OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2018.

Research Article
Cloning and Expression Analysis of a Flowering Gene *FRIGIDA* (*GbFRI*) from *Ginkgo biloba*

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Abstract

Background and Objective: *Ginkgo biloba* is a precious medicinal plant and has a long juvenile phase and spends 15-20 years in the vegetative phase before turning to reproductive phases, which makes breeding and cultivation of *Ginkgo* especially challenging. The *FRI* gene can regulate the *FLC* gene which inhibits flowering and further causes the late flowering of *G. biloba*. Therefore, the cloning and analysis of *FRI* gene can regulate the flowering time of *G. biloba*. **Materials and Methods:** The *GbFRI* gene and the protein sequence were analyzed using the online website of National Center for Biotechnology Information (NCBI), ProtParam and bioinformatic software of Clustal X2.0, Vector NTI 11.5 and MEGA6. The expression of *GbFRI* gene in different tissues of *G. biloba* was studied by quantitative RT-PCR (qRT-PCR). Data were analyzed with one-way ANOVA using SPSS11.0 for Windows. **Results:** The full length cDNA of *GbFRI* gene was 1702 bp (GenBank accession no. KY662058) and the open reading frame (ORF) covered 1602 bp, which encoded a 534 amino-acid protein. The predicted protein showed that a *FRI* superfamily and contain coiled-coil domains in two positions (between amino acids 55-100 and 405-450, respectively). The expression analysis results displayed that the highest *GbFRI* expression was in the male flowers. The *GbFRI* expression was higher in female flowers, stems than in the roots and fruits. The lowest relative expression of *GbFRI* was in the leaves. **Conclusion:** The *GbFRI* gene was isolated and characterized, laying a foundation for further study of vernalization pathway in *G. biloba*.

Key words: *Ginkgo biloba*, juvenile, *FRI*, *Flowering Locus C*., *GbFRI* gene, expression analysis, quantitative RT-PCR

Received: Accepted: Published:

Citation: Jingjing Liao, Xiaomeng Liu, Xian Zhou, Zexiong Chen, Junpin Tan, Jiabao Ye, Weiwei Zhang and Feng Xu, 2018. Cloning and expression analysis of a flowering gene *FRIGIDA* (*GbFRI*) from *Ginkgo biloba*. Biotechnology, CC: CC-CC.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ginkgo biloba is an ancient relict plant in belongs to Ginkgoaceae and Ginkgo. It is a precious tree species for ornamental, wood and medicinal purposes. The extract of *G. biloba* leaves is very complex. The most effective ingredients are flavonoids, terpene lactone compounds and polyisopreno[1]. These compounds can improve blood circulation, treat cardiovascular diseases and protect liver function[2]. However, a long juvenile phase (15-20 years) of *G. biloba* brings serious obstacles to the breeding of *G. biloba* varieties[3]. Therefore, the regulation of flowering is important from an agronomic perspective. However, the molecular mechanisms that regulate flowering are still poorly understood in *G. biloba*.

Plants have evolved complex mechanisms to control the initiation of flowering in response to environmental cues or endogenous signals[4,5]. Flowering time in Arabidopsis depended on resetting and regulation of *FLC* expression during reproductive development[6,7]. In vernalization pathway, *FRI* activated the MADS-box transcription factor, *Flowering Locus C. (FLC)*, a major repressor of this switch regulating flowering time[4,7]. The *FRI* promoted high levels of *FLC* expression and thus inhibited flowering, the action of an active *FLC* allele depended on an active *FRI* allele[8]. In Arabidopsis, the expression level of *FLC* gene determined whether Arabidopsis belonged to annual or biennial or perennial plants[9,10]. While the *FRI* gene has a regulatory effect on the gene of *FLC*, the inactive *FRI* led to a low expression of the *FLC*, which caused Arabidopsis to exhibit an early flowering phenomenon. Winter-annual accessions have functional *FRI*, promoted *FLC* expression and delayed flowering until *FLC* was silenced by a prolonged period of cold.

