ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2017.100.107

Research Article
Molecular Cloning and Expression Analysis of a *AGAMOUS-like 66* Gene (*GbAGL66*) in *Ginkgo biloba*

Jinshuang Dou, Lanlan Wang, Jiaping Yan, Mingyue Fu, Xian Zhang and Feng Xu

College of Horticulture and Gardening, Yangtze University, 434025 Jingzhou, Hubei, China

Abstract

Background and Objective: *Ginkgo biloba* (*G. biloba*) is a precious medicinal plant and has a long juvenile phase. *AGAMOUS LIKE-66* (*AGL66*) gene, an important flowering regulatory gene, belongs to the family of MADS-box gene family. Information on *AGL66* genes in *G. biloba* is relatively lacking. The aim of this study was to characterize a *AGL66* gene from *G. biloba*. **Methodology:** According to the unigene sequences of *G. biloba* transcriptome, a *AGL66* gene was cloned from *G. biloba*, named *GbAGL66* (Genbank accession number is MF443205). Quantitative real-time polymerase chain reaction (qRT-PCR) method was used to analyze the expression level of *GbAGL66* gene. Data were analyzed with one-way ANOVA using SPSS 11.0. **Results:** The full-length cDNA of *GbAGL66* gene was 1,202 bp and its open reading frame (ORF) was 1,146 bp, encoding a deduced protein of 381 amino acids. A homologue search against GenBank showed that *GbAGL66* protein was a homologue of MIKC-type MADS-box proteins and had two typical MADS and K domains. Using bioinformatics software to carry on the analysis, the theoretical molecular weight is 4.33 kDa and the isoelectric point is 5.96. *GbAGL66* had 60, 53 and 52% homology with the *AGL*s from *Prunus persica*, *Cucumis melo* and *Elaeis guineensis*, respectively. The expression of *GbAGL66* gene in roots was the highest. The expressions of *GbAGL66* gene in male and female flowers were higher than that in stems and leaves. **Conclusion:** In this study, a *GbAGL66* gene was cloned and characterized from *G. biloba* for the first time. *GbAGL66* was strongly expressed in roots and flowers. These findings laid the foundation for the molecular regulation of flowering of *G. biloba*.

Key words: *Ginkgo biloba*, *AGAMOUS-like*, MADS-box, gene cloning, qRT-PCR, flowering gene

Citation: Jinshuang Dou, Lanlan Wang, Jiaping Yan, Mingyue Fu, Xian Zhang and Feng Xu, 2017. Molecular cloning and expression analysis of a *AGAMOUS-like 66* gene (*GbAGL66*) in *Ginkgo biloba*. Biotechnology, 16: 100-107.

Corresponding Author: Feng Xu, College of Horticulture and Gardening, Yangtze University, 434025 Jingzhou, Hubei, China Tel: +86 13177040087

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, also known as ginkgo and 'gongsun' tree, is the famous precious plant. It is also a valuable medicinal plant[1,2], wood tree and ornamental tree[3]. The juvenile phase of *G. biloba* is very long, which poses a serious obstacle in breeding of good varieties of ginkgo. A lot of economic and social value of *G. biloba* was greatly limited. With the continuous development and improvement of molecular biology and genetic techniques, a large number of flowering genes are separated and cloned. Plants flowering mechanism research has got great progress in the molecular level. Flowering genes can effectively control the vegetative growth and reproductive growth of plants and change the flowering time[4,5], which can effectively shorten the juvenile phase for plants to grow.

