 74%, 76%, 76%, 77%, 75%, 77% and 75% identities to the counterparts of *PaGGPPS6* from *Picea*

abies, *GbGGPPS1* from *G. biloba*, *LaGGPPS* from *Lepidium apetalum*, *CrGGPPS* from *Catharanthus roseus*, *EuGGPPS* from *Eucommia ulmoides*, *CmGGPPS* from *Cucumis melo*, *CaGGPPS* from *Corylus avellana*, *TkGGPPS1* from *Taraxacum koksaghyz*. Homology analysis with GGPPSs from other plants revealed that *GbGGPPS2* contained five conserved structural domains. Meanwhile, The second and the fifth domain was the first and the second aspartate-rich motifs respectively, which were important for the catalytic activity of GGPPS (Ashby and Edwards, 1990).

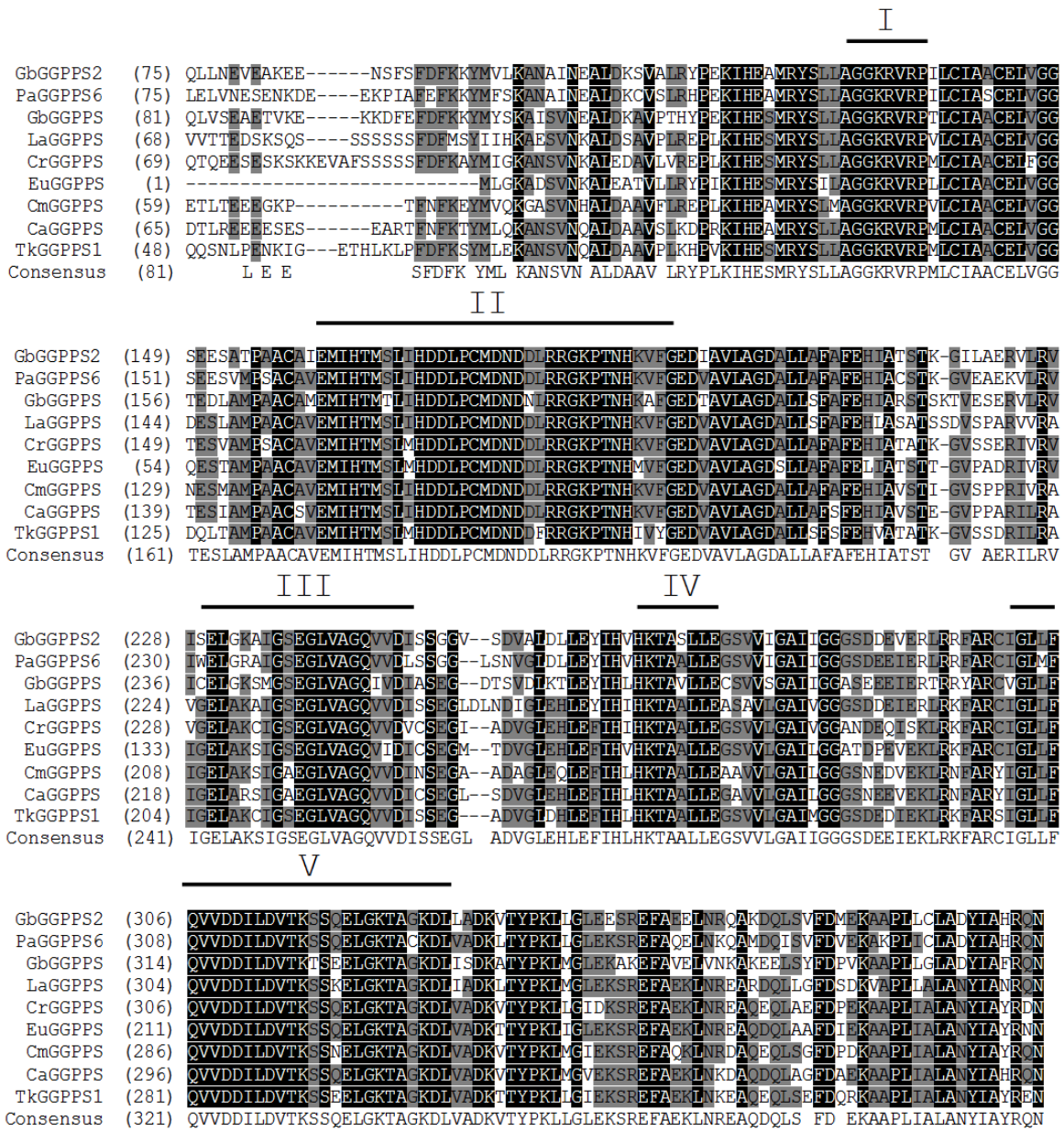

Fig. 3: Multiple alignments of the deduced amino acid sequences of *GbGGPPS2* with other GGPPS proteins. The accession numbers of GGPPS proteins and translation of their names are shown as follows, *GbGGPPS2*: *Ginkgo biloba* (KX756450); PaGGPP6: *Picea abies* (ACA21462.1); GbGGPPS: *Ginkgo biloba* (AAQ72786.1); LaGGPPS: *Lepidium apetalum* (AKQ24577.1); CrGGPPS: *Catharanthus roseus* (AE153622.1); EuGGPPS: *Eucommia ulmoide*s (AGJ03661.1); CmGGPPS: *Cucumis melo* (AGN48013.1); CaGGPPS: *Corylus avellana* ABW06960.1; TkGGPPS1: *Taraxacum koksaghyz* (AMB19719.1). The completely identical amino acids are indicated with white foreground and black background. Shaded in gray are conservative sequences. The five conserved motifs were marked.

