The Journal of Animal & Plant Sciences, 26(1): 2016, Page: 253-260
ISSN: 1018-7081

MOLECULAR CLONING AND EXPRESSION ANALYSIS OF A PUTATIVE E CLASS MADS-BOX GENE, *GbSEP*, FROM *GINKGO BILOBA*

Shui-Yuan Cheng[1], Jun-Huan Cheng[2], Feng Xu[2*],Jia-Bao Ye[2] and Xiao-Hui Wang[2]

[1]School of Biology and Pharmaceutical Engineering, Wuhan Polytechnic University, Wuhan 430023, China;
[2]College of Horticulture and Gardening, Yangtze University, Jingzhou 434025, China;
[*]Corresponding Author: Feng Xu, E-mail address: xufeng198@126.com; Tel: +86-716-8066260

ABSTRACT

MADS-box gene family plays an important role in the development of floral organs in plants. A full-length cDNA of MADS-box gene, named as *GbSEP*(Genbank accession numberKJ630504), was cloned by RT-PCR from female flower of *Ginkgo biloba*. The cDNA sequence of *GbSEP* contained an ORF of 756 bp encoding 252 amino acid residues. The comparison between cDNA and genomic sequences of *GbSEP* indicated no intron in the genomic DNA sequence of this gene. Protein multi-alignment analysis indicated that GbSEP shared high homology with other SEP family proteins from other plants including *Pinus radiate* (PrMADS3), *Pinus resinosa* (PrMADS1), *Arabidopsis thaliana* (AtSEP4), *Alpinia hainanensis* (AhSEP3), *Lotus japonicas* (LjSEP), *Populus tomentosa* (PtSEP), and *Mangifera indica* (MiSEP3). Moreover, GbSEP contained a MADS-box and a K-box domain, typical conservative domains of plant MADS-box proteins. Phylogenetic tree clearly showed that GbSEP is classed into SEP subgroup of E class MADS-box protein family. Real-time PCR analysis revealed that expression levels of *GbSEP* gene in female and male flower were significantly higher than those in root, stem, and leaves, and that *GbSEP* expression level increased along with time of flower development, suggesting that *GbSEP* might be involved in development of reproductive organs in *G. biloba*.

Key words: *Ginkgo biloba*; MADS-box; *GbSEP*; Expression analysis; Real-time PCR.

INTRODUCTION

MADS-box is an allotypic gene family that is widely distributed in most species of the biological world. Plant MADS-box gene has been differentiated from eukaryote ancestor approximately one billion years ago. Most MADS-box genes are of type II with a conserved MIKC structure(Smaczniak *et al*., 2012). This structure consists of four domains (M, K, I, C) with different in conservativeness and functions (Becker andTheiβen, 2003). By binding to specific *cis*-acting elements, the MADS-box gene can mediate gene expression and thus serve an important function in plant growth and development. The primary function of the MADS-box gene is to control the organogenesis and morphogenesis of the floral organ in flowering plants. This gene is thus called the 'molecular constructor' of floral organogenesis (Lalusin *et al*., 2006). On the basis of the study of floral organ mutation in mode plants, such as *Arabidopsis thaliana* and snapdragons. Bowman *et al*. (2012) presented the well-known "ABC model" that controls four cycles of floral organ development. This theory had been widely accepted and subsequently updated to establish the "ABCDE model" of floral organ development. This model divides the functions of the MADS-box gene in floral organ development into five different types. These functions could control the development of sepals, petals, stamens, carpels, ovules and pistils, alone or in combination (Ng and Yanofsky, 2001).

As regards biological metabolism, one important function of MADS is to regulate the biosynthetic pathway of secondary metabolite such as flavonoids as a transcription factor. The ARABIDOPSIS BSISTER (ABS) protein encoded by transparent testa gene *tt16* has been associated with flavonoid synthesis and has the typical structure of a MADS-box protein (Prasad *et al*., 2010). ABS protein is essential for the expression of *BANYULS*, which is a key gene in flavonoid synthesis that could influence the accumulation of proanthocyanidins and carotenoids in testa (Prasad *et al*., 2010). In addition, the MADS-box genes have also been involved in other plant growth and development processes, such as gibberellic acid synthesis (Jackson, 2009), fruit after-ripening (Ireland *et al*., 2013), plant dormancy (Liu *et al*., 2013), root elongation (Zhang and Forde, 1998), differentiation of callus (Fornara *et al*., 2004), and physiological resistance (Lee *et al*., 2008).