Flowering is an important physiological transformation process of higher plants from vegetative growth to reproductive growth, which is influenced by external environmental factors and controlled by inner genes. MADS-box genes play an important role in flower development. MADS-box gene family is divided into two large clades: Type I and type II. Most plant sequences and animal MEF2 genes together form a type II lineage[6]. The type I group contains only the MADS-box conserved domains and the type II group contains MADS-box, I, K and C domains with different degrees of conservatism. Therefore, it is also called MIKC-type MADS-box gene[7-9]. The type II group can be further subdivided into 39 MIKC^C and six MIKC* genes based on the inferred evolutionary history of the family in *Arabidopsis thaliana*[8,9]. MIKC* gene encoding protein with a longer I domain is a distinction between the two groups[10]. Research shows that *Arabidopsis* genome contains 107 MADS-box genes and 71 MADS-box genes exist in the *Oryza sativa*[11]. The study found that the MADS-box family of genes played a very important role in many stages of plant development. Most of the functional studies are related to flower development, including participation in the morphogenesis of floral organs, flowering time control, split block formation and the maturity of the fruit[12,13].

AGAMOUS LIKE-66 (*AGL66*) gene belongs to the family of MADS-box gene. *AGL66* isolated from *Arabidopsis* and other *AGL* genes, such as *AGL30*, *AGL94* and *AGL65* together constituted a regulatory network for regulating the germination of male germ cells[14-15]. The study found that *AGL* gene (C/D gene) was found in all major seed plants but there was no typical *AGL* gene in non-seed plants such as ferns. This fact suggests that the *AGL* gene may have been produced between 300 and 400 million years ago, before the separation of existing gymnospermms and angiosperms[6].

Up to now, other *AGL* genes have been cloned in plants such as *Hosta plantaginea*[16], *Magnolia wufengensis*[17] and *Glycine max*[18], but the study of *AGL66* gene is little. Studies have shown that the MADS-box genes present in the genome of *G. biloba* had diversity[19]. Previously, the MADS-box gene, *AGAMOUS* (*AG*) gene was cloned from *G. biloba* and confirmed that it had genuine C function[20]. In this study, *AGL66* gene, an important flowering gene in *G. biloba*, was first cloned and analyzed by biological information analysis and quantitative analysis of fluorescence to study the function of the gene during flowering of *G. biloba*. This study laid the foundation for studying the molecular regulation mechanism of flowering of *G. biloba* and provide genetic resources for the research of shortening the juvenile phase of *G. biloba*.

MATERIALS AND METHODS

Materials: All the tissues were collected from 31-year-old trees of the *G. biloba* cultivar 'Jiafoshou', in the Ginkgo Science and Technology Garden, Yangtze University. The male and female flowers were sampled in early April. The roots, stems, leaves and young fruits were sampled in end of May. After collecting the roots, stems, leaves, male flowers, female flowers and young fruit of *G. biloba*, these tissues were immediately freezed by liquid nitrogen and refrigerated to -80°C ultra-low temperature refrigerator for later use. Agarose Gel DNA purification Kit Ver.4.0, MiniBEST Plant RNA Extraction kit, PrimeScript™ 1st Strand cDNA Synthesis Kit, *Escherichia coli* DH5α, pMD18-T vector, dNTP, RNase and *Taq* DNA polymerase were purchased from Bao Bioengineering (Dalian) Co., Ltd. Primer synthesis and sequencing commissioned by Shanghai Biotech Bioengineering Company.

Molecular cloning of *GbAGL66* gene: The total RNA was extracted from the female flowers of *G. biloba* according to the RNA extraction kit. The purity and concentration of total RNA were analyzed by agarose gel electrophoresis and spectrophotometer and stored in a refrigerator at -80°C. Refer to the reverse transcription kit (PrimeScript TM 1st Strand cDNA Sythesis Kit),the extracted RNA is reverse transcribed into cDNA.

