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Molecular Cloning and Sequence Analysis of MADS-Box Family Gene (GbMADS6) from *Ginkgo biloba* L.

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Abstract

Based on the results of our research group study on gingko using RNA-seq technology, a full-length cDNA sequence of MADS-box gene from *Ginkgo biloba* was cloned by RT-PCR, which named as GbMADS6 (GenBank accession no. KX061105). The GbMADS6 contains a 735 bp open reading frame(ORF) corresponding to a deduced protein of 245 amino acids, while the estimated isoelectric point (pI) and molecular weight of the putative protein were 5.13 and 59.67 kDa, which had a typical MADS-box domain. Homology analysis indicated that the deduced GbMADS6 protein was highly homologous to other MADS-box Proteins from different species, especially with *Lolium perenne* MADS4 and *Sorghum bicolor* MADS1 identities up to 81%. Predicted protein secondary structure showed that GbMADS6 protein contents of the structure with 51.84% of alpha helix (Hh), extended strand (Ee) of 15.10%, random coil (Cc) of 6.94% and beta turn (Tt) of 26.12%. Phylogenetic analysis showed that GbMADS6 has a closer relationship with *Pinus tabuliformis* MADS protein than with other MADS-box proteins. Those results suggest that GbMADS6 belongs to MADS-box super family and may be involved in regulating the development of the flower.

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Keywords

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Introduction

Ginkgo biloba L., commonly called maidenhair or sun tree, is a dioecious tree native to China. Often described as a "living fossil", it is the world's oldest relic plant. Conventional breeding plays a limited role in this program because ginkgo has characteristics such as highly long juvenile period, complex genetic background as well as usually blossoms and bears fruit 15 to 20 years after growing. Therefore, how to promote blossom of seedling and shorten of the juvenile period on ginkgo development is necessary. MADS gene was an essential gene involved in the photoperiod pathway controlling plant flowering time. MADS genes have been cloned from many plants, such as apple (Sung et al., 2000), *Arabidopsis* (Tapia-López et al., 2008), citrus (Endo et al., 2006), peach (Bielenberg et al., 2008), mango (Pacheco-Sánchez et al., 2014) and tomato (Martel et al., 2011).

The key step of the flowering process was linked to the inflorescence primordium formation and floral organ differentiation, and then the formation of its floral organ have the reproductive functions, of certain shape and specific order of space (Bernier et al., 1974). As a kind of transcription factors, MADS-box gene played a very important role in kinds of cellular processes, especially in the development of floral organs (Shore and Sharrocks, 1995). The ABC model illustrated the molecular mechanism of floral organ development, and explained the floral organ mutants of homeotic gene (Weigel et al., 1994), after Theissen

et al. (2000) structured the latest advances in the genetic ABCDE model of floral organ development. Basing on the ABCDE model, the expression of class A genes command sepal formation and petal development, class B gene activities specifies the development of petals and stamens. In this same way, class C gene expression specifies stamen and pistil formation in the blossom, class D genes determine ovule development, while class E and ABC gene activities regulate the development of floral organ (Adam et al., 2007).

The MADS-box gene was a kind of specific regulation of gene family and widely present in plants, which encode proteins were classification transcription factor. MADS-box genes had a highly conserved DNA binding domain that was a MADS box (Heuer et al., 2000), the MADS-box gene was identified and bound downstream target specific sites, through the binding domain, aimed at regulating the expression of target genes (Jack et al., 1992). The MADS-box gene was the main control factor of the development process, experimental investigation showed that MADS-box gene family were not just regulation of floral organ development, in addition, MADS-box gene in the control of the time of flowering, determine of tissue differentiation (Weigel et al., 1995), control of embryonic development (Perry et al., 1996), promote the formation of root (Purugganan, 1997) and regulate the development of seeds (Ohad et al., 1996) and fruits (Gu et al., 1998) played an important role. In recent years, the research emphasis was on MADS-box gene regulation of reproductive growth, understanding the regulation of the plant from the vegetative to reproductive growth process has important significance for control the growth and development of a flowering plant. The molecular mechanism of floral organ development is a hotspot in plant developmental biology researches. In this study, we characterized the MADS6 gene from the *Ginkgo biloba* for the first time, and GbMADS6 might have played a significant role in the regulation of ginkgo flowering.

Materials and methods

Plant materials

Twenty-year-old grafts of *Ginkgo biloba* were grown in an orchard at Yangtze University, in China. The flowers were collected, immediately put into liquid nitrogen, and kept at Ultra-Low Temperature Freezer-Uplight type (DW-86L729, Haier Co. Ltd., China) prior to total RNA extraction.