Ginkgo biloba, which originates from China, is the oldest plant species that has been extensively applied in greening, landscape, medicine, food, and health care (Bilia, 2002). *G. biloba* is a Mesozoic relict plant with very long juvenile period. The plant usually blossoms and bears fruit 15 to 20 years after cultivation. The blossom habit of *G. biloba* is unique when compared with that of other plants. This property is a factor that limits breeding research on *G. biloba*. Up to now, few studies have been conducted on the molecular biology of *G. biloba*floral

development. To clarify the influence of the MADS-box gene family on the development of *G. biloba*, we here cloned the full sequence of an E-type MADS-box gene, named as *GbSEP*, and then analyzed the structure, function, phylogenetic position, and expression profile of this gene in *G. biloba*. This work aimed to provide the foundation for further studies on the molecular mechanism of this gene in *G. biloba*.

MATERIALS AND METHODS

Plant materials: 15-year-old grafts of *G. biloba* were grown in a greenhouse at Yangtze University, China. To test the spatial expression of *GbSEP*, the roots, leaves, fruits, stems, male and female flowers of *G. biloba* grafts were collected. For determining the time-course expression patter of *GbSEP* in ginkgo flowers, the male and female flower samples on 2, 4, 6, 8, and 10 days after flower bud sprouting. All the samples were immediately frozen in liquid nitrogen after collecting, and kept at –80 °C prior to total RNA extraction.

Cloning of the full-length cDNA and genomic DNA of *GbSEP*: Total RNA was extracted from female flowers four days after flower-bud sprouting using the CTAB method (Cai *et al*., 2007). The quality and concentration of the RNA was all determined by agarose gel electrophoresis and spectrophotometer analysis. The primers SEPFP (5'-CAGAACGCAGGCGATCCAAA-3') and SEPRP (5'-CGGAAGTGACGCTGATAAGATA-3') were designed and synthesized (Shanghai Sangon, China) based on the EST sequence of *G. biloba*. Onestep RT-PCR was performed and a 800 bp fragment was obtained by using the one-step RT-PCR kit (Dalian TaKaRa, China) under the following conditions: 50°C for 30 min and 94°C for 3 min, followed by 35 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. This was followed by extension for 10 min at 72°C. The PCR product was purified, cloned into the pMD18-T vector (Dalian TaKaRa, China), and then sequenced. Subsequent BLAST results confirmed that the amplified product was the sequence of the MADS gene. The primers SEPFP and SEPRP were used to amplify the *GbSEP* genomic sequence using genomic DNA of *G. biloba* as template.

Bioinformatics analysis and molecular evolution analyses: The obtained nucleotide sequence and deduced amino acid sequence were compared through database search using BLAST program (NCBI, http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was performed with the software Vector NTI suit 11.0 program. GbSEP and other MADS box proteins obtained from GenBank were aligned with the software Vector NTI suit 10.0 program. Phylogenetic tree was constructed by were analyzed using CLUSTAL W 1.83 and MEGA 4.0. The reliability of the tree was measured by bootstrap analysis with 100 replicates.

Quantitative Real-time PCR analysis of GbSEP: To investigate the GbSEP expression pattern in different tissues, total RNA of the roots, leaves, fruits, stems, male and female flowers of *G. biloba* were isolated. First-strand cDNA synthesis was carried out in triplicate for each sample according to the instructions of the manufacturer (PrimeScript™ RT Reagent Kit, Dalian TaKaRa, China). Real-time PCR (RT-PCR) was performed using a Perkin-Elmer 7000 thermal cycler with SYBR Premix Ex Taq™ II Kit (Dalian TaKaRa, China) according to the protocol of the manufacturer. Reactions were performed in triplicate using 2 µL of MasterMix, 0.5 M of each primer, 2 µL of diluted cDNA, and nuclease free water to a final volume of 20 µL. The PCR reaction conditions were 95 °C for 3 min, and then 40 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 3 min. Fluorescence was measured at the end of each annealing step. Raw data were analyzed with Light Cycler software, and expression was normalized to *G. biloba* 18S gene (*Gb18S*, accession no. D16448) to minimize the variation in the cDNA template levels. The primers for GbSEP (SEP-S: 5'-GCTCCAGCAACTTGAACGACA-3'; SEP-A: 5'-CGAGGAACAGACTGATACCCA-3') and Gb18S (18S-S: 5'-ATAACAATACTGGGCTCATCG-3'; 18S-A: 5'-TTCGCAGTGGTTCGTCTTTC-3') were designed using the Sequence Detection System software. Real-time PCR data were technically replicated with error bars, representing mean ± SE (n =3).