A pair of primers, the upstream primer G-F and the downstream primer G-R (Table 1), which specifically amplify the *GbAGL66* gene, were designed according to the AG

Table 1: Primers and their primer sequences

Primers	Primer sequence 5'-3'
G-F	CATCCCCTAAAAATCTGTCGG
G-R	TCATACACCCTCTGTGGCGT
P-F	TTCGGAGACAGAGAATTCAGTTGGA
P-R	TTCTGAGACGAACTCTACGCAAAGC
H-F	TTGGTCTCCCGTGCTAATGG
H-R	CGAAGCGTCATCCTAAGACAACA

unigene sequences of the *G. biloba* transcriptome annotated by Xu Feng research group. The synthetic cDNA was used as template to amplify the sequence of *GbAGL66* gene. The PCR conditions were as follows: A 94°C denaturation step for 3 min, followed by cycles of 94°C for 30 sec, 55.4°C for 30 sec and 72°C for 1 min, followed by a final extension of 72°C for 10 min. One percent of agarose gel electrophoresis was used to detect PCR products. After further purification, the recovered fragment was ligated with the vector pMDl8-T and transformed into competent cell *E. coli* DH5α according to the pMDl8-T vector kit instructions. Pick up a single colony for culture and then screen the positive cloned gene. Finally, they were sent to Shanghai Biotechnology Engineering Company sequencing.

Bioinformatics analysis of *GbAGL66* gene: The sequencing of the *GbAGL66* gene sequence was performed by DNAMAN V6 software and vector NTI 11.5 was used to complete the open reading frame (ORF) search, protein translation and *AGL* gene homology amino acid sequence comparison. The comparison of protein sequence similarity and protein sequence analysis was performed on NCBI using Blast-protein online software. The physical and chemical properties of *GbAGL66* protein were predicted by the online tool ExPASy. Finally, the system evolution tree was constructed by Neighbor-Joining (NJ) method using Clustal X2.0 and MEGA 6.0 software.

Expression analysis of *GbAGL66* gene: RNA was extracted from roots, stems, leaves, flowers and fruit samples of *G. biloba* and reverse transcribed into cDNA. Based on the known sequence, Primers P-F and P-R (Table 1) were designed for real-time quantitative experiments of *GbAGL66* gene. *GAPDH* gene was used as the internal reference gene. The *GAPDH* gene upstream primer H-F and the downstream primer H-R (Table 1) were designed according to the known sequence. qRT-PCR was performed with reference to instructions of AceQ® qPCR SYBR® Green Master Mix (Without ROX) kit (Vazyme). Reaction system was 20 μL. PCR conditions were as follows: 95°C for 1 min, followed by cycles of 95°C for 15 sec, 60°C for 1 min with fluorescence signal collection at 60°C. The melting curve program is 95°C for 1 min, 65°C for 1 min, 95°C for 20 sec, 30°C for 1 min. Each sample was set up 3 times to repeat with ultrapure water as a negative control and *GAPDH* gene was an internal reference gene. Data processing used the relative quantitative method, with reference to $2^{-\Delta\Delta ct}$ method for the results of analysis[21].

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

Molecular cloning and sequence analysis of *GbAGL66* gene: After the sequencing of the cloned gene fragments, a cDNA fragment was obtained with a full-length length of 1,202 bp. The Vector NTI 11.5 analysis showed that the *GbAGL66* gene contained an ORF of 1,146 bp in length, encoding 381 amino acids (Fig. 1), named *GbAGL66* (GenBank accession number was MF443205). The online analysis of ExPASy-ProtParam showed that the theoretical molecular weight of the protein was 4.33 kDa and the isoelectric point was 5.96.

The amino acid sequence encoded by the *GbAGL66* gene was subjected to BLAST-protein alignment on the NCBI website. The results showed that the protein had a typical MADS-box domain and the M domains was highly consistent with MEF2-like protein, indicating that the gene belonged to the type II group. Therefore, it was a MIKC type gene. Multiple sequence alignment found that the M domain was highly conserved and the K domains was also conserved (Fig. 2). The GbAGL66 has high homology with AGLs from other plants, which was the highest homology, at 60%, compared with *Prunus persica*. Compared with *Cucumis melo* and *Elaeis guineensis* ,the homology was 53 and 52%, respectively. The homology were both 50%, compared with that of *Nicotiana tomentosiformis* and *O. sativa.* Compared with that of *Phoenix dactylifera* and *Theobroma cacao*, the homology were both 49% (Table 2).