At present, there have been reported on isolated of the *FRI* gene from some *Brassica* Species[11] and *Brassica oleracea*[12]. The study found that the FRI-Ler allele induced high expression levels of *FLC* gene, while the high expression of *FLC* delayed the flowering time of *A. thaliana*, making *A. thaliana* susceptible to vernalization and requiring low temperature induction to flower[13]. A molecular marker for the vernalization gene *FRI* was established and a central domain conserved for FRI and related proteins was discovered[11,14]. Proteasome-mediated degradation of *FRI* modulated flowering time in *Arabidopsis* during vernalization[15]. Research showed that the interactions of strong and weak alleles of the genes *FRI* and *FLC.* in many cases determined the variations in time to flower.

So far, the cloning and expression of the *FRI* gene in *G. biloba* have not been studied. In order to study the key genes regulating the flowering time of *G. biloba*, the *FRI* gene was cloned and the expression level of *GbFRI* gene in different tissues of *G. biloba* was studied by qRT-PCR. The purpose was to find out the relationship between *FRI* gene and long juvenile phase of *G. biloba*. The study of the *GbFRI* gene will help elucidate the molecular mechanisms of *G. biloba* flowering time.

MATERIALS AND METHODS

Materials: The materials were planted in the Ginkgo Science and Technology Garden, Yangtze University, pickted from 31 year *G. biloba* cultivar "Jiafoshou". Different tissues of ginkgo at different developmental stages, including roots, stems, leaves, male flowers, female flowers, fruits were collected before the end of May 2017. All samples were quickly frozen in liquid nitrogen and kept at -80°C until to use. Gel recovery kit (Agarose Gel DNA purification Kit Ver.4.0) RNA extraction kit (MiniBEST Plant RNA Extraction kit) reverse transcription kit (PrimeScript™ 1st Strand cDNA Synthesis Kit) and PCR reagents for ampicillin (AMP), pMD19-T cloning vector and *Escherichia coli* competent cell DH5 were purchased from TaKaRa, Dalian Bao Biotechnology Company. Shanghai Sangon Biological Engineering Company performed sequence and synthesis of the primers.

Cloning of the *GbFRI* gene: Total RNA was extracted using MiniBEST Plant RNA Extraction kit from female flowers of *G. biloba*. The specific primers *GbFRI*-up and *GbFRI*-down (Table 1) for amplification were designed according to the transcriptome sequencing data of *G. biloba*. The PCR system was 25 μL. The amplification program was 94°C for 3 min; 32 cycles of 94°C for 30 sec, 56.5°C for 30 sec, 72°C for 1 min; a final extension at 72°C for 10 min. When the PCR product was successfully tested with 1% agarose gel electrophoresis, the target fragment was recovered according to the instructions of the gel recovery kit. After that, the target gene fragment was ligated into the pMD19-T vector and transformed into *Escherichia coli* strain DH5α. A single colony was picked and cultured. Screened positive clones were sent to Shanghai Sangon Biotech for sequencing.

Table 1: Primer sequences in this study

Primers	Sequence (5'-3')
GbFRI-up	TCTCATTCTCAGCTCTCGGATAC
GbFRI-down	CTCGGAACCTAGCTTTCAGAAT
GbFRI-FP	AGAAGAAACCACCTCCAACTATCG
GbFRI-RP	CTTCCAGAGGCAATCCTGATAGAG
GbGAPDH-FP	TTGGTCTCCCGTGCTAATGG
GbGAPDH-RP	CGAAGCGTCATCCTAAGACAACA

Bioinformatics and molecular evolution analysis: The sequences of *GbFRI* gene were processed and translated into protein using DNAMAN V6. Open reading frame (ORF) can be found by Vector NTI 11.5. Using the online tool ExPASy (http: //web.expasy.org) predicted the molecular weight and theoretical isoelectric point of the protein. According to the deduced amino acid sequence, the highest similarity sequences were obtained by using the BLAST tool (http://www ncbi nlm nih gov /BLAST/) for homologous alignments of the nucleotide. The phylogenetic tree was constructed by CLUSTAL X2.0 and MEGA6.