RESULTS

Cloning of *GbSEP* gene: PCR amplification was performed with cDNA from *G. biloba* as template. Analysis of the PCR product by agarose gel electrophoresis showed a band of approximately 800bp (Fig. 1), consistent with the predicted size of the *SEP* gene sequence. The extracted PCR product was subject to sequencing. The result showed that the cDNA length was 800bp. As shown in Table 1, NCBI BLAST analysis revealed thatGbSEP cDNA sequence was highly homologous to the MADS-box genes in Ginkgo and other plants such as *GbMADS8* (homology of 78%, *G. biloba*, GenBank Accession No. AB029470.1), *GbMADS2* (58%, *G. biloba*, KP260628.1), *GbMADS5* (58%, *G. biloba*, AY114304.1) , *GbMADS10* (56%, *G. biloba*, AB029472.1), *GbMADS9* (49%, *G. biloba*, KP260627.1), *PaMADS*(76%, *Picea abies*, KC347012.1), *PrMADS2* (76%, *Pinus radiate*, PRU42400), *PrMADS* (76%, *P. resinosa*, Y09611), (80%, *Zamia fischeri*, KC899695.1), *GGM9* (76%, *Gnetum gnemon*, AJ132215.1), *GbMADS3* (76%, *G. parvifolium*, AB022665.1), and *WmMADS* (71%, *Welwitschia mirabilis*, KC899694.1), indicating the gene we cloned is a member of MADS-gene family. Therefore, the gene was designated as *GbSEP* (GenBank Accession No.

KJ630504). We obtained the genomic sequence of *GbSEP* with *G*. *biloba* genomic DNA as template. The length of PCR product was consistent with that of *GbSEP* cDNA fragment, indicating that *GbSEP* sequence was intronless. The nucleotide and deduced protein sequences of the *GbSEP* gene are shown in Fig. 2. The *GbSEP* gene had a length of 800bp, including open reading frame of 756bp, encoding 252 amino acids. GbSEP terminator was TGA, and the G+C content was 45.9 %.

Sequence analysis of GbSEP protein: The *GbSEP* gene encoded a protein of 252 amino acids. The isoelectric point and molecular weight predicted by the Computer pI/Mw Tool software were 8.88 and 29.2kDa, respectively, similar to the characteristics of the E class MADS-box SEP protein reported in other plants (Pelaz*et al*., 2000). Multiple-sequence alignment of GbSEP protein and the SEP proteins in other plants was performed with Vector NTI 11.0 software. The results revealed that the GbSEP protein was highly homologous to MADS or SEP protein in other gymnosperms and angiosperms, with a homology more than 50% (Fig. 3). The homology to PrMADS3 from *Pinus radiate* , PrMADS1 from *Pinus resinosa*,AtSEP4 from *Arabidopsis thaliana*, AhSEP3 from *Alpinia hainanensis*, LjSEP from *Lotus japonicus*, PtSEP from *Populus tomentosa*, and MiSEP3 from *Mangifera indica* was 78%, 71%, 51%, 52%, 55%, 54%, and 54%, respectively. Analysis of the conserved domains showed that the GbSEP protein had a MADS-box domain at 1–61 amino acid in the N-terminal and a K-box domain at 89–174 amino acid in the middle region (Fig. 3). MADS-box and K-box were the conserved domains shared by all SEP proteins (Purugganan *et al*., 1995). These results further indicated that *GbSEP* was a member of *SEP* genes in *G. biloba* MADS-box transcription factor family.

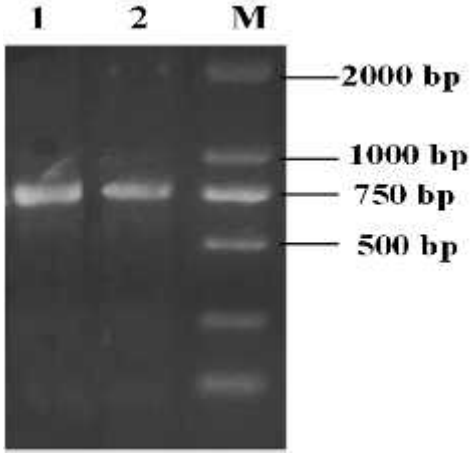

Fig. 1. Elecrtophoresis pattern of amplification of *GbSEP* from *Ginkgo biloba*
M, DNA Marker; 1, Amplified products of GbSEP cDNA; 2, Amplified products of *GbSEP* genomic DNA.