Phylogenetic tree analysis of *GbAGL66*: AGL protein sequences from other species were downloaded from GenBank according to different *AG* gene families (Table 3).

```
1       CATCCCCTAAAAATCTGTCGGTACAGAAAACCATGGGCAGAGTTAAGCTCCCGATTAAGAAAATAGAGAATAGCA
1                                       M  G  R  V  K  L  P  I  K  K  I  E  N  S
76      CTAACAGGCAGGTCACCTTCTCCAAACGGAGAAATGGATTGATCAAAAAGGCATATGAACTCTCAGTTCTGTGCG
25      T  N  R  Q  V  T  F  S  K  R  R  N  G  L  I  K  K  A  Y  E  L  S  V  L  C
151     ACATCGAAATTGCACTCATAATGTTCTCGCCCTCGGGAAGATTGAGCCACTTTTCAGGGAAAAATAGTAGGATAG
50      D  I  E  I  A  L  I  M  F  S  P  S  G  R  L  S  H  F  S  G  K  N  S  R  I
226     AAGATGTTATAGCTCGCTTCGTCAATCTGCCTGAGCACGAGAGGCCAAGGCTTGTCCAAAACCAAGAATATCTCC
75      E  D  V  I  A  R  F  V  N  L  P  E  H  E  R  P  R  L  V  Q  N  Q  E  Y  L
301     TCAGAGCGTTGAAAAAACTCAAGTACGAAAGCGATATCGCCAATCATCTTGCAAGCCCAAACATTGTCGACTCAA
100     L  R  A  L  K  K  L  K  Y  E  S  D  I  A  N  H  L  A  S  P  N  I  V  D  S
376     ATGTGGAGGAACTTCAAATGGATATTCGGAGACAGAGAATTCAGTTGGAGGAAGCCCAGCAGAAGTTAAGGAGTT
125     N  V  E  E  L  Q  M  D  I  R  R  Q  R  I  Q  L  E  E  A  Q  Q  K  L  R  S
451     TTAAAGAGGATCCACTTCTTATAACCTCAATACAAGATGCAGATCAATATGAAAGGACATTGGAGGAGGCTTTGC
150     F  K  E  D  P  L  L  I  T  S  I  Q  D  A  D  Q  Y  E  R  T  L  E  E  A  L
526     GTAGAGTTCGTCTCAGAAAACAACAGCTAGAACACAACCAAATGGCAGTTGCTTTCAACGATGCAAACTTGCAGT
175     R  R  V  R  L  R  K  Q  Q  L  E  H  N  Q  M  A  V  A  F  N  D  A  N  L  Q
601     TTTATATACAAACACAGAATGGATTGCCAAATGGAACAGACACAAGTCAAAATCACCTATACAACTCATGGATGC
200     F  Y  I  Q  T  Q  N  G  L  P  N  G  T  D  T  S  Q  N  H  L  Y  N  S  W  M
676     CACAGGGAGACCCCCATACTAGTGTCCAGAATTTTATGGAGCATGAAAATTCTAATGCCATGCTTGCGATGCGGG
225     P  Q  G  D  P  H  T  S  V  Q  N  F  M  E  H  E  N  S  N  A  M  L  A  M  R
751     AAGCACAATGCATGGCAAAGTGTTTACAAAATGGAACCGTATTTCCGGCACTTCAAGATGCCACTGGAATGCAGC
250     E  A  Q  C  M  A  K  C  L  Q  N  G  T  V  F  P  A  L  Q  D  A  T  G  M  Q
826     TTCCAAATGAGTCTGCAAGTACTCAACCGTATATTCCTACATCACACATGCAGTTTGATTACACTTTAACTGACA
275     L  P  N  E  S  A  S  T  Q  P  Y  I  P  T  S  H  M  Q  F  D  Y  T  L  T  D
901     ACAACAATAATGAACATGCAGAACAGGCCGATATAGCTGCAGCGTTTGACTATGGTTCTGATGCAATGGCATCCG
300     N  N  N  N  E  H  A  E  Q  A  D  I  A  A  A  F  D  Y  G  S  D  A  M  A  S
976     TACATTGGCAAACTTCGTATGGGTCGATGACTCCAATTGTGACAAATCAACAGTATCCTTTGACTAAGGGAATAA
325     V  H  W  Q  T  S  Y  G  S  M  T  P  I  V  T  N  Q  Q  Y  P  L  T  K  G  I
1051    TGCAAAATATTGTTCCGCCTAGTATGTCAATATACCAGCAAGATGGCTCTTCCTCACAGGGCACTCACCATTCAA
350     M  Q  N  I  V  P  P  S  M  S  I  Y  Q  Q  D  G  S  S  S  Q  G  T  H  H  S
1126    CACCTCAAGACAATGCAGGAATGGATGCTTCATTTCAAAGTAACTTGAAATAGCCAAACGCCACAGAGGGTGTAT
375     T  P  Q  D  N  A  G  M  D  A  S  F  Q  S  N  L  K  *
1201    GA
```

