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Cloning and Sequence Analysis of *GBM5* Gene from *Ginkgo biloba*

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Abstract AGAMOUS (AG), a member of *MADS-box* gene family, is a key gene related to regulation of flower development in plants. The *AG* homologous gene *GBM5* (Accession No.: AY114304.1) was obtained from the transcriptome of *Ginkgo biloba*. Primers were designed according to the obtained *GBM5* gene sequence, and the full-length cDNA sequence of *GBM5* gene with 732 bp in size and 243 amino acids in coding product can be synthetized by designing primers. The comparative analysis of evolutionary tree and amino acid sequence showed that ginkgo *GBM5* was closely related to cycads *AG* and black spruce *AG*. qPCR analysis showed that the expression of *GBM5* in different tissues of *Ginkgo biloba* was different at different periods, the expression levels of different tissues are as follows: Female flower>Male flower>Primordia>Foliage; Its expression is high in the undifferentiated stage of flower bud, the expression patterns were: April leaf>May leaf>September leaf, April primordia>May primordia>September primordia.

Keywords *Ginkgo biloba*; AG gene; Flowering regulation; Expression analysis

MADS-box gene family is a group of transcription factors that regulate plant growth and development, especially in flower meristem development, control flowering time and flower organ development (Yao et al., 2018). The N-terminal of the proteins encoded by them all contain *MADS-box* conserved domain composed of 50~60 amino acids (Liu et al., 2010).

Becker and Theissen (2003) proved that *MADS-box* gene formed two lineages of Type I and Type II in evolution. *MADS-box* genes of Type I mainly include *SRF-like/ARG80* genes in animals and fungi and *MADS-box* genes in plants without K domain. Type II *MADS-box* genes were dominated by *MEF2-like* genes from animals and fungi and *MIKC type* genes from plants (Kaufmann et al., 2005). The *MADS-box* gene in higher plants consists of four unique domains: The MADS (M) box, the Intervening (I) region, the Keratin (K) box and the C-terminal (C) terminal (Ma et al., 1991). Among them, region I is a non-conserved region composed of 31-35 amino acid residues, which plays an auxiliary role in the combination of dimer and DNA to form a complex. K region is a unique sequence of *MADS-box* transcription factors in plants, with about 70 amino acid residues (Zhang et al., 2013). C-terminal is a non-conserved region composed of about 30 amino acids, located downstream of K region, which can activate the transcription process (Wang et al., 2015).

AGAMOUS (*AG*) is a gene in *MADS-box* gene family, which is one of the earliest genes found to be closely related to flower development in plants (Gong et al., 2009). Since 1990, Ma et al. (1991) cloned the flower homologous gene *AG* in *Arabidopsis thaliana* for the first time, *AG* homologous genes in many plants have been cloned successively. *AG* homologous genes can be identified as a characteristic structure of Class C genes in the two conserved regions of AG I and AG II at the end of C (Xu and Wang, 2011). Although there have been many studies on *AG* gene, the study of its upstream and downstream genes and the molecular mechanism of action is not very comprehensive.

In recent years, the molecular biology research on the development mechanism of higher plants has been gradually developed, and the flowering process, as the most significant transformation stage of plants, has always been the focus of research. Since *Ginkgo biloba*'s infancy lasts for 20 years, the flowering mechanism of *Ginkgo biloba* is the focus of research. The flowering pathways of higher plants have made great progress since the 1980s, and at least five flowering regulatory pathways have been proposed in Arabidopsis. There are photoperiodic promotion pathway, vernalization promotion pathway and autonomous promotion approach, GA promotion pathway and flowering repression pathway (Zhang, 2002).

