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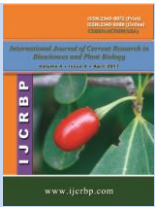

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Cloning and Sequence Analysis of a *Flavonol Synthase* (*FLS2*) Gene from *Ginkgo biloba*

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Abstract

Flavonol Synthase, belongs to 2-oxoglutarate-dependent dioxygenase (2-ODD) superfamily, was involved in flavonol biosynthesis in plants. In this study, we cloned a cDNA encoding flavonol synthase from *Ginkgo biloba* (designated as *GbFLS2*).The cDNA of *GbFLS2* gene is 1251 bp and contains a 1017 bp open reading frame encoding 338 amino acids. The deduced protein of GbFLS2 displays extensive homology to FLS proteins from other plants such as *Pinus radiata*, *Dioscorea alata*, *Camellia sinensis* and *Epimedium sagittatum*. Phylogenetic analysis indicated that the GbFLS2 has a closer relationship with FLS from gymnosperm plants than from other plant species. The results suggest that GbFLS2 is a member of the 2-ODD superfamily, and it is from the same ancestor as FLS proteins of other gymnosperm plants.

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Keywords

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Introduction

Flavonoids is a kind of important secondary metabolites widely distributed in plants. Flavonols is one of the major subclasses of flavonoids and has been identified with the antioxidant, antiproliferative, antiangiogenic, and neuropharmacological properties (Kim et al., 2006; Owens et al., 2008). In addition, flavonols have many important physiological functions in plants, such as auxin transport regulation, flower color modulation, UV protection, and signaling (Böhm et al., 1998; Havsteen, 2002; Toh et al., 2013). Flavonol synthase (FLS), a member of the 2-oxoglutarate-dependent dioxygenase (2-ODD) superfamily, plays a major role in the flavonols biosynthesis. So far, many FLS cDNAs have been isolated from different plant species, such as Tartary buckwheat (Li et al., 2013), *Camellia sinensis* (Lin et al., 2007), *Acacia confusa* (Toh et al., 2013), *Citrus unshiu* (Wellmann et al., 2002), Harosoy (Takahashi et al., 2007), *Zea mays* (Falcone Ferreyra et al., 2010) and *G. biloba* (Xu et al., 2012).

Flavonols are synthesized through the flavonoid pathway. In *G. biloba*, several genes related to the flavonoid pathway, such as *GbCHS1* (Pang et al., 2005), *GbPAL* (Xu et al., 2008), *GbF3H* (Shen et al., 2006), *GbCHS2* (Xu et al., 2007) and *GbCHI* (Cheng et al., 2011) have been cloned and characterized. To figure out biosynthetic pathway of flavonoids in *G. biloba*, each gene must be identified and characterized. In a previous study, we also reported the isolation and characterization of a flavonol synthase gene from *G. biloba*, function analysis indicated that GbFLS is a bifunctional enzyme within the flavonol biosynthetic pathway, recombinant GbFLS1 protein could

catalyze the formation of dihydrokaempferol to kaempferol and the conversion of kaempferol from naringenin (Xu et al., 2012). In the current study, another flavonol synthase gene was isolated from *G. biloba*, the cDNA and protein structure was analyzed via bioinformatics methods.

Materials and methods

Plant material

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

RNA extraction and Isolation of GbFLS2

Total RNA was extracted from leaves tissues of *G. biloba* using the MiniBEST Plant RNA extraction kit (Dalian TaKaRa, China). The cDNA used as template to amplify *GbFLS2* gene was obtained from reverse transcribed of total RNA, which was completed by using the PrimeScript™1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). A pair of specific primers *GbFLS2F* (5′-TGAACACCAACTGATCTGAATCGTA-3′) and *GbFLS2R* (5′-CCAATACACATTTATAAAAGACTTGGTAGG-3′) were designed based on transcriptome sequence, and synthesized by Shanghai Sangon Biotechnology company (In China). PCR reaction was conducted under the following conditions: denaturation at 94°C for 4 min; followed by 32 cycles of 94°C for 30s, 60°C for 30 sand 72°C for 90 s; extension at 72°C for 10 min. The purified PCR product was ligated into pMD18-T cloning vector (Dalian TaKaRa, China) according to the manufacturer's instructions. Then the pMD18-T vector was transformed into *E. coli* TOP10 competent cells. M13 universal primers were used to screen cloned DNA fragments, and the positive clones were sequenced by Shanghai Sangon Biotechnology Company.

