EXCELLENT PUBLISHERS

International Journal of Current Research in Biosciences and Plant Biology

ISSN: 2349-8080 Volume 2 Number 5 (May-2015) pp. 226-233

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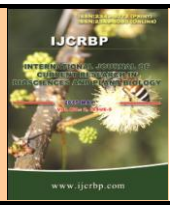

Original Research Article

Cloning and Sequence Analysis of AMADS-Box Gene from *Ginkgo biloba*

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A b s t r a c t

Members of the MADS-box gene family encode transcription factors which play fundamental roles in developmental control and signal transduction in plants. In this report, a gene encoding MADS-box (named as GbMADS10) was isolated by PCR from *Ginkgo biloba*, and the sequence was analyzed by using bioinformatics software. The results revealed that the open reading region of GbMADS10 is 1143bp long, encoding 381 amino acids. The predicted amino acid sequence of GbMADS10 containing a MIKC consensus motif displays extensive homology to MADS amino acids from other plants, including *Eschscholzia california*, *Vitis vinifera*, *Coffea arabica*, *Prunus mume* and *Zea mays*. Phylogenetic tree analysis showed that the GbMADS10 have a closer relationship with AGL11 from *Cucumis melo* than from other plants. The isolation and sequence analysis of GbMADS10 provided basis for further studying the molecular mechanism of flower development in *G. biloba*.

K e y w o r d s

Flowering

Ginkgo biloba

MADS-box

Sequence analysis

Introduction

MADS-domain of the MADS-box transcription factor comprises 60 amino acids which are highly conserved across developmental control genes from yeast, animals, and plants (Shore and Sharrocks, 1995). MADS-box consisting of many homeotic genes with similar intron and exon structures. There are 107 homeotic genes have been identified in Arabidopsis (Paenicova et al., 2003). MADS-box genes encode a family of transcription factors which control diverse developmental processes in flowering plants ranging from root to flower and fruit development. In recent years, the role of MADS-box proteins in controlling floral organ development has been extensively reported (Ma, 1994; Davies and Schwarz-Sommer, 1994). The study on the MADS-box gene family has mainly concentrated on the regulatory effects that it exerts on the development of flower organs differentiation, forming the well-known "ABCDE Floral Model" (Smaczniak et al., 2012). This model divides the MADS-box gene into 5 classes, according to the different roles they play in the development process of the flower meristem. A-class genes

(APETALA1, APETALA2) control the formation of the calyx; B-class genes (APETALA3, PISTILLTA) together with A-class control the development process of the petal; C-class genes (AGAMOUS) control the development of the carpel; D-class genes (AG11, SEEDSTICK, SHATTERPROOF1/2) have very close consanguinity with C-class genes, thus their genetic functions have certain overlap with those of C-class. D-functional genes were first found in the petunia. When FBP7 (FLORAL BINDING PROTEIN7) and FBP11 gene exhibit ectopic expression, the perianth of transgenic plants form an ectopic ovule, thus the two genes are considered as the key to controlling the development of the ovule. Judging from its evolutionary origins, the ovule is the flower's inner organ of the angiosperm and gymnosperm, which can be taken as the fifth-turn organ of the flower (Nitasaka, 2003). E-class genes (*SEPALLA*1/2/3/4) co-function with other classes of genes, they determine the transformation from nutritive organ (leaves) to the reproductive organs (flowers), and participate in the formation of each flower organ. E-class genes regulate the development of sepals, petals, stamens and carpels with the other four classes of genes, and also can prevent the meristem of flowers from indeterminate growth (Itoh and Izawa, 2013).

Apart from determining the characteristics of the meristem and flower organs, the MADS-box gene also participates in the adjustment of vegetative growth (Cohen et al., 2012), ovary development (Favaro et al., 2003), testa development (Rameneni et al., 2014), root formation (Lee et al., 2015), embryonic morphogenesis (Wang et al., 2015), symbiotic induction (Heard and Dunn, 1995), fruit after-ripening (Ireland et al., 2013). Recent research has demonstrated that another important function of this gene's expression is participating in the metabolism of the secondary metabolite (Penn et al., 2014). Currently, the homologous genes of MADS-box have been isolated from most plants, and the analytical research thereof has been done in the function and expression of different genes. Such research has mainly concentrated on model plants such as Arabidopsis, Snapdragon and Petunia. Based on research around such model plants, the results have shown that, during the evolutionary process, the functions of the MADS-box gene have gone through differentiation to various degrees (Alvarez et al., 2014). Thus, not all orthologous genes have similar functions. Therefore, conducting research on the functions of the MADS-box gene in considerable species in different evolutionary statuses can facilitate our understanding of the origins of the MADS-box gene and its functions in diversification. Only a small amount of research is available on the regulatory functions of the MADS-box in the growth, development and flowering of gymnosperm.