Molecular evolution analysis

To investigate the evolutionary relationships among GGPPS proteins including *GbGGPPS2*, a phylogenetic tree was constructed based on the deduced amino acid sequences of predicted GbGGPPS2 and GGPPS proteins from other plant species (Fig. 4). The GGPPSs of the analyzed plants were divided into two main groups: angiosperms and gymnosperms. Fig. 4 shows that the *GbGGPPS2* belongs to gymnosperm and has the closest relationship with *PaGGPPS6*. As expected, *GbGGPPS2* belonged to gymnospermous GGPPS family.

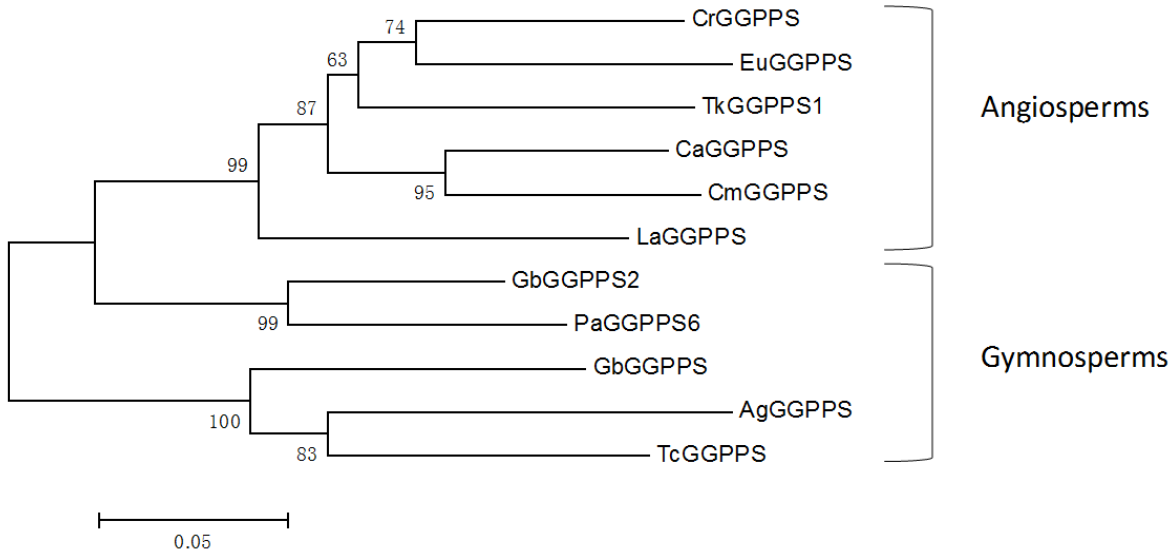

Fig. 4: Phylogenetic tree of the sequences of GbGGPPS2 and other plants GGPPS protein. Bootstrap value are expressed in percentages and placed at the nodes in the tree. The GenBank accession numbers of the GGPPS proteins and translation of their names are shown, CrGGPPS: *Catharanthus roseus* (AE153622.1); EuGGPPS: *Eucommia ulmoides* (AGJ03661.1); TkGGPPS1: *Taraxacum koksaghyz* (AMB19719.1); CaGGPPS: *Corylus avellana* (ABW06960.1); CmGGPPS: *Cucumis melo* (AGN48013.1); LaGGPPS: *Lepidium apetalum* (AKQ24577.1); *GbGGPPS2*: *Ginkgo biloba* (KX756450); PaGGPP6: *Picea abies* (ACA21462.1); GbGGPPS: *Ginkgo biloba* (AAQ72786.1); AgGGPPS: *Abies grandis* (AAL17614.2); TcGGPPS: *Taxus canadensis* (AAD16018.1).

Conclusion

In this study, we have successfully isolated and characterized of the gene *GbGGPPS2* encoding GGPPS involved in the biosynthesis of diterpenes in *G. biloba*. The multiple sequences alignment by using bioinformatics analysis software indicated that *GbGGPPS2* had high identity with other *GGPPS* genes isolated from other plants. The phylogenetic analysis also showed that *GbGGPPS2* might keep a strong conservation during the molecular evolution. The cloning and characterization of *GbGGPPS2* will provide a theoretical basis for enhancement ginkgolide in *G. biloba* through genetic engineering.

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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