Bioinformatics analysis

The obtained nucleotide sequence and deduced amino acid sequence were compared through database search using online bioinformatics tools (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). Software Vector NTI Suite V 11.5 and DNAMAN8 was used to analyze cDNA sequence of *FLS* gene. Physical and chemical parameters of GbFLS2 protein were analysed using ExPASy (http://www.expasy.org/). The secondary structure of GbFLS2 protein was analyzed by SOPMA tool. GbFLS2 and other FLS proteins obtained from GenBank were aligned through the program Align X (Vector NTI Suite V 11.5). Phylogenetic tree of FLS proteins from different plants was constructed with Clustal X 2.0 and MEGA 6.0 software using the neighbor-joining (NJ) method (Tamura et al., 2013).

Results

cDNA cloning of GbFLS2

Leaves of *G. biloba*, which contain abundant secondary metabolites such as flavonols and flavonols, were used to clone *GbFLS2*. PCR was done with specific primers and the cDNA as template. The length of obtained *GbFLS2* cDNA is 1251 bp, and the open reading frame is 1017 bp encoding 338 amino acids. The 5′-untranslated region and 3′-untranslated region of *GbFLS2* cDNA were 66 bp and 168 bp, respectively (Fig. 1).

```
                10        20        30        40        50        60
1       tgaacaccaactgatctgaatcgtaagagttgcccactgactaactgtgtgcatattttg
61      gttgagATGGCAGCCGCAGCAGCGCGAGTGCAGTCAATTGCAAAGAGTGGCATACAAGCC
21            M  A  A  A  A  A  R  V  Q  S  I  A  K  S  G  I  Q  A
121     ATTCCTCCTCAGTTCATTAGACCTCTTCACGAGAGGCCTATTATAACTACGGTTTCCAAT
41       I  P  P  Q  F  I  R  P  L  H  E  R  P  I  I  T  T  V  S  N
181     GGCCCTCAGATGCCCCTAATTGATCTCTCCAATTTGGAGGAACCCCAACTCCGCCAACAA
61       G  P  Q  M  P  L  I  D  L  S  N  L  E  E  P  Q  L  R  Q  Q
241     ACTCTGAGAGATATTGCAGACGCTTGTGAAGAATGGGGTATTTTCCAAGTGCTCAATCAT
81       T  L  R  D  I  A  D  A  C  E  E  W  G  I  F  Q  V  L  N  H
301     GGAGTTTCCGAAGAGCTTATCCAACGCCTCCAGACTGTTGGCAAACAATTCTTCGATCTT