Ginkgo biloba is the only surviving member of one of the oldest living gymnosperm plant groups with medicinal, spiritual and horticultural importance worldwide. The *G. biloba* is of dioecism, whose flower organs have unique features of morpho-differentiation (e.g., miniaturized dispersed gametophyte or pollen, modified female ovule or sporangium, containing the female gametophyte and becoming an embryo-containing seed (Jager et al., 2003), making it an undoubtedly good material for studying the MADS-box gene. Up until now, data are lacking about MADS-box genes in Ginkgo. In this study, the full-length sequence of the GbMADS10 gene was cloned from *G. biloba*, and the molecular characterization of GbMADS10 was analyzed. The results do some preparation for transgenic research of GbMADS10, and lay the foundation for facilitating the study of *G. biloba* flowering molecular mechanisms.

Materials and methods

Plant materials

Twenty-year-old grafts of *G. biloba* were grown in an orchard at Yangtze University, in China. the leaves were collected, immediately frozen in liquid nitrogen, and kept at -80°C prior to DNA and RNA extraction.

Isolation of the full-length cDNA of GbMADS10

Total RNA was extracted from ginkgo leaves using the CTAB method (Cai et al., 2007). Genomic DNA was extracted from the leaves of *G. biloba* following the CTAB method described by Xu et al. (2008). The quality and concentration of the RNA and DNA were determined by agarose gel electrophoresis and spectrophotometer analysis. The primers MADS10UP (5′-ATGGGCAGAGTTAAGCTCCCGAATA-3′) and MADS-10DP (5′-TTTCAAGTTACTTTGAAATGAAGCATCC-3′), were designed and synthesized (Shanghai Sangon, China) to obtain the full-length cDNA and DNA. One-step reverse transcription PCR (RT-PCR) was performed 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, 54°C for 1 min, and 72°C for 1 min; followed by an extension for 10 min at 72°C. The PCR product was purified, cloned into the pMD18-T vector (Dalian TaKaRa, China), and then sequenced.

Bioinformatics analysis and molecular evolution analyses

The obtained sequences were analyzed using online bioinformatics tools (http://www.ncbi.nlm.nih.gov and http://www.expasy.org). The software DNA man 6.0 was used to analyze GbMADS10 gene sequence and amino acid composition. The two-dimensional structure of GbMADS10 protein was analyzed using online bioinformatics tools (https://prabi.ibcp.fr/htm/ index.php). The software Clustal x 2.0 was used was used for sequence multi alignment, and the software MEGA 4.0 was used to construct the evolution tree.

Results

cDNA cloning of GbMADS10

Using One-step reverse transcription PCR method, the full-length cDNA sequence of GbMADS10 gene was finally obtained from *G. biloba*. Conventional PCR was used for GbMADS10 genome DNA amplification. The length of the open reading frame is 1143bp without intron and encoding 381 amino acids (Fig. 1). The cDNA sequence of GbMADS10 had high similarity with other MADS-box genes.