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1      CAGAACGCAGGCGATCCAAATGGGACGAGGTCGAGTCCAGCTGAGGCGCATCGAGAACAA
1                          M  G  R  G  R  V  Q  L  R  R  I  E  N  K
61     AATAAATCGCCAAGTCACGTTTTCAAAGCGGCGAAATGGACTGTTAAAGAAGGCTTACGA
15       I  N  R  Q  V  T  F  S  K  R  R  N  G  L  L  K  K  A  Y  E
121    ACTTTCTGTGTTGTGCGACGCCGAAGTGGCATTGATTGTCTTCTCCACCAGAGGCAAACT
35       L  S  V  L  C  D  A  E  V  A  L  I  V  F  S  T  R  G  K  L
181    CTACGAGTTCGCCAGTTCCAGCATGAACAAGACGCTCGAGAGGTATGAAAAGTGCTCGTA
55       Y  E  F  A  S  S  S  M  N  K  T  L  E  R  Y  E  K  C  S  Y
241    TGCAGTGCAAGATACAAATGTCTCAAACCGGGAAGCACAGAATTGGCATCAAGAGGTTAC
75       A  V  Q  D  T  N  V  S  N  R  E  A  Q  N  W  H  Q  E  V  T
301    AAAACTGAAGTCTAAGGTTGAGCTCCTACAACAGTCACAAAGGCATCTGTTGGGGGAAGA
95       K  L  K  S  K  V  E  L  L  Q  Q  S  Q  R  H  L  L  G  E  D
361    TCTTGGCCCACTCAGTGTGAAGGAGCTCCAGCAACTTGAACGACAGCTGGAGATTGCACT
115      L  G  P  L  S  V  K  E  L  Q  Q  L  E  R  Q  L  E  I  A  L
421    GAATCATGTTAGGTCGAGAAAGAGTCAAGTTATGATGGACTTGATTGATGAGCTTCGGAA
135      N  H  V  R  S  R  K  S  Q  V  M  M  D  L  I  D  E  L  R  K
481    AAAGGAAAGGCTGCTACAGGAAGTGAACAAATCTCTGCACAAGAAGCTTTCAGAATCAGA
155      K  E  R  L  L  Q  E  V  N  K  S  L  H  K  K  L  S  E  S  E
541    GGGACGAAATGCAACCCATGATATGCGGCATCCTACCGACGATAACGGACCTTGGAACCC
175      G  R  N  A  T  H  D  M  R  H  P  T  D  D  N  G  P  W  N  P
601    ATCTGTAAACGGTGGATATGCCCTCCCATCGACCCAGCAAAACACCAACCTCCACCCTGT
195      S  V  N  G  G  Y  A  L  P  S  T  Q  Q  N  T  N  L  H  P  V
661    GGATTGTGAACCGACACTACAAATTGGGTATCAGTCTGTTCCTCGTGAAAGCATTGAGCC
215      D  C  E  P  T  L  Q  I  G  Y  Q  S  V  P  R  E  S  I  E  P
721    TCCACAGGAGCAAACTCACAACCAGCCCCAGGATAACTACACGGGGTGGTGGGTTTGATA
235      P  Q  E  Q  T  H  N  Q  P  Q  D  N  Y  T  G  W  W  V  *
781    TCTTATCAGCGTCACTTCCG
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Fig. 2. The nucleotide sequence and deduced amino acid sequence of *GbSEP*
The specific primers were underlined.

Phylogenetic analysis of GbSEP protein: To further investigate the relationship between *GbSEP* and MADS-box genes of other plants, typical MADS-box genes were selected from five subfamilies of plant MADS-box gene (A, B, C, D and E type gene families) from GenBank. The phylogenetic tree of protein sequence was constructed using the NJ method with Clustal X 2.0 and MEGA 5.1 software. As could be seen in Fig. 4, all MADS-box proteins were clustered in five subfamilies, that is AP1/APE (A function genes), AP3 (B function

gene), PI-like (C function genes), AGL11 (D function genes), and SEP-like (E function genes). Phylogenetic analysis showed that GbSEP is more closely related to SEP proteins, including *Gossypium hirsutum* SEP3 and *P. tomentosa* SEP proteins, than to other MADS-box proteins, such as PI, APE, AGL and AP1 (Fig. 4). Our results suggested that GbSEP shared a common evolutionary origin with other plant SEP proteins based on conserved structure and sequence characteristics, such as amino acid homologies and conserved motifs, respectively. From the above results, it can be speculated that *GbSEP* is a member of the E class MADS-box genes.

