

Molecular Cloning and Expression Analysis of a MADS-Box Gene (*GbMADS2*) from *Ginkgo biloba*

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Abstract

As a kind of transcription factors gene family, MADS-box genes play an important role in plant development processes. To find genes involved in the floral transition of *Ginkgo biloba*, a MADS-box gene, designated as *GbMADS2*, was cloned from *G. biloba* based on EST sequences by RT-PCR. Sequence analysis results showed that the cDNA sequence of *GbMADS2* contained a 663 bp length ORF encoding 221 amino acids protein, which displayed typical structure of plant MADS-box protein including MADS, I, and K domains and C terminus. The sequence of *GbMADS2* protein was highly homologous to those of MADS-box proteins from other plant species with the highest homologous to AGAMOUS (CyAG) from *Cycas revoluta*. The phylogenetic tree analysis revealed that *GbMADS2* belonged to AGAMOUS clade genes. Real-time PCR analysis indicated that expression levels of *GbMADS2* gene in female and male flower were significantly higher than those in root, stem, and leaves, and that *GbMADS2* expression level increased along with time of flower development. The spatial and time-course expression profile of *GbMADS2* implied that *GbMADS2* might be involved in development of reproductive organs. The isolation and expression analysis of *GbMADS2* provided basis for further studying the molecular mechanism of flower development in *G. biloba*.

Keywords: bioinformatics analysis, *GbMADS2*, spatial expression pattern, time-course expression pattern

Introduction

Plant flower formation and development is a complex process that is controlled by environmental and endogenous signals (Brambilla and Fornara, 2013), with four main pathways: autonomous, photoperiod, vernalization, and gibberellin-mediated (Bernier and Périlleux, 2005). Many genes involved in flower development act in concert with physiological signals to establish complex gene regulatory networks that regulate flowering and floral organ development. Many of these genes belong to the MADS-box gene family, which is a large transcription factor family with diverse roles, especially floral development (Hemming and Trevaskis, 2011). Also, MADS genes are great significant for studying the system evolution of plant groups because of their universality and highly conservatism in the plant kingdom (Purugganan *et al.*, 1995). In flowering plants, MADS-box genes control diverse developmental processes in organs ranging from roots to flowers and fruit development (Ng and

Yanofsky, 2001). In regard to biological metabolism, researcher have found an important function of MADS which as transcription factors regulate the biosynthetic pathways of secondary metabolites flavonoids (Lalusin *et al.*, 2006). Moreover, MADS-box genes involve in plant growth and development process, such as synthesis of gibberellin (Jackson, 2009), the fruits ripening (Ireland *et al.*, 2013), plant dormancy (Saito *et al.*, 2013), the root elongation (Zhang and Forde, 1998), callus differentiation (Fornara *et al.*, 2004), shoot development (Lee *et al.*, 2008).

Ginkgo biloba, the only surviving genus and species of the Ginkgoaceae family, is a dioecious tree, native of China. Resistant to city smoke and industrial fumes, it has become a favorite ornamental tree in parks and streets of numerous towns all over the world. Moreover, *G. biloba* accumulates, mainly in its leaves, two types of compounds of pharmaceutical importance, that are terpenes (ginkgolides

and bilobalide) and flavonoids. The leaf extracts are widely used for the treatment of peripheral or cerebral circulatory disorders, especially in geriatric practice (Van Beek and Montoro, 2009). *G. biloba* is a kind of Mesozoic precious plants with a special long juvenile phase, general 15 to 20 years to bear fruit after planting. As a unique relict species belonging to a plant lineage known as Ginkgophyta, *G. biloba* has many unique flowering habits compared with other plants (Chen et al., 2008). Therefore, it is important theoretical and practical significance to study floral organ development and fruit formation on *G. biloba*. However, up to now, little literature reported isolation and function of the genes involved in *G. biloba* flower organ development. To explore the molecular mechanism of *G. biloba* flower organ development, this study isolated a MADS-box gene, designated as *GbMADS2*, from *G. biloba* and analyzed the gene expression patterns in different *G. biloba* organs. This work aimed to provide the theoretical basis for further studies on the molecular mechanism of this gene participating in flowering 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 pattern of *GbMADS2*, the roots, leaves, fruits, stems, male and female flowers of *G. biloba* grafts were collected. For measurement of the time-course expression pattern of *GbMADS2* 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 collected and immediately frozen in liquid nitrogen, and kept at -80 °C prior to total RNA extraction.