Quantitative real-time PCR analysis: Total RNA was extracted from roots, stems, leaves, male flowers, female flowers, fruits of *G. biloba*. First-strand cDNA was synthesized by using PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). The primers *GbFRI*-FP and *GbFRI*-RP (Table 1) were designed for quantitative real-time PCR (qRT-PCR) amplification. *GAPDH* was used as the quantified internal reference gene. The upstream and downstream primers of *GAPDH* were *GbGAPDH*-FP and *GbGAPDH*-RP, respectively (Table 1). Referring to TaKaRa Company's AceQ® qPCR SYBR® green master mix (Without ROX) kit (Vazyme), real-time fluorescence was performed on Bio-Rad CFX. PCR programs were as follows: 95°C for 5 min, 40 cycles of 95°C for 10 sec, 60°C for 30 sec, 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. Three repeated experiments at each sample. The relative expression fold of each sample was calculated by its Ct value normalized to the Ct-value of reference gene using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen[16].

Statistical analysis: Data were analyzed with one-way ANOVA using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). The means were compared with Duncan's multiple range tests. p-value of<0.05 was considered to be statistically significant.

RESULTS

Cloning and sequence analysis of *GbFRI*: The specific primers *GbFRI*-up and *GbFRI*-down designed by transcriptome sequencing data of *G. biloba*, amplified by PCR. The sequence analysis results showed that the full-length cDNA of *FRI* gene from *G. biloba* was 1,704 bp in length (Fig. 1). It contained a 1,604 bp ORF, encoding a putative protein of 534 amino acids (Fig. 1), namely *GbFRI-1* (GenBank accession no. KY662058).

Analysis of GbFRI protein: Online analysis of ExPASy-ProtParam showed that predicted theoretical molecular weight and isoelectric point of GbFRI protein were 57.70 and 9.1 KDa, respectively. The analysis of phosphorylation sites using NetPhos 3.0 server indicated that many phosphorylation sites of serine and a few phosphorylation sites of threonine and tyrosine existed. The nucleotide sequence of the *GbFRI* gene exhibited highly homologous to the *FRI* gene sequences of other plants (Table 2) by BLAST-protein on NCBI, their similarities ranged from 42-60%.

The conserved functional domain of the GbFRI protein was analyzed online using BLAST (https://blast.ncbi. nlm.nih.gov/), which predicted that it had a conserved *FRI* superfamily domain. The GbFRI protein sequence was compared with FRI protein sequences of other plants (Fig. 2). The GbFRI protein had a highly conserved Frigida superfamily domain, indicating that *GbFRI* was a member of *FRI* gene family and was relatively conservative in evolution.

Molecular evolution analysis of *GbFRI*: To clarify the evolutionary relationship between GbFRI protein and FRI from other plants, the phylogenetic tree of FRI was constructed by CLUSTAL X2 and MEGA6 software using NJ method. As shown in the Fig. 3, the phylogenetic tree of FRI was divided into two major branches: Gymnosperms and Angiosperms. *G. biloba* was more closely related to gymnosperms than angiosperms. It was highly related to the Gymnosperms and *G. biloba*, which embodied the evolution of the *GbFRI* gene in the Gymnosperm. The *FRI* gene existed in Pinaceae, Gramineae, Arecaceae, Solanaceae and Leguminosae, which reflected the evolutionary diversity of the gene, which was consistent with the taxonomy of plant morphology.

Expression patterns analysis of GbFRI: The samples of *G. biloba* roots, stems, leaves, male flowers, female flowers,

Table 2: Protein sequence of *GbFRI* similarity to the *FRI's* of other plant species

Species	Genbank accession no.	Identity (%)	Query cover (%)	E-value
Picea sitchensis	ABR17868.1	60	98	0
Marchantia polymorpha	OAE19925.1	53	94	1.00E-142
Physcomitrella patens	XP_001752883.1	47	71	1.00E-123
Selaginella moellendorffii	ABK252091	45	100	1.00E-154
Nelumbo nucifera	XP_010270166.1	44	97	2.00E-122
Elaeis guineensis	XP_010930297.1	42	89	1.00E-123
Musa acuminata subsp.	XP_009392592.1	42	89	1.00E-199
Juglans regia	XP_018817942.1	42	100	2.00E-135