Expression analysis of *GbSEP:* As shown in Fig. 5, the *GbSEP* gene is constitutively expressed in the female flowers, male flowers, roots, stems and leaves of *G. biloba*. The expression levels in the female and male flowers were significantly higher than those in roots, stems, and leaves. The organ-specific expression profile indicated that *GbSEP* may regulate the development of specific floral organs. For examine the role of *GbSEP* during flower development, we measured the time-course expression pattern of *GbSEP* in flowers. The results showed that the transcript level of *GbSEP* significantly increased along with the development of male and female flowers (Fig. 6), further implying that *GbSEP*is likely involved in flower development.

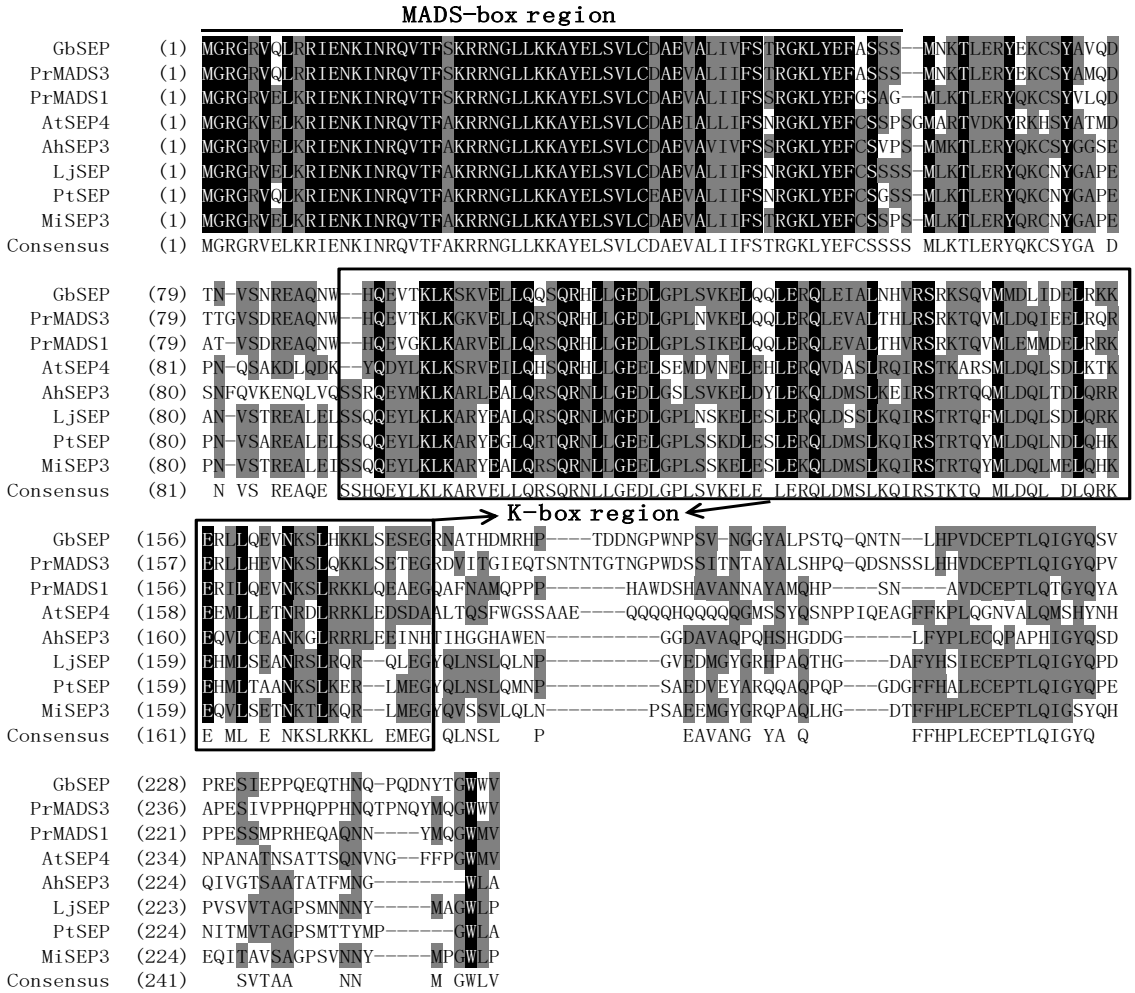

Fig. 3. Mulit-alignment of the deduced sequences of GbSEP protein with those of MADS-box or SEP orthologs from other plant species