Cloning of the full-length cDNA of *GbMADS2*

Total RNA was extracted from female flowers 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 MADSFP (5'-TGCTCTAAATTCCAACAGAAAAG-3') and MADSRP (5'-GTAATATATCCCGCCATAAACT-3') were designed and synthesized (Shanghai Sangon, China) based on the EST sequence of *G. biloba*. A 712 bp-length fragment was obtained 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, 51 °C for 1 min, and 72 °C for 1 min. 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.

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 10.0 program. The protein sequences of *GbMADS2* and other MADSs were obtained from GenBank and aligned with the software Vector NTI suit 10.0 program. Phylogenetic tree was constructed 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 *GbMADS2*

To investigate expression pattern of *GbMADS2* gene 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 (Real-Time PCR Reagent Kit, Dalian TaKaRa, China). Quantitative Real-time PCR (qRT-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 Master Mix, 0.5 M of each primer, 2 µL of diluted cDNA template, and nuclease free water to a final volume of 20 µL. The PCR reaction conditions were 95 °C for 3 min, and then 30 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 level was normalized to *G. biloba* house-keeping gene *18S* (*Gb18S*, GenBank accession no. D16448) to minimize the variation in the cDNA template levels. The primers for *GbMADS2* gene (MADS2-S: 5'-GTGGCTCTCATCGTCTTCTC-3'; MADS2-A: 5'-ATTGCTGTCTCAGTTTTCC-3') and *Gb18S* gene (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 and characterization of *GbMADS2*

The amplified products of RT-PCR were separated on 1% agarose gel stained with ethidium bromide. The result showed that the PCR products size was about 700 bp (Fig. 1), which had a same size with the expected MADS-box gene sequence. The recyclable PCR products were sequenced, and showed that the cDNA sequence of PCR products contained 712 bp. The results of BlastN analysis on NCBI showed that this cDNA sequence had high similarity with that of other MADS genes. The nucleotide sequence of *GbMADS2* was 84% and 80% identical to MADS-box gene from *Cycas edentata* and *Rafflesia cantleyi*, and 82%, 82%, and 81% identical to *AGAMOUS* gene from *Macrozamia spiralis*, *Ceratozamia mexicana*, *Zamia integrifolia*, and 74% identical to *AGAMOUS-like* gene from *Magnolia denudata*, respectively (Table 1), indicating the gene we isolated

Table 1. Nucleotide sequence of *GbMADS2* similarity to the *MADS-box* genes from other plant species

Species	Gene ID	GenBank Accession No.	Homology
<i>Cycas edentata</i>	<i>MADS-box</i>	AF492455	84%
<i>Macrozamia spiralis</i>	<i>AG</i>	HM596862	82%
<i>Ceratozamia mexicana</i>	<i>AG</i>	HM596861	82%
<i>Zamia integrifolia</i>	<i>AG</i>	HM596866	81%
<i>Rafflesia cantleyi</i>	<i>MADS-box</i>	KF730033	80%
<i>Magnolia denudata</i>	<i>AGAMOUS-like</i>	JQ326231	74%

According to the modular domain structure of type II MADS protein, GbMADS2 could be divided into four conservative domains (Fig. 3), including MADS domain at 1-57 amino acids, K domain at 91-156 amino acids, I domain containing 33 amino acids, and a C terminus. However, GbMADS2 did not show high homology with the sequence of N-terminus extension of AG proteins of *Arabidopsis thaliana*, but similar to the AG subfamily of reported MADS-box protein from other plants. Compared the different domains similarities of GbMADS2 with CyAG, DAL2 and AGL11, respectively, they had the highest homology in MADS domain. It was found that GbMADS2 exhibited 94.74%, 100%, and 100% similarities in MADS domain as well as 8.5%, 86.4% and 69.7% similarities in K domain of CyAG, DAL2 and AGL11, respectively (Fig. 3).