101      G  V  S  E  E  L  I  Q  R  L  Q  T  V  G  K  Q  F  F  D  L
361     CCACAAGAGGAAAAGGAAGCCTATGCCAACAATGCCGGTGCTGGGATTCTGGAGGGCTAC
121      P  Q  E  E  K  E  A  Y  A  N  N  A  G  A  G  I  L  E  G  Y
421     GGTACCAAGTTGGCTCATAACATCGATGGAAAGATGGAGTGGATTGATTACTACTTCCAT
141      G  T  K  L  A  H  N  I  D  G  K  M  E  W  I  D  Y  Y  F  H
481     CTGCTCTGGCCGCCTTCTCACAGAAATTTCAATACATGGCCTAAAAACCCACCTTCTTAC
161      L  L  W  P  P  S  H  R  N  F  N  T  W  P  K  N  P  P  S  Y
541     ATAGAGGTTACTGATGAATACGGCAGACGGCTATTGGAAGTGGTGAATAAGCTGCTGGCT
181      I  E  V  T  D  E  Y  G  R  R  L  L  E  V  V  N  K  L  L  A
601     GTGCTTTCCATCAATCTTGGGCTGCAAGAATCTGGACTGAAAGATGCATTGGGAGGTGAA
201      V  L  S  I  N  L  G  L  Q  E  S  G  L  K  D  A  L  G  G  E
661     AATCTGGAAATGGAAATGAAAATTAATTATTATCCGACATGCCCACAACCAGAGCTTGCT
221      N  L  E  M  E  M  K  I  N  Y  Y  P  T  C  P  Q  P  E  L  A
721     CTCGGCGTCGAATCTCATACAGACATGAGCGCCTTAACAGTTCTTATACCCAATGATGTG
241      L  G  V  E  S  H  T  D  M  S  A  L  T  V  L  I  P  N  D  V
781     CCAGGACTCCAAGTATACAAGGATGGCAACTGGGTTACTGCCGATTATGTTCCCAATGCA
261      P  G  L  Q  V  Y  K  D  G  N  W  V  T  A  D  Y  V  P  N  A
841     TTGGTTATCCATATCGGTGATCAGCTACAGATATTGAGCAACGACAAATACAAGAGCGTT
281      L  V  I  H  I  G  D  Q  L  Q  I  L  S  N  D  K  Y  K  S  V
901     TTACACAGGAGTTTGGTGAGCAAAGATAAGGTGAGGATGTCGTGGCCGGTCTTCTGCACT
301      L  H  R  S  L  V  S  K  D  K  V  R  M  S  W  P  V  F  C  T
961     CCTCCTCCTGATGCTGTCATCGGCCCTTTGAAAGAGCTTATCGATGAGAAACACCCTCCC
321      P  P  P  D  A  V  I  G  P  L  K  E  L  I  D  E  K  H  P  P
1021    TTGTTCAATGCCAAAACATACAAAGAGTTCAAGCATCGCAAAATCAACAAATTGAGCCAA
341      L  F  N  A  K  T  Y  K  E  F  K  H  R  K  I  N  K  L  S  Q
1081    TAGaaaccgctacaaggattggataaggcatttatttcagataggggttgtaaccgggaac
1141    tcttgaatcaaattattgttttaaaattatgtatgctttttataattattaaaaaaatata
1201    accgcggttattgtttttgcgcctaccaagtcttttataaatgtgtattgg