The identical amino acids are indicated with white foreground and black background. The conserved amino acids are indicated with black foreground and gray background. Non-similar amino acids are indicated with black foreground and white background. MADS-box and K-box conserved domains indicated with overline and box, respectively. The GenBank accession numbers of these sequences are below: PrMADS3, *Pinus radiata*, AAB58907; PrMADS1, *Pinus resinosa*, CAA70822; AtSEP4, *Arabidopsis thaliana*, NP_849930; AhSEP3, *Alpinia hainanensis*, ACO72983; LjSEP, *Lotus japonicus*, AAX13298; PtSEP, *Populus tomentosa*, ABE02212; MiSEP3, *Mangifera indica*, AEO45959.

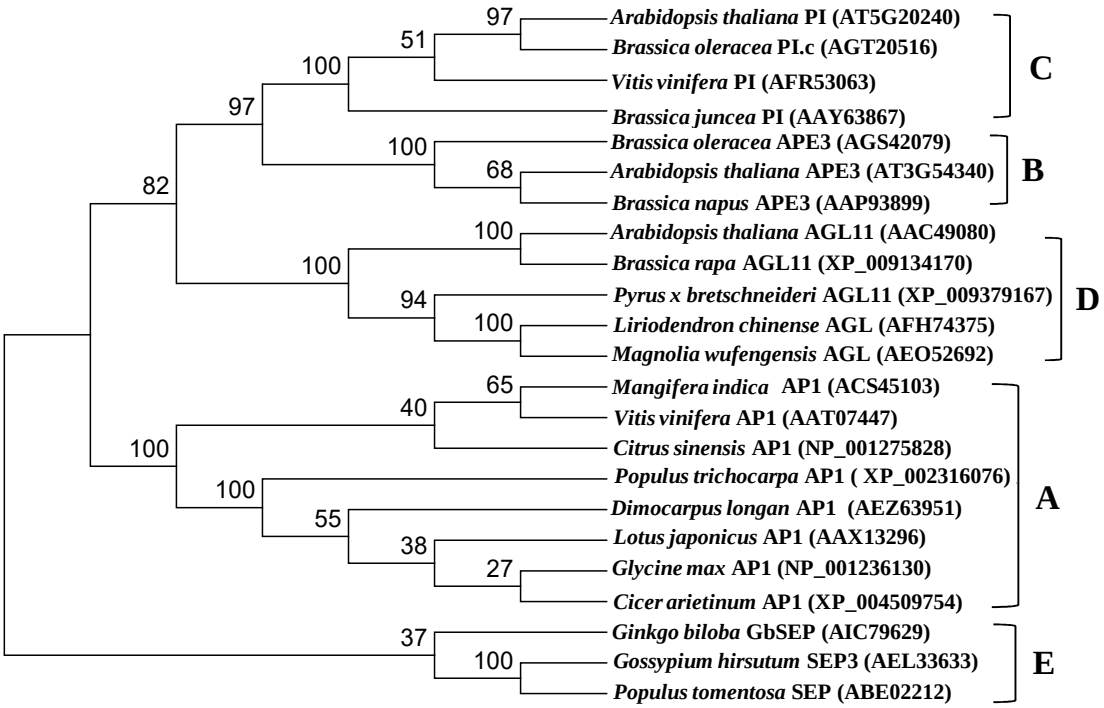

Fig. 4. The phylogenetic tree of GbSEP and otherMADS-boxhomogeneous protein

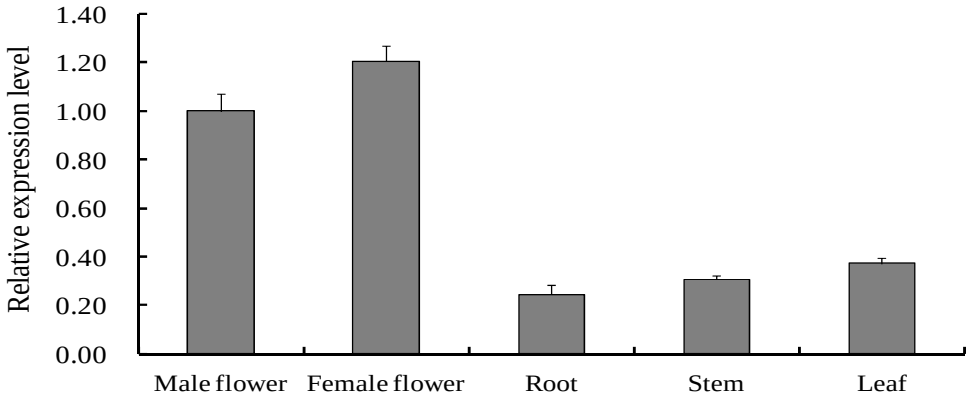

Fig. 5. The relative expression of *GbSEP* in different tissues from *Ginkgo biloba*