CO gene is an important flowering time regulation gene in the photoperiodic pathway of plants. Studies have shown that regulating the expression of *FT* can regulate flowering of plants (Jager et al., 2003). *GbCO* gene was first cloned from *Ginkgo biloba* in 2010, encoding a protein composed of 373 amino acids. It contains two B-box zinc finger molds (Zhang, 2002). As a ubiquitous transcription factor, *FLC* gene in MADS-box gene family plays an important role in the vernalization process of plants (Hong and Cao, 2002). The expression level of MADS-box protein is closely related to the flowering time of plants. At the same time, *FLC* gene is also regulated by related genes in the autonomic flowering pathway, and acts as rheostat in both flowering pathways (Michaels et al., 1999). *EMF* gene, the main suppressor of flowering repression pathway, negatively regulates with *AP1* and *AG* to inhibit flowering, while *CO* and *GI* negatively regulate *EMF* gene (Yang et al., 1995). In addition, *AG* gene and *APETALA2* (*AP2*) gene inhibit *APETSLA1* (*AP1*) gene expression in the third and fourth round of flower organs (Jack et al., 1997). Several flowering regulation pathways are interrelated and jointly regulate the flowering of higher plants.

Ginkgo biloba L. is the oldest relict plant in the surviving gymnosperms and is recognized as a "living fossil" (Wen et al., 2010). *Ginkgo biloba* in China accounts for more than 90% of the total resources in the world, and is a multi-purpose and treasured species unique to China, with unique morphological characteristics, systematic evolution and taxonomic status. Ginkgo is loved by people for its unique ecological value, economic value, medicinal value, ornamental value and scientific research value. In the 1960s, the important medicinal value of *Ginkgo biloba* for cardiovascular and cerebrovascular diseases was discovered. In 1965, German doctor Willmar Schwabe introduced *Ginkgo biloba* extract (EGB761) into clinical medicine (van Beek, 2000), which subsequently led to the research and development of the medicinal value of *Ginkgo biloba* in academic circles (Cao et al., 2007). The flowering of *Ginkgo biloba* seedlings after 15~20 years seriously affected the selection and breeding of *Ginkgo biloba* superior varieties. Therefore, this study intends to further study the role of *AG MADS-Box* transcription factors in the regulation of ginkgo flowering through sequence analysis and expression analysis of *GBM5* gene, so as to analyze the molecular regulation mechanism of ginkgo flowering, to promote the flowering of *Ginkgo biloba* from the molecular level and accelerate the breeding of superior varieties.

1 Results and Analysis

1.1 Cloning of *GBM5* gene

Using cDNA of *Ginkgo biloba* stem as template and specific primers *Gb16301*-F and *Gb16301*-R as primers, DNA fragment with length of 732 bp was obtained by PCR amplification (Figure 1). Sequence comparison was conducted on NCBI. It was found that it had a high ratio of comparison with *AGAMOUS-like* gene *GBM5* (Registration number: AY114304.1) in MADS-box family of *Ginkgo biloba*, so the cloned sequence was temporarily named *GBM5*.

1.2 Sequence analysis and tertiary structure prediction of GBM5 protein

Basic physical and chemical parameters such as *GBM5* isoelectric point and gene protein size of *Ginkgo biloba* were analyzed by ProtParam tool in ExPASy program (Figure 2). The predicted results showed that the isoelectric point (pI) of GBM5 protein was 8.55, the molecular weight was about 27 kD, the molecular formula was $C_{1188}H_{1923}N_{357}O_{366}S_{12}$, and the instability coefficient was 56.48, suggesting that GBM5 protein was an unstable protein. The mean value of overall hydrophilicity is -0.519, which belongs to hydrophilic protein. The secondary and tertiary structures of GBM5 protein were predicted online (Figure 3), and the proportions of α-helix(H), β-sheet(E), β-turn (T) and random coil (C) were 70.78%, 7.82%, 3.70% and 17.70%, respectively.