Fig. 1: Nucleotide sequence and deduced amino acid sequence of *GbAGL66*
Protein sequence analysis of *GbAGL66*

Table 2: Protein sequence of *GbAGL66* similarity to *AGL*s of other plants

Species	No. of accession	Identity	E-value
Prunus persica	XP_020410352.1	60	1e-63
Cucumis melo	XP_008442144.1	53	1e-64
Elaeis guineensis	XP_010939774.1	52	1e-71
Nicotiana tomentosiformis	XP_009629661.1	50	3e-65
Oryza sativa	XP_015649547.1	50	9e-62
Phoenix dactylifera	XP_008790798.1	49	5e-69
Theobroma cacao	XP_007018574.2	49	1e-65

The *AGL* phylogenetic tree of different species was constructed with NJ method by using software ClustalX2.0 and MEGA6.0. The AGL system phylogenetic tree was divided into two groups, MADS type I and type II. These genes, (*Arabidopsis*) *AGL38*, *AGL54*, *AGL51*, *AGL57*, *AGL64*, *AGL28*, (*Helianthus annuus*) *AGL28*, (*Aquilegia coerulea*) *AGL73*, (*Turritis glabra*) *AGL45*, together form type I, which

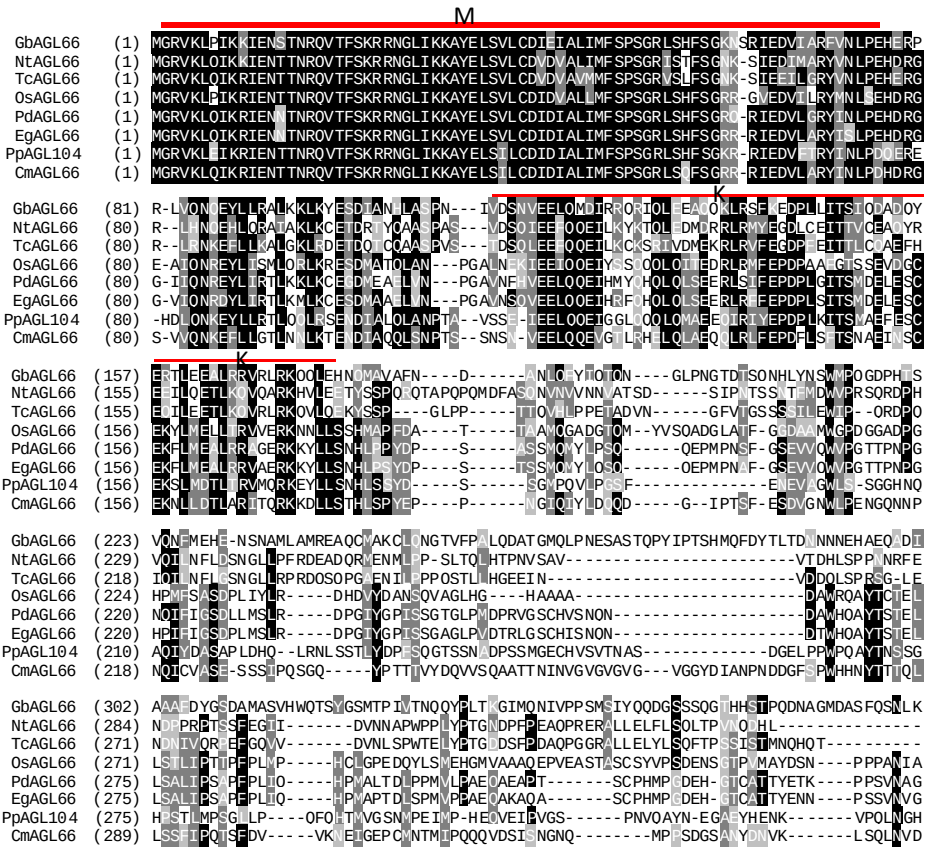

Fig. 2: Similarity analysis of *GbAGL66* coding protein and other known *AGL* proteins *GbAGL66*: *Ginkgo biloba*, *NtAGL66*: *Nicotiana tomentosiformis*, *TcAGL66*: *Theobroma cacao*, *OsAGL66*: *Oryza sativa*, *PdAGL66*: *Phoenix dactylifera*, *EgAGL66*: *Elaeis guineensis*, *PpAGL104*: *Prunus persica*, *CmAGL66*: *Cucumis melo*. M domain marked with a red bold line and K domain marked with red lines

Table 3:Gene sequences used in the phylogenetic tree

Names	Specie	No. of accession	Name	Specie	No. of accession
AtAGL30	*Arabidopsis thaliana*	OAP09118.1	*HbAGL30*	*Hevea brasiliensis*	XP_021660586.1