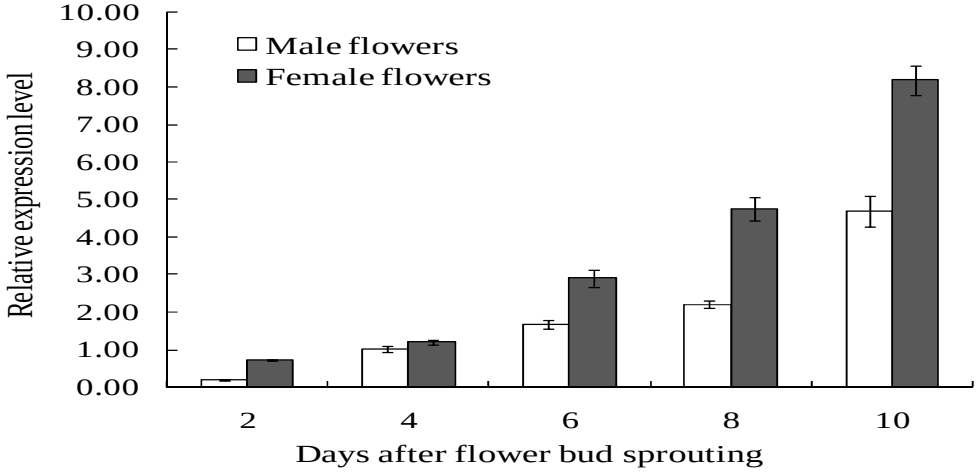

Fig. 6. Time-course expression pattern of *GbSEP* during flower development in *Ginkgo biloba*

Table 1. Nucleotide sequence of *GbSEP* similarity to the MADS gene from other plant species.

Species	Gene names	GenBank accession No.	Homology of nucleotide sequence	E-value
Ginkgo biloba	*GbMADS8*	AB029470.1	78%	2e--116
Ginkgo biloba	*GbMADS2*	KP260628.1	58%	5e--79
Ginkgo biloba	*GbMADS5*	AY114304.1	58%	6e--158
Ginkgo biloba	*GbMADS10*	AB029472.1	56%	3e--65
Ginkgo biloba	*GbMADS9*	KP260627.1	49%	6e--143
Picea abies	*PaMADS*	KC347012.1	76%	7e--103
Pinus radiata	*PrMADS2*	PRU42400	76%	1e--100
Pinus resinosa	*PrMADS*	Y09611	76%	8e--162
Zamia fischeri	*ZfMADS*	KC899695.1	80%	7e--128
Gnetum gnemon	*GGM9*	AJ132215.1	76%	3e--100
Gnetum parvifolium	*GpMADS3*	AB022665.1	76%	2e--98
Welwitschia mirabilis	*WmMADS*	KC899694.1	71%	5e--67

DISUSSION

G. biloba is the most ancient relic plant of dioecism. The male flower has only one floral organ, whereas the female flower is composed of an exposed ovule with a long stalk (Jager*et al*., 2003). The development of the floral organ of *G. biloba* has typical gymnospermous characteristics, different from normal four-whirlfloral organs, and has unique characteristics of morphological structure (Haworth and Raschi, 2014). MADS-box genes have been The differentiation of MADS-box gene was realized 1,300 million years ago or more (Theiβen *et al*., 1996), earlier than the differentiation of angiosperm from gymnospserm (Becker and Theiβen, 2003). Several molecular mechanisms of MADS-box gene diversification were used to a quite different extent during angiosperm and gymnosperm evolution (Theiβen *et al*., 1996; Shindo *et al*., 1999;Gramzow *et al*., 2014). Given that the floral organ of *G. biloba* has unique developmental characteristics, this study on cloning and expression analysis of a MADS-box gene is significant for exploring physiological mechanism of flower development at molecular level in *G. biloba*.