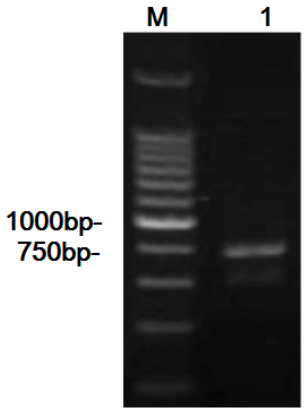

Figure 1 PCR product of *GBM5*

Note: M: MD116 DNA Maker; 1: Target gene

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                10        20        30        40        50        60
1     ATGGACGAGCTGTACAAGTCCGGAGCTGCGGCCGCTGCCGCTGCGGCAGCGGCCGAATTC
1      M  D  E  L  Y  K  S  G  A  A  A  A  A  A  A  A  A  E  F

               70        80        90       100       110       120
61    CCCGGGATGGGCCGTGGGAAGATTGAGATAAAGAGGATTGAGAACACTACAAACCGACAG
21     P  R  M  G  R  G  K  I  E  I  K  R  I  E  N  T  T  N  R  Q

              130       140       150       160       170       180
121   GTCACTTTCTGCAAGCGTCGAAATGGTCTGTTGAAGAAAGCGTATGAATTATCCGTGCTT
41     V  T  F  C  K  R  R  N  G  L  L  K  K  A  Y  E  L  S  V  L

              190       200       210       220       230       240
181   TGTGATGCGGAAGTGGCTCTCATCGTCTTCTCCAGTCGCGGGAGACTCTATGAGTTTGCT
61     C  D  A  E  V  A  L  I  V  F  S  S  R  G  R  L  Y  E  F  A

              250       260       270       280       290       300
241   AATAACAGTGTGAAGAGAACAATCGACAGATACAAGAAGACTTGCGCTGACAACTCTCAG
81     N  N  S  V  K  R  T  I  D  R  Y  K  K  T  C  A  D  N  S  Q

              310       320       330       340       350       360
301   GGTGGAGCCATTTCAGAGTGTAATTCTCAGTACTGGCAACAGGAGGCAGGAAAACTGAGA
101    G  G  A  I  S  E  C  N  S  Q  Y  W  Q  Q  E  A  G  K  L  R

              370       380       390       400       410       420
361   CAGCAAATTGATATTCTGCAAAATGCGAATAGACACTTGATGGGGGACGCGCTTACATCT
121    Q  Q  I  D  I  L  Q  N  A  N  R  H  L  M  G  D  A  L  T  S

              430       440       450       460       470       480
421   TTAAGTGTAAAGGAGCTTAAGCAGCTAGAAATTCGACTTGAGAGGGGCATTAGCAGGGTT
141    L  S  V  K  E  L  K  Q  L  E  I  R  L  E  R  G  I  S  R  V

              490       500       510       520       530       540
481   CGATCAAAGAAGAATGAAATGTTGCTTGAGGAGATAGAGATTATGCAAAGAAGGGAACAC
161    R  S  K  K  N  E  M  L  L  E  E  I  E  I  M  Q  R  R  E  H

              550       560       570       580       590       600
541   ATATTACTGGCGGAGAACCAGTTTCTGCGCACTAAGATAGCTGAATGTGAAAGCAGCCAA
181    I  L  L  A  E  N  Q  F  L  R  T  K  I  A  E  C  E  S  S  Q

              610       620       630       640       650       660
601   AATGCAAATATGCTGCCGGGTCCCGAATTCGATTCATTGCCTGGATTTGATTCTCGACAT
201    N  A  N  M  L  P  G  P  E  F  D  S  L  P  G  F  D  S  R  H

              670       680       690       700       710       720
661   TTCCTACATGCAAGTATAATGGATGCTCACCATTATGCACAACAGGATCAAACGGCCCTT
221    F  L  H  A  S  I  M  D  A  H  H  Y  A  Q  Q  D  Q  T  A  L

              730
721   CAACTTGGATGA
241    Q  L  G  *
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Figure 2 Deduced amino acid sequence of *GBM5* and overall length cDNA sequence