RNA extraction and isolation of GbMADS6

Total RNA was isolated from frozen plant tissues using the CTAB method (Cai et al., 2007). The products of total RNA were separated on 1.5% agarose gel (Sangon Biotech, Shanghai, Co. Ltd.) stained with ethidium bromide. The process for inverse transcription of cDNA through reverse transcriptase byusing PrimeScript™1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) with RNA as a template. The specific primer GbMADS6-F (5′-AGGTGCAGTACCGTTTCTGTGTGC-3′) and reverse primer GbMADS6-R (5′-TTTTTTTCTA CCAAGAAAGT CATTAGCG-3′) were designed with the EST sequence of the Ginkgo MADS6 gene. The PCR reaction was carried out in a final volume of 50µl with a reaction mixture containing 2µl cDNA products, 5.0 µl 10×buffer, 4.0 mmol l^{-1} $MgCl_2$, 1.0µl TaqDNA polymerase, 1.0 µl dNTP mix (10 mmol l^{-1}) and 33.5µl ddH_2O up to 50 µl. The reaction was performed at 95℃ for 3 min, and then subjected to 34 cycles of 95℃ for 20s, 58℃ for 30 s and 72℃ for 90s, plus a final extension at 72℃ for 10 min. The amplified products were analysed by 1% gel electrophoresis and purified by a AxyPrep DNA Gel Extraction Kit. The purified product was cloned into the pMD18-T vector (Dalian TaKaRa, China), and then sequenced (Sangon Biotech. Shanghai Co. Ltd.).

Bioinformatic analysis

Protein and DNA homology searches were performed by using the BLAST program in website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment was performed by using the website http://multalin.toulouse.inra.fr/multalin/. Phylogenetic analysis of GbMADS6 from ginkgo and other MADS from other plants was performed by using software Clustal X 2.0 and MEGA6.0, The dependability of the tree was determined by bootstrap analysis with 1000 replicates.

Results

cDNA cloning of GbMADS6

Based on the sequences of EST, the full-length cDNA fragment of GbMADS6 was amplified by PCR using a pair of specific primer. The length of the cDNA sequence of GbMADS6 is 1291bp (GenBank accession no. KX061105), and the open reading frame is 735bp encoding 245 amino acids (Fig. 1). The cDNA sequence of GbMADS6 had high similarity with other MADS-box genes, and the G+C content of GbMADS6 sequence is 42.8%.

```
1       aggtgcagtaccgtttctgtgtgcatgcatatcaaggatttaattgcttgccccctcccg
61      cacaagcgaattttcagtagatcggaaaaaaaaataagtagagatttccctagatgtagcg
121     tttcttggtcaagcctacacatcaatgaatatcaagaaaatccaaacacgaaaaccagaa
181     aaaggaggaagcagatcatcATGGGGAGGGGTCGAATCCAGCTGAAGCGGATCGAGAACA
                          M  G  R  G  R  I  Q  L  K  R  I  E  N
241     AGATCAATCGCCAAGTCACCTTTTCAAAGCGCCGAAATGGGCTCCTTAAAAAGGCTTGTG
        K  I  N  R  Q  V  T  F  S  K  R  R  N  G  L  L  K  K  A  C
301     AGCTGTCCATCTTGTGCGATGCGGAGGTGGCCTTGATCATCTTCTCCAACAGAGGCAAAC
        E  L  S  I  L  C  D  A  E  V  A  L  I  I  F  S  N  R  G  K
361     TCTACGAGTACGCCAGTTCCAGCATGAGCAAGACGTTGGAGAGGTACCAAAAGAGCTTGC
        L  Y  E  Y  A  S  S  S  M  S  K  T  L  E  R  Y  Q  K  S  L
421     ATGTAATCCCAGATACAAACGTCACAACTAGGGAGGCACAGAATTGGCATCAAGAGGTCA
        H  V  I  P  D  T  N  V  T  T  R  E  A  Q  N  W  H  Q  E  V
481     CAAAATTGAAGGGCAAGGTTCAGATCCTACAACAGTCACAAAGGCATTTGTTGGGTGAAG
        T  K  L  K  G  K  V  Q  I  L  Q  Q  S  Q  R  H  L  L  G  E
541     ATCTTGGTATGTTAAGTCTTAAAGAGTTGCACCAATTAGAGCATCAAGTAGAAGTTGCTT
        D  L  G  M  L  S  L  K  E  L  H  Q  L  E  H  Q  V  E  V  A
601     TGAAGCATCTAAGGTCGAGAAAGACTCAGATAATGCTGGACCAGATTGATGATCTACGCA
        L  K  H  L  R  S  R  K  T  Q  I  M  L  D  Q  I  D  D  L  R
661     AAAAGGAGCGCATGTTACAGGAAGTAAACAAATCTTTGCACAAGAAGTTTTTGGAGGCCG
        K  K  E  R  M  L  Q  E  V  N  K  S  L  H  K  K  F  L  E  A
721     ATGGACAAAATGCATGTAATTTTGGGCAATTCAGTCGACCGTGGGACTCTGCTGTAGGAA
        D  G  Q  N  A  C  N  F  G  Q  F  S  R  P  W  D  S  A  V  G
781     ACCCTGCCTACGGTGTAAATGAACCTGACAGTCACGTCCAGCCAGCACATCGCGAACCGA
        N  P  A  Y  G  V  N  E  P  D  S  H  V  Q  P  A  H  R  E  P
841     CTCTACACATTGGGTATCGCGAGGCTGCCCATCCTGTAAGCACTGTTCATAGACAGAAGC
        T  L  H  I  G  Y  R  E  A  A  H  P  V  S  T  V  H  R  Q  K
901     AAAGCGCGAATCATTATACGCAGGACTGGATGGTATGaaaattttcatgttccgagtcca
        Q  S  A  N  H  Y  T  Q  D  W  M  V  *
961     ttcttggatcgcagattatataaatagcatggcagcatcaacttttgctggatatcccca
1021    atttcacctttctattcacagcatcaagtgatgatgctttcgtttttaaagtgaattaaa
1081    tggggatgacaatcagtaattcctcgttaacctgtttacattgtttgagatctgtctgat
1141    aaaattctcaaacattttggttacctctctgtggcacaatggtaaacacactagaattag
1201    ctggtaatttcctatatatgtactaactttggtgtgttcttttgaatcgttcataaatgt
1261    gcacgctaatgactttcttggtagaaaaaaa
```