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Fig. 1: cDNA sequence of *GbFLS2* gene and its deduced amino acids. The start codon (ATG) and the stop (TAG) are underlined, noncoding regions are indicated in lowercase.

Characterization of the deduced GbFLS2 protein

The putative GbFLS2 protein contains 338 amino acids. Computer pI/Mw analysis showed the molecular weight and isoelectric point of GbFLS2 protein were 38.1 kDa and 5.72, respectively. The secondary structure of GbFLS protein was predicted by using SOPMA tool. It was found that the percentages of alpha helix, extended strand, random coil and beta turn in the secondary structure were 37.57%, 18.64%, 35.80% and 7.99%, respectively (Fig. 2).

Homology analysis of GbFLS2 protein

Homology analysis was completed with BLASTP (NCBI) and Align X (Vector NTI 11.5). The results showed the deduced GbFLS protein belongs to OG-Fe (II) dioxygenase superfamily, conserved DIOX_N and 2OG-FeII_oxy domains were found in the protein. 2OG-FeII_oxy superfamily contains members of the 2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily. DIOX_N is the highly conserved N-terminal region of proteins with 2-oxoglutarate/Fe (II)-dependent dioxygenase activity. Sequence alignment found that the putative GbFLS2 was 67% identical to FLS1 from *G. biloba* (ACY00393), 61% identical to FLS from *Pinus radiata* (AGY80773), 57% identical to FLS from *Dioscorea alata* (AIY60790), and 56% identical to FLS from *Camellia sinensis* (ABM88786), *Epimedium sagittatum* (ABY63659), *Theobroma cacao* (EOY09743), and *Tricyrtis sp. Shinonome* (BAU20368) (Fig. 3). The homologous sequence of FLS among different species indicated the FLS proteins might keep a conservative during the molecular evolution, and GbFLS2 was a member of the OG-Fe (II) dioxygenase superfamily.

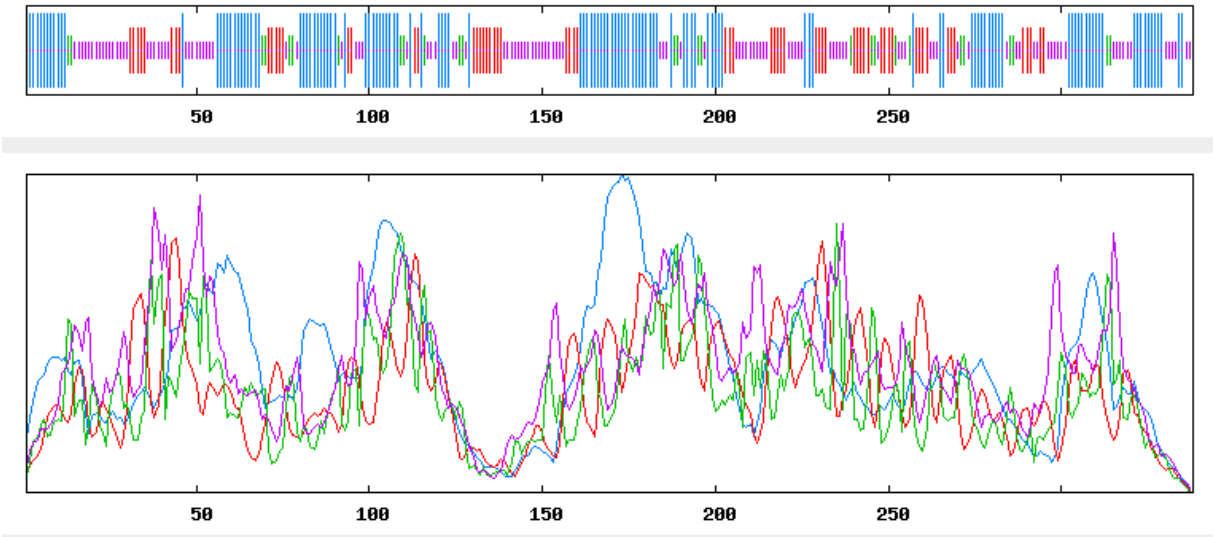

Fig. 2: The predicted secondary structures of GbFLS2 protein.

Phylogenetic analysis of FLS proteins