Note: The red boxes represent the starter and terminator

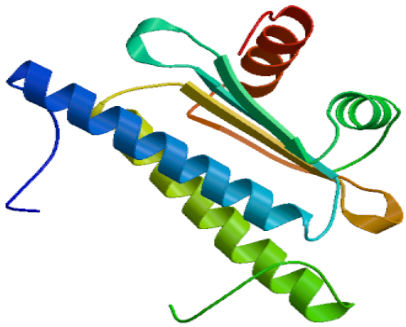

Figure 3 Tertiary structure prediction of GBM5 protein

1.3 Phylogenetic analysis of *GBM5* gene

In order to study the evolutionary relationship between *GBM5* gene of *Ginkgo biloba* and other plant genes, MEGA 7.0 software was used for cluster analysis of *GBM5* gene of *Ginkgo biloba* and *AG* gene of *Arabidopsis thaliana*, Camphor oil-free and Cycad (Figure 4). Cluster analysis showed that the *GBM5* gene of *Ginkgo biloba* was most closely related to *Cycas Revoluta AG* gene, followed by *Picea mariana AG* gene in Pinaceae, and *Amborella trichopoda AG* gene in relict plants. The *AG* gene of *Prunus mume*, *Arabidopsis thaliana*, and *Camellia japonica* has longer evolutionary relationship, and the *AG* gene of *Oryza sativa* has the longest evolutionary relationship. The amino acid sequence encoded by GBM5 was compared with protein in NCBI (Figure 5), and the result showed that *GBM5* belonged to *AG* gene. The results showed that *AG* gene plays an important role in regulating flowering time and determining floral organ development. It is speculated that *GBM5* gene is related to flowering time and floral organ development.

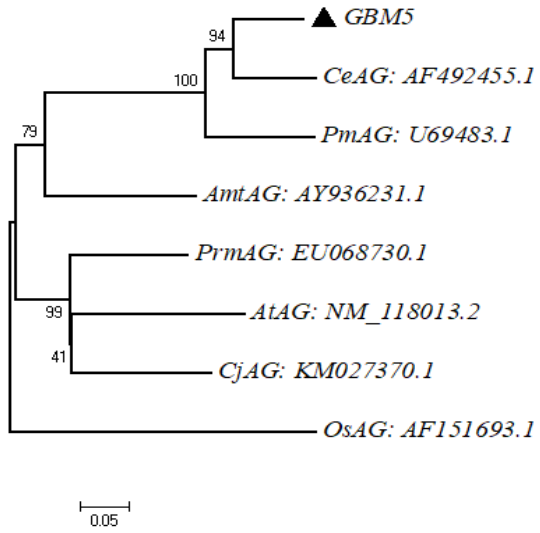

Figure 4 Analysis diagram of the phylogenetic tree of *GBM5* and *AG* gene in other species

Note: At: *Arabidopsis thaliana*; Amt: Camphor oil-free; Ce: Cycad Cj: Camellia; Os Rice; Pm: Black spruce; Prm: Plum

1.4 The relative expression levels of GBM5 gene in different tissues of *Ginkgo biloba*

Studies have shown that *MADS-box* family genes play an important role in the development of reproductive organs, so the expression level of *GBM5* gene in *Ginkgo biloba* leaves, stem tips, female flowers and male flowers was detected by qPCR. The results showed that *GBM5* was expressed in female flowers, male flowers and stem tips of reproductive organs, and the highest expression level was found in female flowers, followed by male flowers, stem tips and leaves, and the lowest expression level was found in leaves (Figure 6). There was extremely significant difference in the expression level among different tissues (<0.05). *GBM5* gene is highly expressed in female and male flowers of *Ginkgo biloba*, which is the same as *AG* gene expression pattern of tea tree, plum and

other plants 21 (Hou, 2009), which is speculated to be the possible reason for the high expression level of *GBM5* gene in reproductive organs.