Fig. 1: The nucleotide acid sequence and deduced amino acid sequence of GbMADS6.

Characterization of the deduced GbMADS6 protein

Through analysis with the Computer pI/Mw (http://web.expasy.org/compute_pi/), we found the molecular weight and isoelectric point of predicted GbMADS6 amino acid were 5.13 and 59.67 kDa, respectively. Blastp aligning analysis found the deduced GbMADS6 protein has MADS_MEF2_like, K-box region, and conserved MADS-box domain. The cloned GbMADS6 belongs to MADS super family. The secondary structures of MADS proteins were predicted by using SOPMA tool. It was found that GbMADS6 contents extensive alpha helix (Hh) 51.84%, extended strand (Ee) 15.10%, random coil (Cc) 6.94% and beta turn (Tt) 26.12% (Fig. 2). The secondary structures of deduced GbMADS6 had high similarities with *Lilium formosanum* and *Dendrocalamus latiflorus* MADS-box proteins (Table 1).

Table 1. Secondary structure of MADS6 proteins (unit: %).

Species	Alpha (Hh)	Extended strand (Ee)	Random coil (Cc)	Beta turn (Tt)
Ginkgo biloba	51.84%	15.10%	6.94%	26.12%
Lilium formosanum	55.37%	11.98%	8.68%	23.97%
Dendrocalamus latiflorus	54.92%	17.21%	8.61%	19.26%