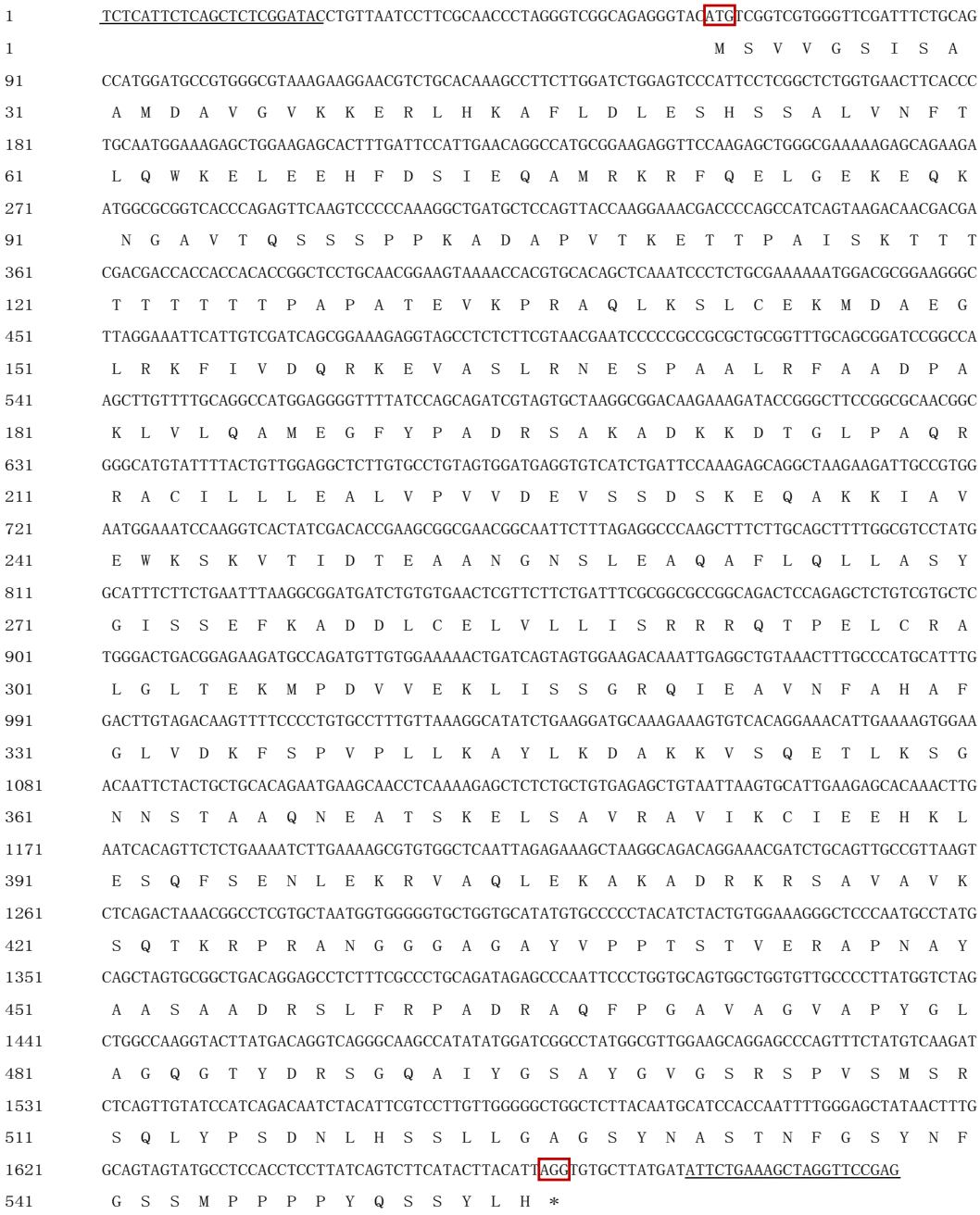

Fig. 1: Nucleotide sequence and deduced amino acid sequence of *GbFRI-1*

Start codon and termination codon are indicated by the box, the primers were indicated by the underline