Fig. 1: Nucleotide sequence and deduced amino acid sequence of GbMADS10.

```
1    ATGGGCAGAGTTAAGCTCCCGATTAAGAAAATAGAGAATAGCACTAACAGGCAGGTCACCTTCTCCAAACGGAGA
       M  G  R  V  K  L  P  I  K  K  I  E  N  S  T  N  R  Q  V  T  F  S  K  R  R
76   AATGGATTGATCAAAAAGGCATATGAACTCTCAGTTCTGTGCGACATCGAAATTGCACTCATAATGTTCTCGCCC
       N  G  L  I  K  K  A  Y  E  L  S  V  L  C  D  I  E  I  A  L  I  M  F  S  P
151  TCGGGAAGATTGAGCCACTTTTCAGGGAAAAATAGTAGGATAGAAGATGTTATAGCTCGCTTCGTCAATCTGCCT
       S  G  R  L  S  H  F  S  G  K  N  S  R  I  E  D  V  I  A  R  F  V  N  L  P
226  GAGCACGAGAGGCCAAGGCTTGTCCAAAACCAAGAATATCTCCTCAGAGCGTTGAAAAAACTCAAGTACGAAAGC
       E  H  E  R  P  R  L  V  Q  N  Q  E  Y  L  L  R  A  L  K  K  L  K  Y  E  S
301  GATATCGCCAATCATCTTGCAAGCCCAAACATTGTCGACTCAAATGTGGAGGAACTTCAAATGGATATTCGGAGA
       D  I  A  N  H  L  A  S  P  N  I  V  D  S  N  V  E  E  L  Q  M  D  I  R  R
376  CAGAGAATTCAGTTGGAGGAAGCCCAGCAGAAGTTAAGGAGTTTTAAAGAGGATCCACTTCTTATAACCTCAATA
       Q  R  I  Q  L  E  E  A  Q  Q  K  L  R  S  F  K  E  D  P  L  L  I  T  S  I
451  CAAGATGCAGATCAATATGAAAGGACATTGGAGGAGGCTTTGCGTAGAGTTCGTCTCAGAAAACAACAGCTAGAA
       Q  D  A  D  Q  Y  E  R  T  L  E  E  A  L  R  R  V  R  L  R  K  Q  Q  L  E
526  CACAACCAAATGGCAGTTGCTTTCAACGATGCAAACTTGCAGTTTTATATACAAACACAGAATGGATTGCCAAAT
       H  N  Q  M  A  V  A  F  N  D  A  N  L  Q  F  Y  I  Q  T  Q  N  G  L  P  N
601  GGAACAGACACAAGTCAAAATCACCTATACAACTCATGGATGCCACAGGGAGACCCCCATACTAGTGTCCAGAAT
       G  T  D  T  S  Q  N  H  L  Y  N  S  W  M  P  Q  G  D  P  H  T  S  V  Q  N
676  TTTATGGAGCATGAAAATTCTAATGCCATGCTTGCGATGCGGGAAGCACAATGCATGGCAAAGTGTTTACAAAAT
       F  M  E  H  E  N  S  N  A  M  L  A  M  R  E  A  Q  C  M  A  K  C  L  Q  N
751  GGAACCGTATTTCCGGCACTTCAAGATGCCACTGGAATGCAGCTTCCAAATGAGTCTGCAAGTACTCAACCGTAT
       G  T  V  F  P  A  L  Q  D  A  T  G  M  Q  L  P  N  E  S  A  S  T  Q  P  Y
826  ATTCCTACATCACACATGCAGTTTGATTACACTTTAACTGACAACAACAATAATGAACATGCAGAACAGGCCGAT
       I  P  T  S  H  M  Q  F  D  Y  T  L  T  D  N  N  N  N  E  H  A  E  Q  A  D
901  ATAGCTGCAGCGTTTGACTATGGTTCTGATGCAATGGCATCCGTACATTGGCAAACTTCGTATGGGTCGATGACT
       I  A  A  A  F  D  Y  G  S  D  A  M  A  S  V  H  W  Q  T  S  Y  G  S  M  T
976  CCAATTGTGACAAATCAACAGTATCCTTTGACTAAGGGAATAATGCAAAATATTGTTCCGCCTAGTATGTCAATA
       P  I  V  T  N  Q  Q  Y  P  L  T  K  G  I  M  Q  N  I  V  P  P  S  M  S  I
1051 TACCAGCAAGATGGCTCTTCCTCACAGGGCACTCACCATTCAACACCTCAAGACAATGCAGGAATGGATGCTTCA
       Y  Q  Q  D  G  S  S  S  Q  G  T  H  H  S  T  P  Q  D  N  A  G  M  D  A  S
1126 TTTCAAAGTAACTTGAAA
       F  Q  S  N  L  K
```

Characterization of the deduced GbMADS10 protein

The deduced amino acid sequence for the GbMADS10 polypeptide was also shown in Fig. 1. By using the software of Computer pI/Mw tool at www.expasy.org, the calculated pI and molecular weight of the deduced GbMADS10 polypeptide were predicted to be about 6.38 and 43.2 kDa , respectively. Through Blastp aligning analysis, the deduced GbMADS10 protein was found to have a conserved MADS-box domains, and MADS_MEF2_like, K-box region, was also found. The GbMADS10 gene is MIKC-type MADS protein, belonging to the MEF2 (myocyte enhancer factor 2)-like/Type II MADS subfamily of MADS-box family eukaryotic transcriptional regulators. The secondary structural of GbMADS10 polypeptide was predicted by using GOR method (Fig. 2). The result showed GbMADS10 content alpha helix (H) (38.32%), beta-sheet (E) (17.06%), and random coil (C) 44.62%. The grand average of GbMADS10 hydropathicity (GRAVY) is -0.672, indicating that the protein is hydrophilic.