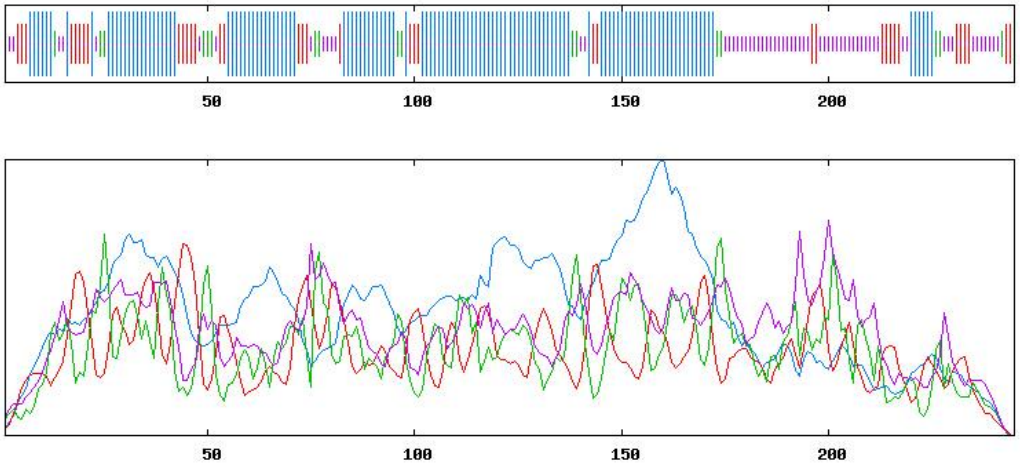

Fig. 2: The predicted secondary structures of GbMADS6 protein.

```
           1       10        20        30        40        50        60        70        80
           |--------+---------+---------+---------+---------+---------+---------+---------|
GbMADS6                                MGRGRIQLKRIENKINRQVTFSKRRNGLLKKACELSILCDAEVALIIFSNRGKLYE
AlsMADS                              IEMGRGRVELKRIENKINRQVTFSKRRNGILKKAYELSVLCDAEVALIIFSSRGKLYE
LpMADS4   LARADQWCFAWSIECSKEEEEEEIMGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYE
ZmMADS5                                MGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSGRGKLYE
MalMADS2                               MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLFE
AfSEP2                                 MGRGKVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYE
SbMADS1                       LEGLGAMGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYE
BdMADS                                 MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYE
Consensus                     ......MGRGr!#LKRIENKINRQVTFaKRRNGlLKKAyELS!LCDAEVALIIFSnRGKL%E

           81      90        100       110       120       130       140       150       160
           |--------+---------+---------+---------+---------+---------+---------+---------|
GbMADS6   YASS-SMSKTLERYQKSLHVIPDTNV-TTRE--AQNWHQEVTKLKGKVQILQQSQRHLLGEDLGMLSLKELHQLEHQVEV
AlsMADS   FGSA-GTSKTLERYQRCCYTSQDTNA-IDRE--TQNWYQEMSKLKAKFESLQRAQRHLLGEDLGPLSVKELQQLERQLES
LpMADS4   FGSA-GTTKTLERYQHCCYNAQDSSS-ALSE--TQSWYQEMSKLKAKFEALQRTQRHLLGEDLGPLSVKELQQLEKQLEC
ZmMADS5   FGSA-GVTKTLERYQHCCYNAQDSNNGALSE--SQSWYQEISKLRAKFEALQRTQRHLLGEDLGPLSVKELQQLEKQLEC
MalMADS2  FCSSSSMLKTLERYQKCNYGAPETNVMSRET---QNSQQEYLKLKARVEALQRSQRNLLGEDLGPLNIKELEQLERQLDA
AfSEP2    FCSSSSMFKTLERYQKCNYGAPETTVTNRET---QSSHQEYLKLKARVEALQRSQRNLLGEDLGPLSGKELEQLERQLDV
SbMADS1   FCSGQSITKTLERYEK--HMRPDTAVQNKENELVQSSRNEYLKLKARVDNLQRTQRNLLGEDLGSLGIKELEQLEKQLDS
BdMADS    FCSGQSMPKTLERYQKCSYSGPDTAVQNKENELVQSSRNEYLKLKARVENLQRTQRNLLGEDLGSLGIKELEELEKQLDS
Consensus %cS..sm.KTLERY#kc.y..p#tnv...e....Qss.#EylKLKarv#.LQr.QRnLLGEDLGpLs.KELe#LE.Ql#.

           161     170       180       190       200       210       220       230       240
           |--------+---------+---------+---------+---------+---------+---------+---------|
GbMADS6   ALKHLRSRKTQIMLDQIDDLRKKERMLQEVNKSLHKKFLEADGQNACNFGQFSRPWDSAV-------GNPAYGVNEPDSH
AlsMADS   ALAQARQRKTQLMLDQMEELRKKERHLGEINKQLKNK-LEAEGANLRALQGSWESEAVAG-------GNAFPMHQ---IQ
LpMADS4   SLSQARQRKTQLMMEQVEELRKKERQLGEINRQLKHK-LDAEGSSSNSFRAMQQITWAAGTVYDEGAGAYHMHHQQQPNH
ZmMADS5   ALSQARQRKTQLMMEQVEELRRTERHLGEMNRQLKHK-LEAEGCSNYTTLQHAACWPAPGGTIVEHDGATYQVHP--PAH
MalMADS2  SLRQIRSTRTQYMLDQLTDLQRREQMLCEANKALKRRLEESNQANQQQLWDPNT---Y----------GRQQPQPQGDGF
AfSEP2    SLRQIRSTRTQYMLDQLADLQRKEQMLSEANKALKRRLEEGNPANPHQAWDPNQHALF----------GRQPGPPQGDGF
SbMADS1   SLRHIRSTRTQHMLDQLTDLQRREQMLCEANKCLRRKLEETSN-QVHSKCGARCQLTR----------LRAATPPHSRPH
BdMADS    SLRHIRSTRTQHMLDQLTDLQRKEQMLCEANRCLXRKLEESSQVHGHMWEHAANLLGY----------DQRQSPQQQAPH
Consensus sLrqiRstrTQ.M$#Ql.#LqrkEqmL.EaNk.LkrkleE....n.....................................h

           241     250       260       270       280       289
           |--------+---------+---------+---------+---------|
GbMADS6   VQPAHREPTLHIGY--REAAHPVSTVHRQKQSA----NHYTQDWMV
AlsMADS   SSAMDTEPTLQIGY--HPFIPQDA--NLQRNN--GGENNFMLGWVL
LpMADS4   SAAMDCEPTLQIGYPHQFAAAEQAANNIPRSSAPGGENNFMLGWVLGAN
ZmMADS5   SVAMDCEPTLQIGYPHHQFPPPEAANNIPRSAATG-ENNFMLGWVL
MalMADS2  FQPIDCEPTLQIGY-HPDKMAIAAAAAAAAAAGPSVSN-YVPGWLA
AfSEP2    FHPLECEPTLHIGY-QPEQITIAAP-------GPSVNNNFMQGWLA
SbMADS1   H-----ILAMDCSF-IPWKLQQSQPCRS----GSLLNI
BdMADS    HGGNGFFHPLDAAS-EPTLQIGFTPEQM----SSSCVTAFLPTWLP
Consensus ......ept$.igy..p......a.............n.f..gw..
```