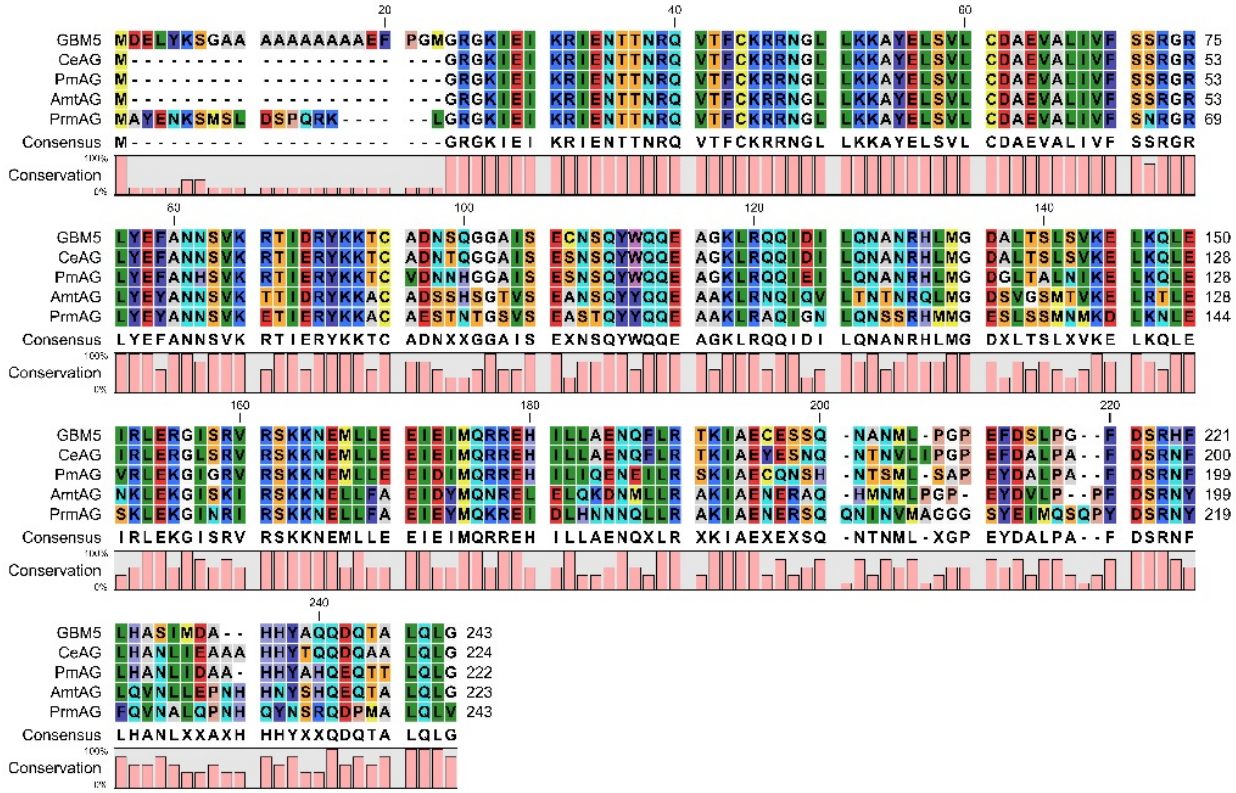

Figure 5 Alignment of *GBM5* protein with other *AG* proteins

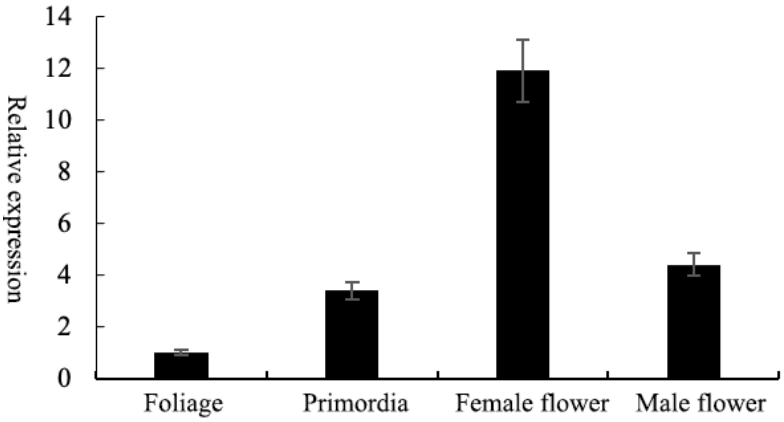

Figure 6 Expression levels of *GBM5* gene in different tissues

Note: Different lowercase letters above each column indicate significant differences at the 0.05 level