fruits were selected for qRT-PCR experiments to analyze further the specific expression of *GbFRI* in different tissues of *G. biloba* (Fig. 4). The qRT-PCR results showed that *GbFRI* was expressed in roots, stems, leaves, male flowers, female flowers and fruits. The highest *GbFRI* expression was in the male flowers. Furthermore, the *GbFRI* expression was higher in female flowers and stems than in the roots and fruits. The lowest relative expression of *GbFRI* was in the leaves.

DISCUSSION

In Brassica, genomes A and C of *FRI* was represented by two loci: *FRI.a* and *FRI.b*[17-19]. Up to now, *FRI* in Brassica genome B was rarely investigated[15]. In Brassica genomes A and C, all FRIGIDA protein sequences contained the conserved central region corresponding to the *Frigida* domain characteristic for the superfamily of proteins FRIGIDA and

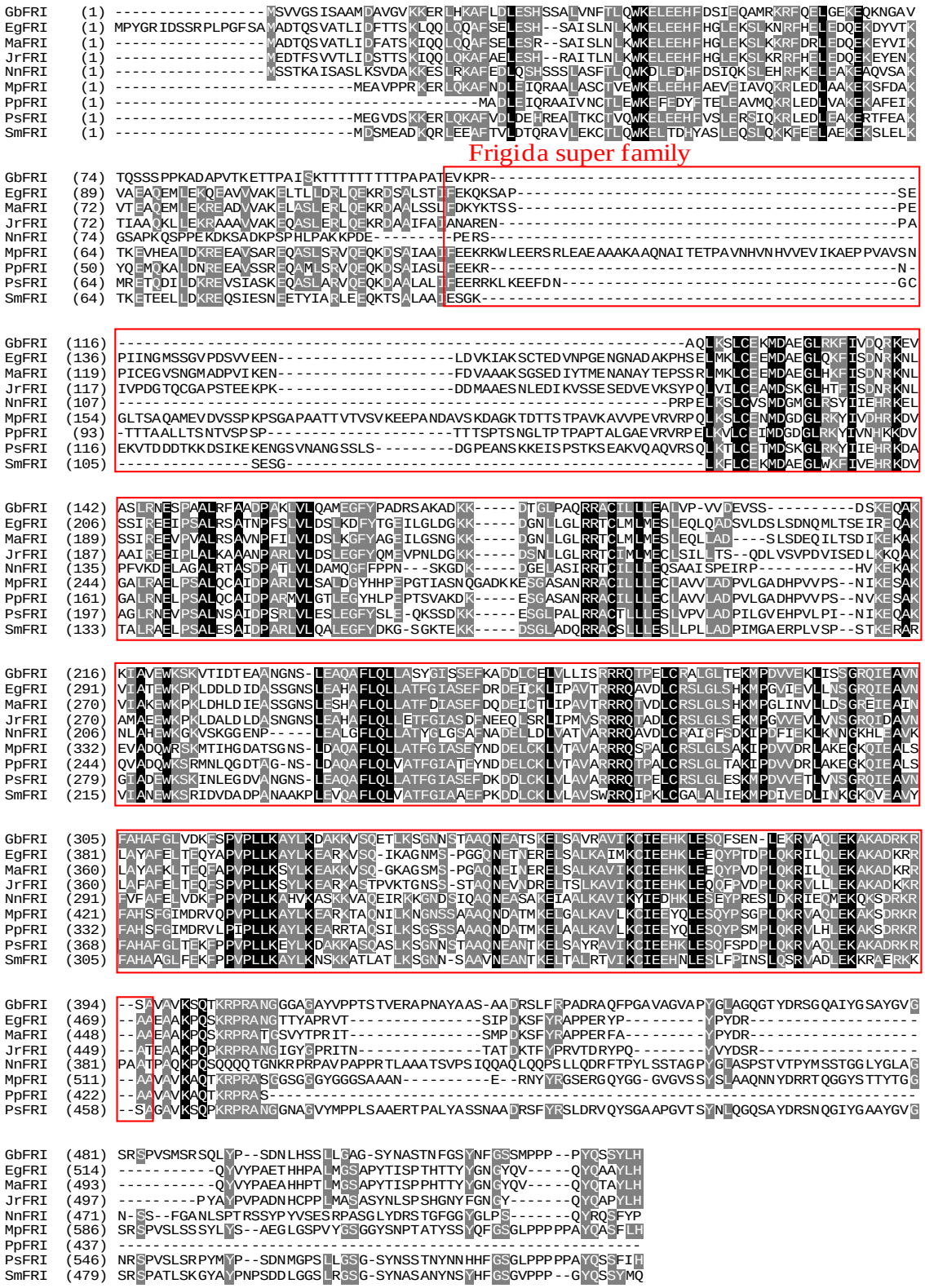

Fig. 2: Similarity analysis of *GbFRI*-coding protein and other known FRI proteins

GbFRI: *Ginkgo biloba* (KY662058), *EgFRI*: *Elaeis guineensis* (XP_010930297.1), *MaFRI*: *Musa acuminate* (XP_009392592.1), *JrFRI*: *Juglns regia* (XP_018817942.1), *NnFRI*: *Nelumbo nucifera* (XP_010270166.1), *MpFRI*: *Marchantia polymorpha* (OAE19925.1), *PpFRI*: *Physcomitrella patens* (XP_001752883.1), *PsFRI*: *Picea sitchensis* (ABR17868.1), *SmFRI*: *Selaginella moellendorffii* (ABK252091)