A database search with Blast P (NCBI) and multialignment by Vector NTI 10 showed that the deduced GbMADS10 polypeptide had a certain similarity with other MADS from other plant species (Fig. 3). The amino acid sequence of GbMADS10 was 56%, 57%, 53%, 62%, 6%, 56%, 58% and 50% identical to MADS from *Eschscholzia californica*, *Vitis vinifera*, *Beta vulgaris* subsp. *vulgaris*, *Coffea arabica*, *Fragaria vesca* subsp. *Vesca*, *Prunus mume*, *Zea mays* and *Pyrus* × *bretschneideri*. All of these protein have conservative MADS area and K area.

Fig. 2: The secondary structural of GbMADS10 protein.

```
    10        20        30        40        50        60        70
     |         |         |         |         |         |         |
MGRVKLPIKKIENSTNRQVTFSKRRNGLIKKAYELSVLCDIEIALIMFSPSGRLSHFSGKNSRIEDVIA
Rccccceeceeccccccchhhhhhccchhhhhhhhhcccceeeeeeeccccccceeccccccchhhhh
hh
FVNLPEHERPRLVQNQEYLLRALKKLKYESDIANHLASPNIVDSNVEELQMDIRRQRIQLEEAQQKLRS
Fhccccccchhhhhhhhhhhhhhhhhhhhhhhhhhcccccccccchhhhhhhhhhhhhhhhhhhhhh
hh
KEDPLLITSIQDADQYERTLEEALRRVRLRKQQLEHNQMAVAFNDANLQFYIQTQNGLPNGTDTSQNHL
Yccccceeechhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhhhccccccccccccccce
ee
NSWMPQGDPHTSVQNFMEHENSNAMLAMREAQCMAKCLQNGTVFPALQDATGMQLPNESASTQPYIPTS
Hceeccccccceeeeeeeeeechhhhhhhhhhhheeeccccccccccccccccccccccccccccccc
cc
MQFDYTLTDNNNNEHAEQADIAAAFDYGSDAMASVHWQTSYGSMTPIVTNQQYPLTKGIMQNIVPPSMS
Icceeeeeeccccccchhhhhhhhhhhhhccchhhhhheeeeccccceeeeeccccccccccccceeccccc
ee
YQQDGSSSQGTHHSTPQDNAGMDASFQSNLKeeccccccceeccccccccccceeeeeeeec
```

Molecular evolution analysis

The research of MADS-box genes in the gymnosperm focused on conifers and gnetales (Katahata et al., 2014), such as *Picea* and *Pinus*. To investigate the evolutionary relationships among GbMADS10 and other MADS proteins, a phylogenetic tree was constructed based on the deduced amino acid sequences of GbMADS10 and other MADS family proteins from other plant species. MADS family proteins sequence formed five distinct function specific clusters (Fig. 4). The GbMADS10 protein together with *Cucumis*, *Nicotiana*, *Solanum*, *Pyrus*, and *Brachypodium* were grouped into D functional cluster, suggesting GbMADS10 having the closest relationship with those plant species.

Fig. 3: Multiple alignments of GbMADS10 protein with those of MADS-box from proteins other plant species.

The completely identical amino acids were indicated by white letters on a black background ,whereas those conservative amino acids were indicated by white letters on a grey background and those non-similar amino acids were indicated by black letters on a white background.The GenBank accession numbers of these sequences are below: ECAGL66, *Eschscholzia californica* (CAX16992.1); VvAGL8, *Vitis vinifera* (XP_010652831.1); BVMADS27, *Beta vulgaris* subsp. *vulgaris* (XP_010680585.1); CAMADS, *Coffea arabica* (ADU56838.1); FSMADS6, *Fragaria vesca* subsp. *vesca* (XP_011465524.1); PBAGL66, *Pyrus × bretschneideri* (XP_009354141.1); PrAGL, *Prunus mume* (XP_008233985.1).