1.5 The relative expression levels of GBM5 gene in *Ginkgo biloba* at different periods

The results showed that the expression level of *GBM5* gene in leaves and stem tip showed a decreasing trend from the undifferentiated stage of flower buds in April, the beginning stage of flower buds differentiation in May to the peak stage of flower buds differentiation in September. The expression level of *GBM5* gene was higher at the undifferentiated stage of flower buds. The results showed that the expression level was highest in April, followed by May, and lowest in September in leaves (Figure 7A); the expression level was highest in April, followed by May, and lowest in September in stem ends (Figure 7B). The expression levels of samples in different periods were significantly different ($P<0.05$).

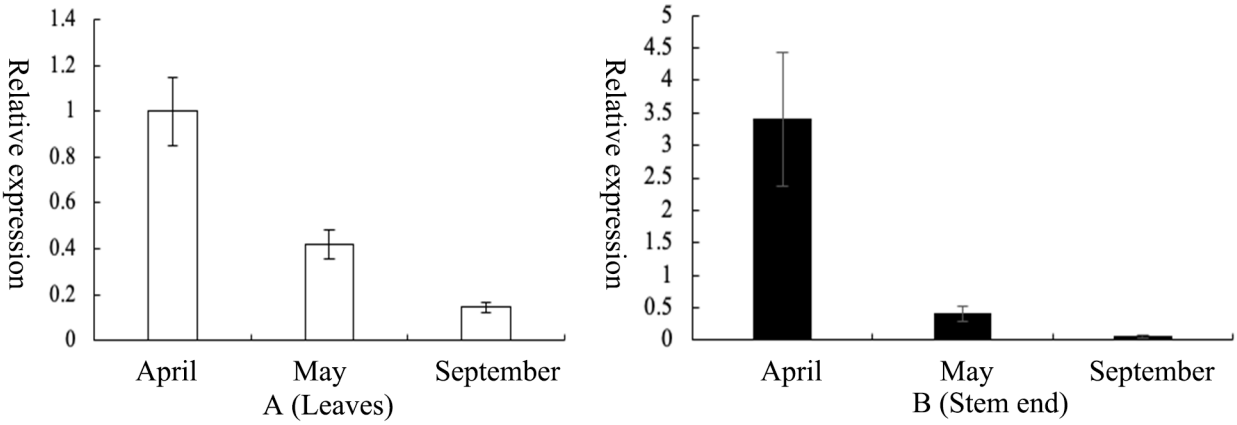

Figure 7 Expression levels in leaves and stems of *GBM5* genes at different time

Note: A: Ginkgo *GBM5* gene expression level in leaves; B: Ginkgo *GBM5* gene expression level in the stem end; Horizontal axis represents different periods, April: Flower bud undifferentiated period; May: Flower bud differentiation stage; September: Flower bud differentiation period; Different lowercase letters above each column indicate significant differences at the 0.05 level

2 Discussion

In this study, a specific primer was designed according to the measured ginkgo transcriptome data, and 732 bp *GBM5* gene was cloned from *Ginkgo biloba* by RT-PCR technology. Protein sequence analysis showed that the protein encoded by *GBM5* gene contained the MADS conservative domain and K region, with typical plant *MADS-box* gene structure. Phylogenetic analysis showed that *GBM5* gene was highly homologous with *AG* gene, so it was speculated that *GBM5* gene belonged to the *AG* gene in *MADS-box* gene family.