HbAGL65	*Hevea brasiliensis*	XP_021642368.1	*AtAGL65*	*Arabidopsis thaliana*	AAN37407.1
AtAGL94	*Arabidopsis thaliana*	NP_177113.3	*GbAGL66*	*Ginkgo biloba*	MF443205
EgAGL66	*Elaeis guineensis*	XP_010939774.1	*PdAGL66*	*Phoenix dactylifera*	XP_008790798.1
CaAG	*Coffea arabica*	AHW58037.1	*NaAG*	*Nuphar advena*	AAY25576.1
CsAGL3	*Camelina sativa*	XP_010425169.1	*ThAGL3*	*Tarenaya hassleriana*	XP_010527982.1
NoAGL6	*Nymphaea* odorata	ADD25208.1	*CaAGL6*	*Coffea arabica*	AHW58046.1
MhAGL6	*Monotropa hypopitys*	AQM52295.1	*AtAGL38*	*Arabidopsis thaliana*	AAN52809.1
TgAGL45	*Turritis glabra*	BAN63747.1	*AtAGL54*	*Arabidopsis thaliana*	AAN52782.1
AtAGL51	*Arabidopsis thaliana*	OAO98799.1	*AtAGL57*	*Arabidopsis thaliana*	NP_187060.1
AtAGL64	*Arabidopsis thaliana*	NP_001077625.1	*HaAGL28*	*Helianthus annuus*	OTF97168.1

contains only a conservative MADS-box conservative domain. Type II was divided into two small branches: MIKC* type with longer I domain and MIKC^c type with shorter I domain (Fig. 3). Through the analysis of phylogenetic tree, it can be found that *GbAGL66* belongs to MIKC* type in type II, indicating that I domain of *GbAGL66* is longer.

Expression of *GbAGL66* gene in different tissues: To characterize the function of *GbAGL66*, we determined transcript level of *GbAGL66* in different tissues. qRT-PCR results showed that transcripts of *GbAGL66* accumulated in all tested tissues, including roots, stems, leaves and male and female flowers (Fig. 4). However, the transcript levels of *GbAGL66* varied greatly among different tissues.