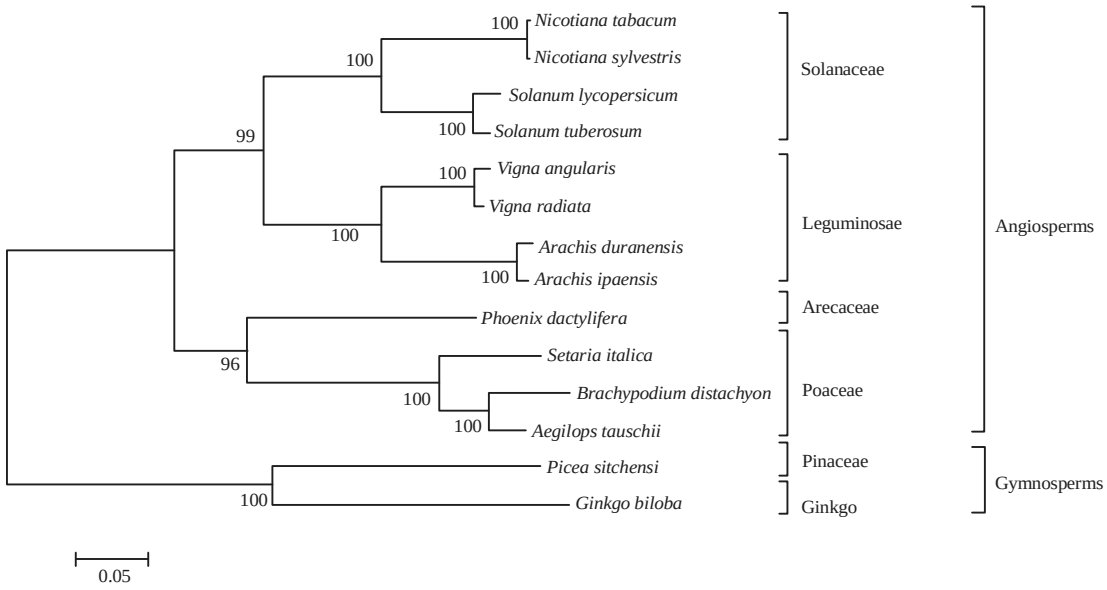

Fig. 3: Phylogenetic tree of genes in plant *FRI* gene family

Expression patterns analysis of *GbFRI*

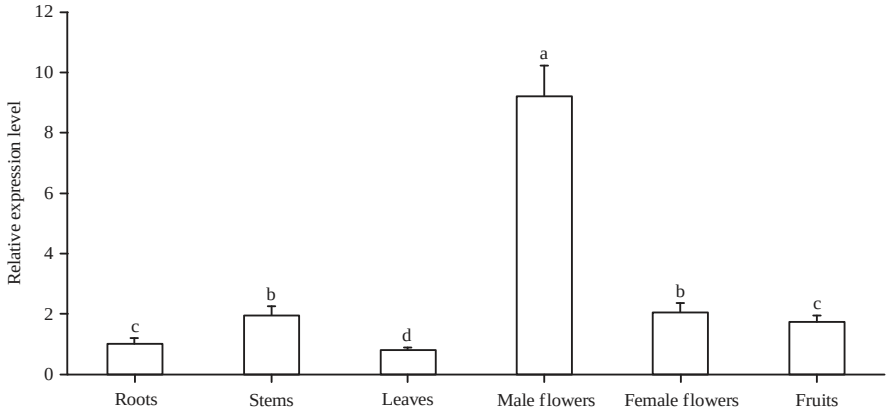

Fig. 4: Expression analysis of *GbFRI* in different tissues of *G. biloba* with qRT-PCR

Data are Means±SE for n = 3 biological replicates. Means with the different letters are significantly different at p<0.05 by Duncan' s multiple range tests

FRIGIDALIKE[19] 1. Comparing the *FRIGIDA* sequences, the characteristic repetitive signatures were distinguished similar to the MEEKARSLS vernaliza repeat in *FRI.a* from *A. thaliana*. Genomes A had three MEEARSIS repeats and genomes C had two MEEARSIS repeats. Furthermore, MEGEARSIS and MQGEARSIS repeats in *FRI.b* from genomes A and C, respectively[20]. These repeats overlap form the *Frigida* domain and the coiled coil domain at the C-terminus of FRIGIDA protein[14,17,18]. In this study, the protein sequences of *G. biloba* had no repetitive signatures as them but found two α-helical coiled coil, which implied that *GbFRI* had a highly conserved FRIGIDA superfamily domain, this finding was consistent with the experimental result obtained in the present study. The result tentatively put forward that nucleotide and amino acid sequences of plants had mutated during the evolution, different plants might had different classification of the *FRIGIDA* gene. Each locus manifested genome specific polymorphisms, the *FRIGIDA* gene also possibly mutated in the evolution process and performing different functions. Whether the predicted coiled coils in the FRI protein were important for this function remains to be tested.