Members of *MADS-box* gene family play crucial roles in the reproductive development of higher plants. At present, the *AG* genes in *MADS-box* gene have been well studied in terms of structure, function, homologous gene cloning and gene evolution (Gao et al., 2008). Sieburth and Meyerowitz (1995) showed that the *AG* allele can deal with the role of Arabidopsis genes that determine the specificity of round 3 and round 4 during flower determination, and believed that the *AG* gene requires a corresponding critical value in order to perform the desired function. However, due to the diversity of *MADS-box* gene expression patterns and great differences in function, the mechanism of the interaction of *AG* gene on upstream and downstream genes to jointly regulate flower development has not been determined. Xu and Wang (2011) found that *AG* homologous gene is related to the development of sporophyll in gymnosperms, and the main role of *AG* in the reproductive organs of gymnosperms is to determine the differentiation and development of sporophyll.

In this study, cluster analysis of *GBM5* gene and *AG-like* genes showed that *GBM5* gene of *Ginkgo biloba* and Cycas *AG* had the closest genetic relationship, that is, the gene function was more similar. It was found that the gene expression level in the main reproductive organs of *Ginkgo biloba* was much higher than that in the vegetative organs. The gene expression level in female and male flowers was the highest, followed by the stem end, and the leaf was the least. The content of *GBM5* in the stem and leaf of plants decreased significantly with the degree of differentiation, and the decrease degree of *GBM5* in the stem was greater than that in the leaf. The results showed that *GBM5* gene was highly expressed in female flowers and male flowers of *Ginkgo biloba*, which was the same as *AG* gene expression pattern of tea tree, plum and other plants. Therefore, *GBM5* gene expression was basically consistent with *MADS-box* gene expression pattern, and it was inferred that *GBM5* gene function was similar to *MADS-box* gene function. That is, it plays a certain role in regulating the growth and development of *Ginkgo biloba*. At present, *LFY*, *AG*, *FT* and *AP1* are the main flowering regulation genes in woody plants. There are few reports on the use of transgenic technology to shorten the cloning and expression of woody plants.

In the future, more undiscovered new *AG* genes will be cloned, and more mutants will be identified, so as to realize in-depth research and progress in the related gene function field. Through cloning, sequence analysis,

bioinformatics analysis and phylogenetic analysis of *GBM5* gene obtained by transcriptome sequencing, this study provides a theoretical basis for further research on key flowering genes of *Ginkgo biloba* and analysis of flowering regulation mechanism.

3 Materials and Methods

3.1 Experimental material

The stem end of *Ginkgo biloba* was obtained from adult ginkgo trees in the Botanical Garden of Central South University of Forestry and Technology. The stem end of *Ginkgo biloba* was frozen with liquid nitrogen and stored in a refrigerator at -80℃. Then RNA extraction and sequencing were performed. EX Taq Polymerase, dNTP Mixture, Buffer, 5×PrimeScript RT Master Mix (Perfect real time) Reverse Transcription Kit (TaKaRa), MD116 DNA Marker (Beijing TransGen Biotech Co., Ltd.), E.Z.N.A. TM Plant RNA Kit (Omega Bio-Tek), High purity Plasmid Microextraction Kit (ComWin Biotech Co., Ltd.), SanPrep Column DNA Gel Recovery Kit (Sangon (Shanghai) Biotech Co., Ltd.), other conventional reagents used in domestic analytical pure or imported packaging.