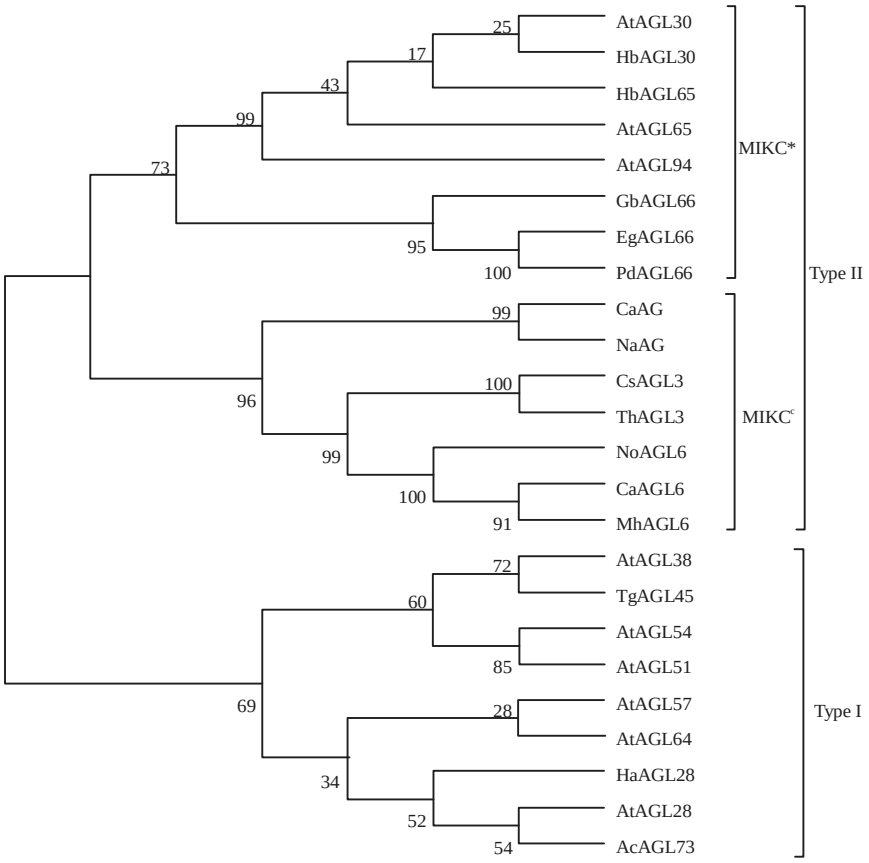

Fig. 3: Phylogenetic tree of *AGL* using Neighbor-Joining method

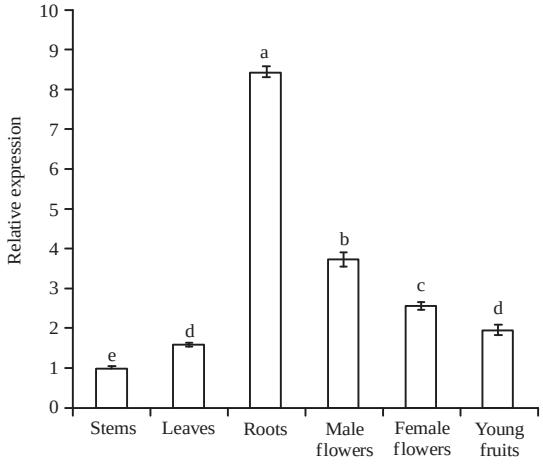

Fig. 4: Expression of *GbAGL66* in different tissues. The expression level of *GbAGL66* in the stem was set to 1 and those of *GbAGL66* in other tissues were accordingly accounted and presented as the relative fold changes, respectively

Data from qRT-PCR were shown as the mean±SD (standard deviation) of three biological replicated assays. Means with different letters were significantly different by $p<0.05$ by Duncan's multiple rang test

The highest transcript level of *GbAGL66* was found in roots, followed by male and female flowers. The transcript levels of *GbAGL66* in young fruits and stems were significantly ($p<0.05$) lower than in male and female flowers. The lowest transcript level was observed in stems and significantly ($p<0.05$) lower than other tissues.