In order to analyze evolutionary relationships of FLS proteins among the various species. Using Clustal X 2.0 and MEGA 6.0 software, the phylogenetic tree was constructed by the neighbor-joining method. As shown in Fig. 4, FLS proteins were clustered into two group gymnosperm and angiosperm, and the putative GbFLS2 protein together with GbFLS1 and PrFLS (*Pinus radiata*) was clustered into the branch of gymnosperm plants. FLS proteins from closely related species shared the same subclades. FLS proteins from Rosaceae such as *Rosa rugosa*, *Fragaria×ananassa*, *Malus domestica* and *Prunus persica* were grouped into the same cluster, while FLS proteins of *Arabidopsis thaliana* and *Brassica rapa* that from Cruciferae were grouped into another cluster. The phylogenetic tree indicated that GbFLS shared a common evolutionary origin with the gymnosperm species FLS proteins.

Discussion

Flavonol Synthase, as a key enzyme involved in the flavonol biosynthetic pathway, has been studied in many plants (Li et al., 2013; Toh et al., 2013; Takahashi et al., 2007; Falcone Ferreyra et al., 2010; Xu et al., 2012; Mahajan et al., 2011). In plants, flavonol synthase could catalyse flavonoids synthesis.

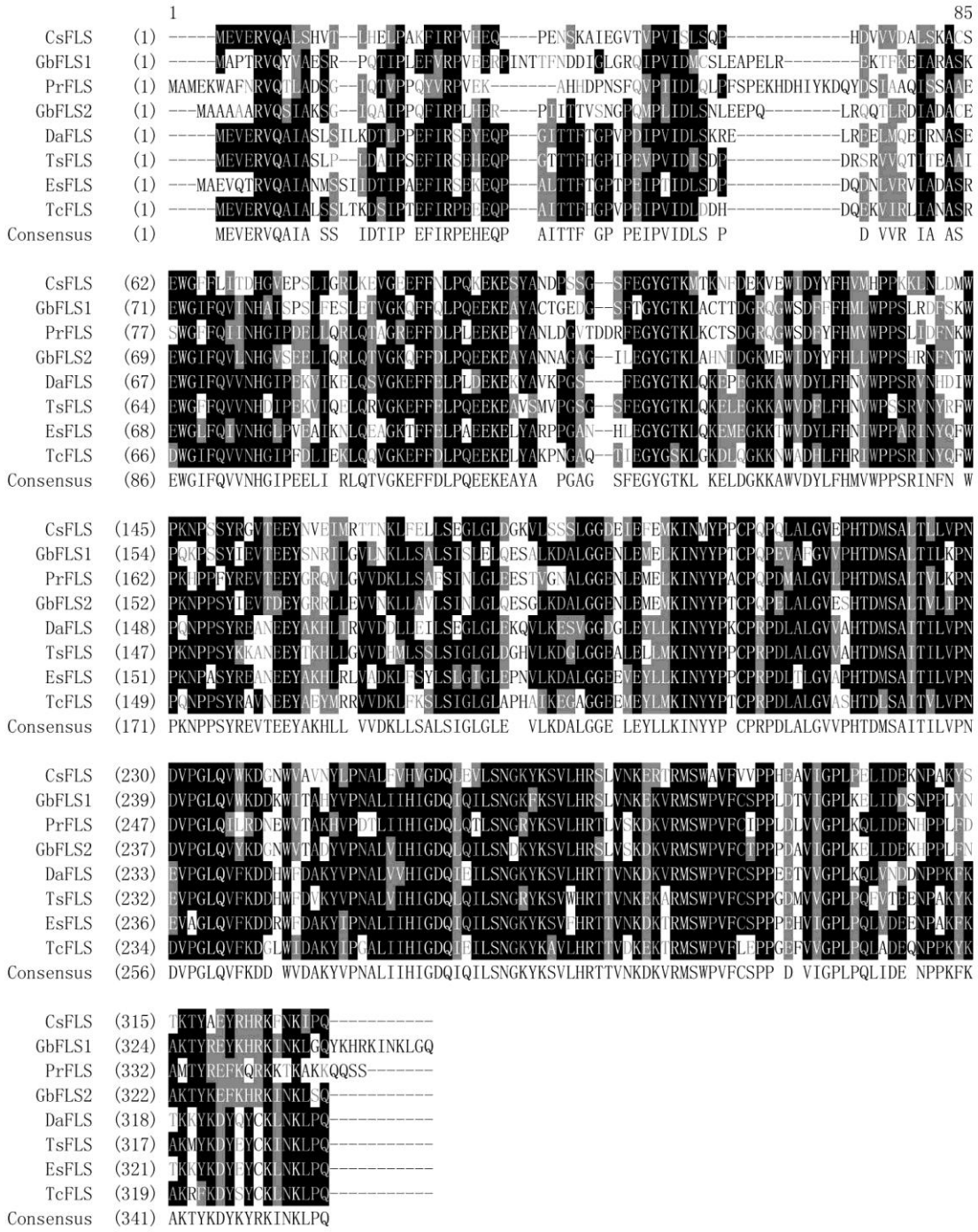

Fig. 3: Multiple sequence alignment of the deduced FLS with other proteins from other plants. The completely identical amino acids are indicated with black background. The conserved amino acids are indicated with grey background. Non-similar amino acids are indicated with white background.

In *Acacia confusa*, AcFLS could enzymatically transform dihydromyricetin, dihydroquercetin, and naringenin into the flavonols myricetin, quercetin, and kaempferol, respectively (Toh et al., 2013). In maize, ZmFLS1 was also able to convert DHK and DHQ to produce the flavonols K and Q, though it fails to convert Nar to DHK (Falcone Ferreyra et al., 2010). Flavonol synthase also could play an important role in the physiological activities of plants. In tartary buckwheat, different FtFLS isoforms of buckwheat have different functions in the response to environmental stress; transcription of FtFLS1 was inhibited by the exogenous