3.2 Total RNA extraction and synthesis of the first strand of cDNA

E.Z.N.A. TM Plant RNA kit was selected for rapid extraction of total RNA from *Ginkgo biloba* stem. The first strand of cDNA was reversely transcribed according to the procedure and introduction of the 5×PrimeScript RT Master Mix kit: total RNA 8 μL, Oligo (dT) 18 primer 1 μL, the total volume of rnASE sterile water to reagent 12 μL. Incubate at 65℃ for 5 min and cool down on ice. Then 5×Reaction buffer 4 μL, Ribolock RNase Inhibitor (20 U/μL) 1 μL, 10 mmol/L dNTP Mix 2 μL and RevertAid M-mulv RT were added in a certain order (200 U/μL) 1 μL, 20 μL reverse transcription system, incubated at 42℃ for 60 min, water bath at 70℃ for 5 min, and the reaction was terminated. The cDNA concentration was detected by UV spectrophotometry. The obtained samples were stored in a refrigerator at -20℃ for later use.

3.3 PCR amplification and recovery, cloning and determination of the product

According to the different stages of plant stem end database related gene sequence length, by NCBI, clear the corresponding gene sequences, through Primer5.0 create the required specific primers, amplification primer F: TCCCCCGGGATGGGCCGTGGGAAGATT; R: CGCGGATCCTCATCCAAGTTGAAGGGCC. 20 μL PCR was prepared under the following conditions: DNA 0.5 μL (about 100 ng/μL), primer on both sides corresponding to 0.5 μL, dNTP Mixture 2 μL, $MgCl_2$ (25 mmol/L) 1.5 μL, 10×PCR Buffer 2 μL, Ex Taq 0.2 μL, dH_2O 12.8 μL. PCR reaction conditions were as follows: 95℃ for 3 min, 95℃ for 30 s, 60℃ for 30 s, 72℃ for 60 s, 40 cycles; Extension at 72℃ for 5 min and preservation at 4℃.

SanPrep Column DNA gel recovery kit was used to recover the target DNA fragment of PCR amplification product detected by agarose gel electrophoresis. The recovered part remained connected to the vector and was transformed into *E. coli* DH5α for blue and white spot screening. PCR detection was conducted after culture of the bacteria liquid. The positive bacteria solution was selected and transported to Shanghai Beauchamp to achieve the expected sequencing effect.

3.4 Bioinformatics analysis of *GBM5* gene

The open reading frame (ORF) corresponding to *GBM5* gene sequence was obtained by NCBI online platform. DNAMAN software was used to predict the amino acid sequence encoded by the target gene sequence. The BLAST program on NCBI was used to analyze the homology of protein and DNA, and the Conserved Domains program on NCBI was used to predict the conserved domains. MEGA7.0 software was used for clustering analysis of *GBM5* gene.

3.5 Fluorescence quantitative PCR (qRT-PCR)

The expression level of *GBM5* gene in *Ginkgo biloba* leaves and stem tips of short branches was detected by cDNA template using QRT-PCR method. GAPDH was used as an internal reference gene. *GBM5* specific primers: upstream primer: 5 'AGAGGATTGAGAACAATAC 3'. Downstream primer: 5 'GCACGGATAATTCATACG 3'.

Qrt-pcr was performed with SuperReal PreMix Plus (SYBR Green) of Tiangen Company. 20 μL reaction system: 2×SuperReal PreMix Plus (with SYBR Green I) 10 μL, upstream primer (10 μmol/L) and downstream primer (10 μmol/L) 0.8 μL, ROX Reference Dye II 0.4 μL, CDNA4.0 μL and dH2O 4.0 μL. Three biological replicates were set for each tissue sample. The reaction conditions were as follows: 95℃ 15 min, 95℃ 10 s, 60℃ 30 s, 40 cycles. At the end of amplification, the temperature gradually increased from 60℃ to 95℃ at the rate of 0.5℃/10 s, and the sample was collected by fluorescence and analyzed by $2^{-\Delta\Delta Ct}$ method.

Authors' contributions

LYL and DJJ were the experimental designers and executors of this experiment. YTT completed data analysis; LYL completed the first draft of her thesis; WYQ is the architect and principal of the project, guiding data analysis, paper writing and revision. LM, YG, FZ, ZP, WGB and YWW all gave a lot of help in this research process. All authors read and approved the final manuscript.

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