Fig. 3: The homology comparison of GbMADS6 amino acid sequence with other plant MADS proteins. *Alstroemeria ligtu* subsp. MADS (AB694896.1); *Musa acuminata* MADS2 (KM261780.1); *Lolium perenne* MADS4 (AY198329.1); *Sorghum bicolor* MADS1 (U49734.1); *Zea mays* MADS5 (KJ726929.1); *Aristolochia fimbriata* MADS1 (KT957081.1); *Brachypodium distachyon* MADS (HQ588325.1). High consensus is indicated in red colour (default: 90%); low consensus is indicated in blue colour (default: 50%, must be less than first value); non-similar amino acids are indicated with black colour; white is the background colour.

Multiple alignments of GbMADS6 protein

Sequence comparison analyses by Blast P Search (http://www.ncbi.nih.gov) showed that GbMADS6 had higher homology with other MADS proteins. The amino acid sequences multiple sequence alignment showed that GbMADS6 had high identities with *Alstroemeria ligtu* subsp. MADS (73% identities, AB694896.1), *Musa acuminata* MADS2 (79% identities, KM261780.1), *Lolium perenne* MADS4 (81% identities, AY198329.1), *Sorghum bicolor* MADS1 (81% identities, U49734.1), *Zea mays* MADS5 (80% identities, KJ726929.1), *Aristolochia fimbriata* MADS1 (78% identities, KT957081.1) and *Brachypodium distachyon* MADS (77% identities, HQ588325.1) (Fig. 3). All of the data mentioned above indicate that GbMADS6 was a member of the MADS family.

Molecular evolution analysis of MADS proteins

In order to study the evolutionary relationships between GbMADS6 and MADS proteins from other plant species, a phylogenetic tree was constructed by using MEGA 6.0. Phylogenetic analysis of MADS showed that GbMADS6 have a closer relationship to *Pinus tabuliformis* MADS protein than to other MADS-box proteins.

As shown in Fig. 4, the GbMADS6 protein together with *Alstroemeria ligtu* subsp., *Lolium perenne*, *Zea mays* and *Setaria italica* were grouped into a functional cluster. From the above results, it can be suggested that GbMADS6 belonged to MADS-box gene families, GbMADS6 may have similar functions with other MADS proteins of the same branch.