application of ABA, SA and NaCl, while FtFLS2 was not affected by ABA but up-regulated by SA and NaCl (Li et al., 2013). In *A. thaliana*, 6 FLS isoforms were identified from *A. thaliana* that exhibited tissue or cell type-specific promoter activities. However, only AtFLS1 was shown to play a primary role in flavonol biosynthesis in *A. thaliana* (Owens et al., 2008). In *G. biloba*, GbFLS1 is a bifunctional enzyme within the flavonol biosynthetic pathway, but function of other FLS isoforms is still unknown (Xu et al., 2012).

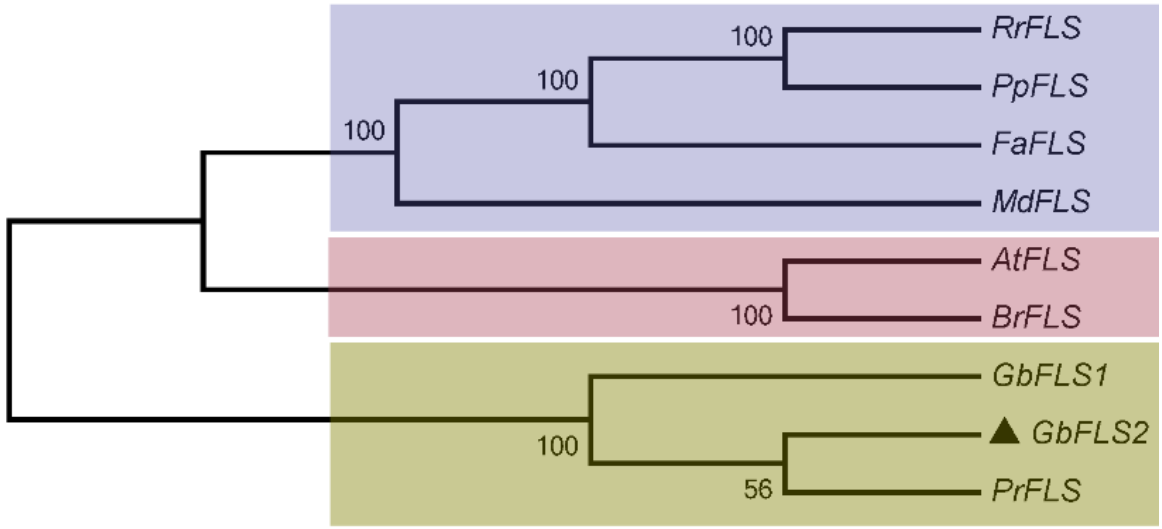

Fig. 4: Phylogenetic tree of FLS proteins from different species. The numbers at each node represent the bootstrap values (with 1,000 replicates). GenBank accession number are as follows: PrFLS, *Pinus radiata*, AGY80773; GbFLS2, *Ginkgo biloba*,ACY0039; AtFLS, *Arabidopsis thaliana*, AAB41504;BrFLS, *Brassica rapa*, XP_009122482;RrFLS, *Rosa rugosa*, AIS22436; FaFLS, *Fragaria×ananassa*, AAZ78661; MdFLS, *Malus domestica*, AAD26261; PpFLS, *Prunus persica*, AJO70134.

In this study, one *FLS* gene, *GbFLS2*, was isolated from *G. biloba*. The ORF of *GbFLS2* was 1017 bp, encoding 338 amino acids. Homology analysis found that the deduced GbFLS2 belong to 2-ODD superfamily, and was high identity with FLS proteins from other plants. Phylogenetic analysis suggested that the flavonol Synthase of gymnosperm plants may be from the same ancestor. Above all, the cDNA sequence and protein structure of *GbFLS2* was similar to other *FLS* genes that function had been identified. Therefore, we hypothesized that the *GbFLS2* maybe also involved in regulation of flavonol biosynthesis, which need to be further studied. The isolation and structural analysis of *GbFLS2* gene provided a foundation for further studying the function of FLS proteins in *G. biloba.*

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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