Floral organ identity genes have been subdivided into five different classes, termed class A, B, C, D, and E genes, which provide five different 'homeotic functions'. With the exceptions of APETALA2 (AP2), all genes involved in the ABCDE model are MADS-box genes (Aceto and Gaudio, 2011). Some MADS-box genes have been reported in *G. biloba*. Jager *et al*. (2003) isolated 33 MADS-box genes from *G. biloba* for the first time . Phylogenetic analyses revealed that one of these genes, GBM5, is an orthologue of the *AGAMOUS* (AG) MADS-box gene of *A. thaliana*. Lovisetto *et al*. (2013) also identified a B-sister MADS-box gene*GBM10* from *G. biloba*. *GBM10* overexpression analyses from tobacco have demonstrated that *GBM10* played important role in ovule/seed development. Recently, our group reported a AG clade gene, termed as *GbMADS2*, might be involved in development of reproductive organs (Wang *et al*., 2015). This study isolated an E class SEP gene, i.e., MADS-box gene *GbSEP*, from *G. biloba*. The alignment of the protein sequence indicated that the GbSEP protein was highly homologous to the SEP subfamily of proteins in other plants and had typical MADS-box and Keratin-box conserved domains. Phylogenetic analysis showed that *GbSEP* was clustered in the SEP branch of the E class gene, forming a subfamily branch with the *SEP* homologous gene of other plants. The alignment of protein sequence and phylogenetic analysis indicated that *GbSEP* was the predicted E class MADS-box gene in *G. biloba* and a member of the SEP subfamily. Our results provide theory basis for clarifying the GbSEP function involved in the development of floral organ in *G. biloba.*

The *SEPALLATA*-likegene is an important subfamily of the MADS-box genes and is responsible for floral organogenesis. Four genes, i.e., *SEP1/2/3/4*, are present in most plants. The *SEP4* gene is only expressed in the calyx meristem, whereas the other three genes are mainly expressed in the stamen, pistil, and petal (Uimari *et al*., 2004). The *SEP* gene can promote reciprocal transformation between floral organogenesis and the genesis of nutrition leaf during plant development (Sun *et al*., 2014; Acri-Nunes-Miranda and Mondragón-Palomino, 2014). In addition, the *SEP* gene is involved in the development of almost all floral organs, including the reproductive organ sporophyte embryos (Honma and Goto, 2001). The present study showed that the expression level of *SEP* gene in male and female flowers is significantly higher than in other vegetable organs, consistent with the expression profile of the SEP gene in other plants (Uimari *et al*., 2004; Kobayashi *et al*., 2012). Similar to the time-course expression pattern of another MADS-box gene *GbMADS2* in Ginkgo (Wang *et al*., 2015), we also found the expression level of *GbSEP*

increased along with the flower development. The time and spatial expression profile of *GbSEP* in our data suggested *GbSEP* might play important role in flower development in *G. biloba*. Moreover, *GbSEP* expression was observed in *G. biloba* vegetable organs, such as root, stem, and leaf. This finding indicated that the function of *GbSEP* gene in *G. biloba* is pluripotent and may be associated with plant growth and metabolism processes in which the MADS-box gene is involved. A recent study demonstrated that the homologous gene of the *SEP2* gene in apple, *MADS8/9* gene, can serve as the transcription factor of 1-amino-cyclopropane-carboxylate (ACC) synthase (ethylene biosynthesis activating enzyme) and thus determine the development result and maturation (Ireland *et al*., 2013).

Few literaturehas reported on the regulation mechanism of flowering time and genes for floral organogenesis in *G. biloba* (Brenner *et al*., 2005). The studies on the E class gene have been limited to a few model plants. The function of the E class gene and the reciprocal relationship between this gene and other genes associated with floral organ development remain unclear. The *GbSEP* gene is a member of MADS-box gene family, and its function would be further investigated through *in situ* hybridization, yeast two-hybrid, and transgenetic technology relative to morphology and cytology. The individual development of a plant is a systemic process. To understand the genesis of *G. biloba* flower and the morphological structure of the floral organs, further study on *GbSEP* function and other MADS-box gene of *G. biloba* is being conducted in our lab. Findings will further reveal the regulation of flower bud differentiation and the continued development of male and female organs and fruits from the germination to full-bloom periods by the MADS-box gene, as well as provide important evidence for the molecular mechanism of floral genesis in higher plants.

Acknowledgements: The present work was supported by National Natural Science Foundation of China (No. 31370680), the Natural Science Foundation of Hubei Province (No. 2013CFA039), the Key Projects of Chinese Ministry of Education (No. 212112 and 210137).

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