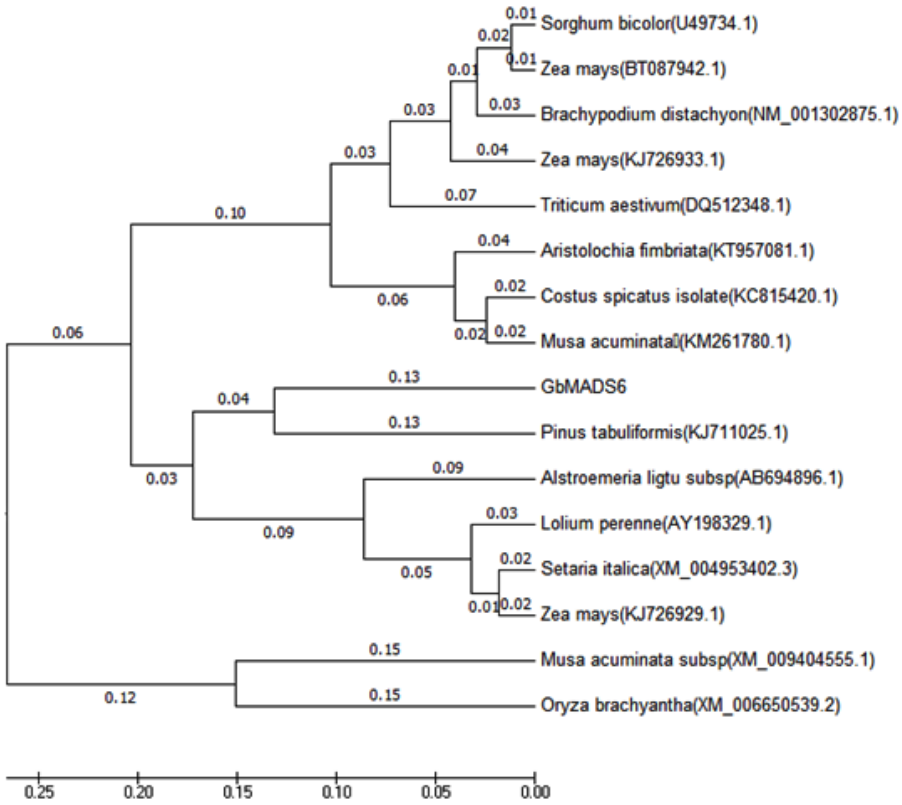

Fig. 4: Phylogenetic tree of MADS amino acid sequences from *Ginkgo biloba* and other plant species. The numbers at each node represent the bootstrap values (with 1,000 replicates).

Discussion

MADS-box gene family played an important role in controlling flower development, the gene family had a conservative DNA-binding domain (MADS-box).The functional diversity within MADS-box genes, not only determined the meristem and floral organ specificity, but also involved in regulation of vegetative growth, ovary development, formation of the root, completion of the embryo morphological, and the gene expression is also associated with resistance (Liljegren et al., 2000). 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.

MADS-box gene family is divided into type I and type II genes according to sequence and structure characteristics (Alvarez-Buylla et al., 2000).Type I genes functions are still not clear in plants and in the current research, expression patterns and function of type II genes are known (De Bodt et al., 2003). Type II genes contain MADS (M), Intervening (I), Keratin-like (K) and C-terminal (C) inordinately of conservative domain, it was also known as the MIKC-type (Nam et al., 2004). MADS-box gene GbMADS6 was amplified from ginkgo by RT-PCR. Sequence analysis indicated that its coding region was 735 bp, with an open reading frame encoding 345 amino acids, which displayed the structure of a typical plant MADS-box gene, including M, I, K and C area, consequently, GbMADS6 gene attributed to MIKC type. It is inferred that GbMADS6 might adjust the differentiation process of flower and fruit development in *Ginkgo biloba*. The genetic transformation of GbMADS6 in tobacco is in progress by *Agrobacterium*-mediated transformations for functional identification of GbMADS6 gene.

Conclusion

GbMADS6 with an ORF about 735bp, encoding 245 amino acids was isolated by RT-PCR from ginkgo. The amino acids of GbMADS6 contain a conserved MADS-box domains and displays extensive homology to MADS amino acids from other plants that will enable us to conclude the flower development model of *Ginkgo biloba*. The phylogenetic tree analysis demonstrated the GbMADS6 protein was grouped with *Pinus tabuliformis*. The GbMADS6 is likely to participate in regulating the differentiation process of flower and fruit development in *Ginkgo biloba.*

Conflict of interest statement

Authors declare that they have no conflict of interest.

Acknowledgement

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