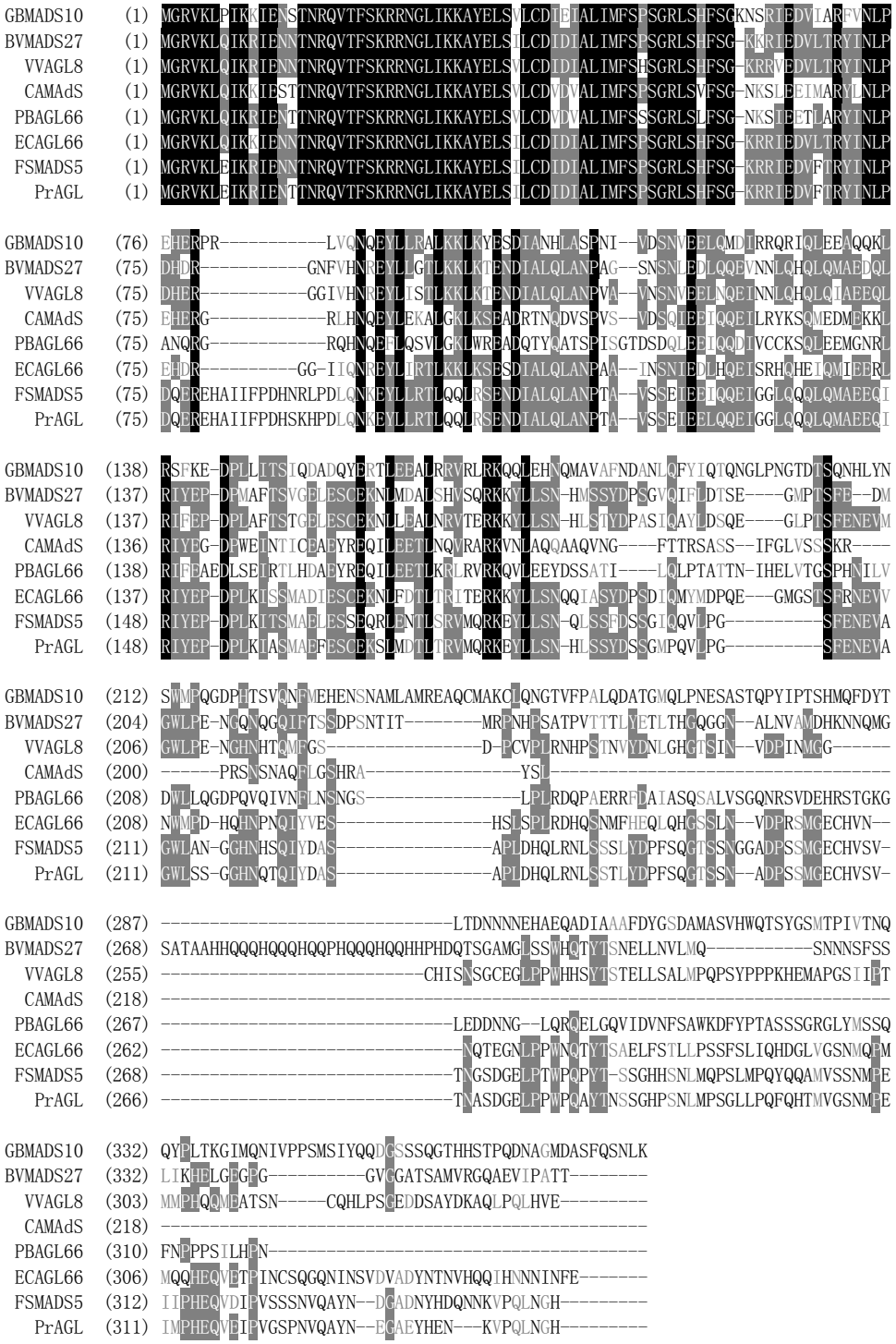
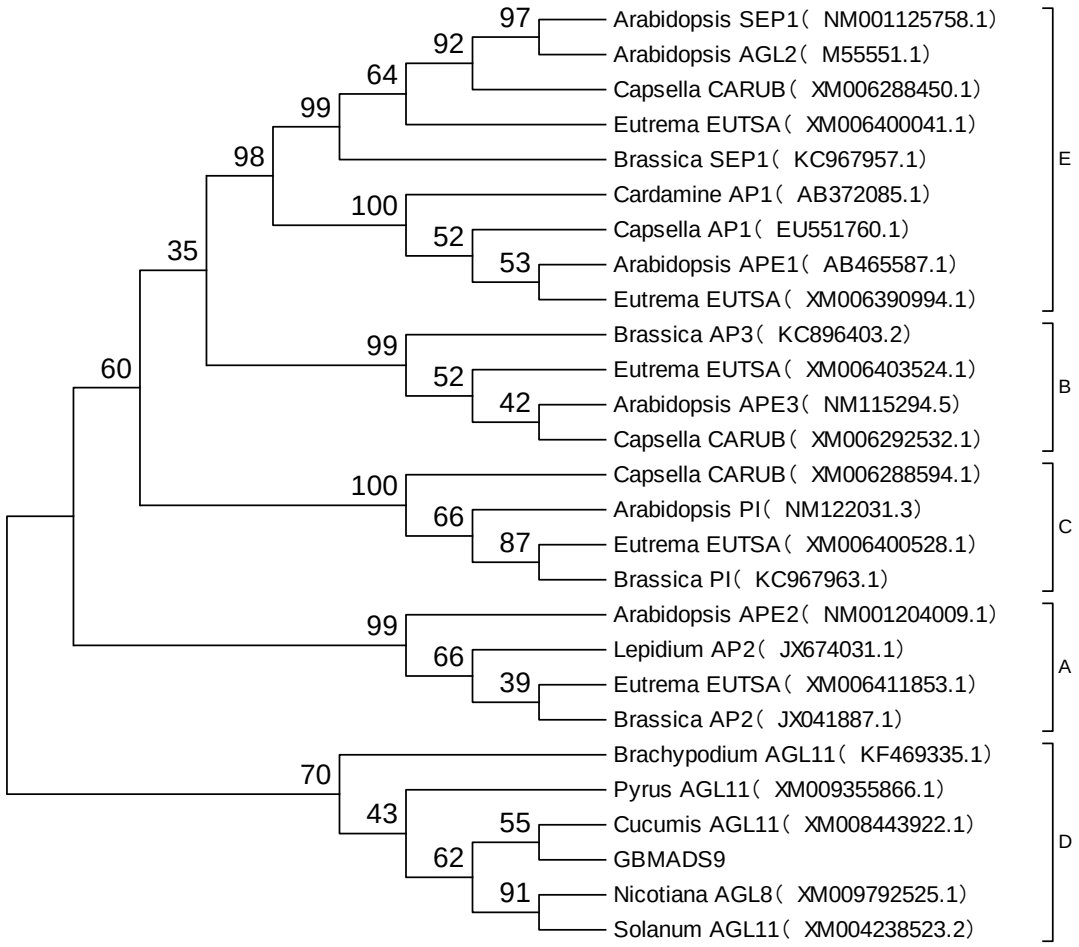