Consistent with previous studies that the expression of *FRI* gene existed in different tissues[21]. The qRT-PCR results indicated that *GbFRI* expression was higher in female flowers,

stems than in the roots and fruits. The lowest relative expression of *GbFRI* was in the leaves. *FRI* promoted high expression levels of *FLC*, but the expression analysis of gene *FLC* in different organs and development periods have greater fluctuation[22]. The results may be due to the influence of *FRI* on *FLC* was regulated by season, temperature, different growth cycle or other factors.

CONCLUSION

In this paper, the *FRI* gene of *G. biloba* was coloned at the first time and found that a highly conserved *FRIGIDA* superfamily domain was contained in *GbFRI* protein, FRI gene was existed in different tissues in *G. biloba*. *GbFRI* plays an important role in flowering, this study will help researcher explore the long juvenile phase of *G. biloba* and develop Ginkgo industry in pharmacology, food, health care and other fields.

SIGNIFICANCE STATEMENTS

This study discovers the flowering regulation mechanism of *GbFRI* gene that can be beneficial for the development of ginkgo industry. The *GbFRI* is a key gene regulating the flowering time of *G. biloba*, which is useful for shortening the long juvenile phase of *G. biloba* through *FRI* gene silencing. The study will help the researcher to uncover the critical areas of *FRIGIDA* genes from *G. biloba* that many researchers were not able to explore. At the same time, it lays a theoretical foundation for shortening the infancy of woody plants by means of genetic engineering.

ACKNOWLEDGMENT

This work was supported by National Natural Science Foundation of China (No. 31670608).

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