DISCUSSION

The MADS domain family was characterized by the highly conserved DNA-binding MADS domain[22]. This study showed that the protein encoded by the *GbAGL66* gene had a typical MADS-box domain, one I domain and one K domain. It was determined that the gene was a typical MADS family gene. Compared with the amino acid sequence of *AGL* gene from other species, it was found that *GbAGL66* had high homology with AGL protein from other species. The phylogenetic tree analysis showed that the *AGL* gene from different species originated from the same ancestor, which was consistent with the conclusion that the *AGL* gene was produced before the separation of existing gymnosperms and angiosperms[6]. In

addition, *GbAGL66* was found in the MIKC* group. It was determined that the gene belonged to the MIKC* type in the MADS-box gene family. There were studies found that MIKC* genes retained a conserved role in the gametophyte during land plant evolution[23] and the function of heterodimeric MIKC*-type protein complexes in pollen development has been conserved since the divergence of monocots and eudicots, roughly 150 million years ago[24]. Therefore, *GbAGL66* gene was likely to participate in pollen development of *G. biloba*.

Previous studies have shown that some *AG* genes were found to be expressed not only in the reproductive structures but also in various vegetative tissues[25] and that the *AGL66* gene was detected in both embryonic and inflorescence tissues in *Arabidopsis* but was not detected in the seed[26]. In the *Betula platyphylla*, the expression of *AGL* gene was significantly different in female and male inflorescence. Compared with female inflorescence, the expression level of *AGL* gene was very low in the development stage of male inflorescence and there was no significant change[27]. Similarly, the expression of *AGL6* gene can be detected in different tissues of *Cymbidium goeringii*[28]. The expression level of *AGL6* gene was higher in petals, flower buds and ovary than that in petals and sepals. The expression level was the lowest in roots, leaves and cores[28]. In this study, it has found that the *GbAGL66* gene in different tissues have different levels of expression. The expression of *GbAGL66* gene in male and female flowers was high, which is consistent with the function of *AGL66* gene that it can regulate the development of male flower pollen[14,15]. In comparison with the results of previous studies, there was a difference that the expression of *GbAGL66* gene of in root was the highest. However, the same results were found in the results of *AG* homologous gene expression in *Medicago truncatula*[29]. At present, a MADS-box family gene, *AG* gene, named GBM5, has cloned in *G. biloba* with a genuine C function and it could be involved in the formation of the Ginkgo fleshy fruit-like structure surrounding the seed[20]. Many literatures have shown that the suite of MADS-box genes involved in the development of the fleshy fruit habit was already active in Gymnosperms as ancient as the Ginkgoales[20,30]. *AGL* gene belonged to *AG* subfamily and *GbAGL66* gene was detected in fruit organization of *G. biloba*, suggesting that *GbAGL66* gene may also regulate the development of fruit. At present, there were few reports on *AGL66* gene and the mechanism of regulation of flowering of *GbAGL66* gene needs further research to verify.

CONCLUSION

It is concluded that, an *AGAMOUS-like 66* (*GbAGL66*) gene was cloned from *G. biloba*. The full-length cDNA of *GbAGL66* was 1,202 bp and encoded a deduced protein of 381 amino acids. GbAGL66 protein was a homologue of MIKC-type MADS-box proteins and had two typical MADS and K domains. The results of phylogenetic tree analysis showed that GbAGL66 protein had a longer I domain. *GbAGL66* gene was expressed in various tissues of *G. biloba*, the highest in the root, followed by male and female flowers. These could lay a good foundation for the use of genetic engineering technology to shorten the juvenile phase of *G. biloba*.

SIGNIFICANCE STATEMENT

AGL66 gene is an important gene for the regulation of floral organ development and is also a member of the MADS-box family of genes. This study may offer some reference for researchers to study the evolution of members of the MADS-box gene family and also provide important genetic resources for the study of shortening the juvenile phase of *G. biloba.*

ACKNOWLEDGMENT

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

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