Fig. 4: Neighbor-joining phylogenetic tree of the sequences of GbMADS10 and other MADS-box proteins.



Discussion

In this report, a novel GbMADS10 cDNA sequence was presented and characterized. Multiple alignments showed that the deduced GbMADS10 was homologous with other known MADS-box proteins, and it contained conserved MADS-box domains belong to MADS-box protein family. Most MADS-box genes participate in different phases of floral development and form a complicated gene regulating network with each other to determine the characteristics of a flower's primordium and organs. As showed in the phylogenetic tree, the GbMADS10 protein was grouped into D functional cluster. D-functional genes can control the development of ovule. Floral binding protein 7 (FBP7) and Floral binding protein11 (FBP11) belong to D-functional genes that control the development of ovule in petunia (Colombo et al., 1995), both of them were expressed in the process from ovule primordium to ovule maturation, and the ovule will be converted to a carpel-shaped structure when the express of FBP7 and FBP11 genes were suppressed. Different from model plants, the formation of floral primordium and the development of flower organs in gymnosperm are uniqueness. The female flower bud of the *G. biloba* has more than one leaf primordia and flower primordia, and it has a naked ovule. Some researchers call it ovule instead of the female flower. The cloned GbMADS10 gene belong to D-functional genes, therefore, we can speculate that GbMADS10 might regulate the differentiation process of female flower together with other regulatory factor in *G. biloba.* The functional characterization of GbMADS10 gene was also been recently proceeding.

Conclusion

GbMADS10 with a ORF about 1143bp, encoding 381 amino acids was isolated by PCR from *G. biloba*. The amino acids of GbMADS10 contain a conserved

MADS-box domains and displays extensive homology to MADS amino acids from other plants. The phylogenetic tree showed the GbMADS10 protein was grouped into D functional cluster. The GbMADS10 might involve in regulating the differentiation process of female flower in *G. biloba*.

Acknowledgement

The present work was supported by National Natural Science Foundation of China (31400603), the Key Projects of Chinese Ministry of Education (No. 210137 and 212112), and the Natural Science Foundation of Hubei Province (No. 2013CFA039 and 2009CDB232), and International Science and Technology Cooperation Project of Hubei Province (2013BHE029 and 2013BHE039).

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