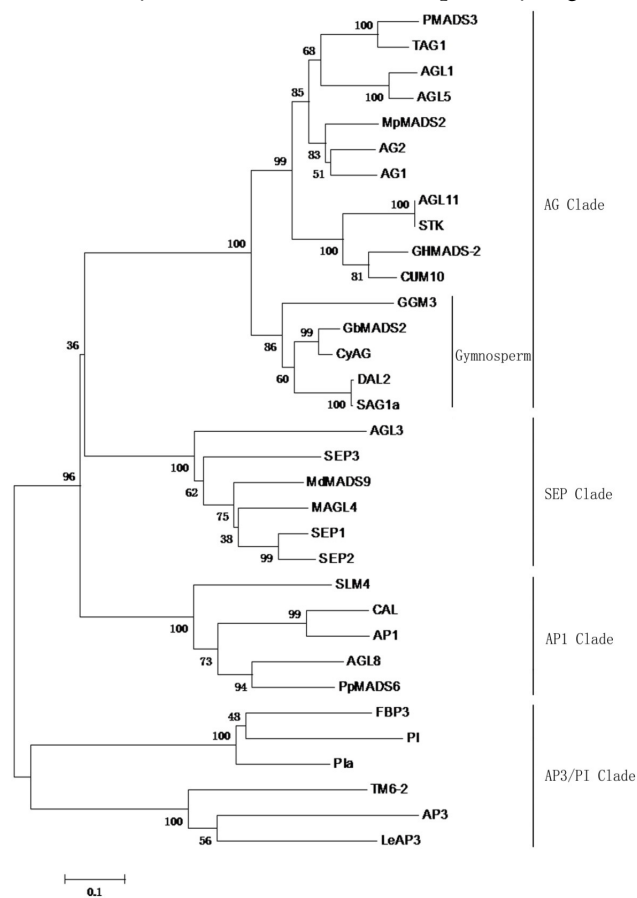


Fig. 4. Phylogenetic tree analysis of protein encoded by MADS-box genes. The specie and protein name and GenBank accession number are as following: *Arabidopsis thaliana*: STK (NP_001078364), CAL (NP_564243), AP1 (CAA78909), AGL8 (Q42429), AGL3 (P29383), SEP1 (P29382), SEP2 (P29384), SEP3 (O22456), PI (NP_197524) and AP3 (NP_191002); *Petunia hybrida*: PMADS3 (BAB79434) and FBP3 (CAA50549); *Lycopersicon esculentum*: TAG1 (Q40168) and LeAP3 (AAC42583); *Eschscholzia californica*: AG2 (AAZ53206) and AG1 (AAZ53205); *Magnolia praecoxisima*: MpMADS2 (BAB70737); *Gossypium hirsutum*: GHMADS-2 (AAN15183); *Cucumis sativus*: CUM10 (AAC08529); *Prunus persica*: PpMADS6 (AAU29514); *Malus domestica*: MdMADS9 (CAA04920); *Populus tremuloides*: MAGL4 (AAL08423); *Lotus corniculatus* var. *japonicus*: PIa (AAX13299); *Carica papaya*: TM6-2 (ABQ51322); *Silene latifolia*: SLM4 (CAA56658)

Molecular evolution analysis

To investigate the evolutionary relationships in GbMADS2 and MADSs from other plant species, we choose some typical MADS-box genes from GenBank to analyze molecular evolution of GbMADS2 on the basis of the four subfamilies (AP1, AP3/PI, SEP and AG) of flowers homologous alien genes of MADS-box gene. A phylogenetic tree was constructed by using NJ method of MEGA software. As shown in Fig. 4, GbMADS2 belonged to the subfamily of AG genes in the branch of AP1, AP3/PI, SEP, AG, and had the closest relationship with CyAG of *C. revolute*. Taken into account together with high sequence homologies of GbMADS2 with other plant AG protein, our data suggested that GbMADS2 belonged to AG subfamily of MADS-box gene families. These results suggested that GbMADS2 shared a common evolutionary with other plant AG proteins based on conserved structure and sequence characteristics, such as amino acid homologies and conserved motifs, respectively.

Expression analysis of GbMADS2

To investigate the expression of *GbMADS2* gene in different tissues of *G. biloba*, qRT-PCR was carried out using gene-specific primers and cDNAs from different ginkgo tissues as templates. As shown in Fig. 5, GbMADS2 was strongly expressed in male and female flowers, but negligible amounts of its transcripts were detected in roots, stems, and leaves vegetative tissues of *G. biloba*. For further examine the role of *GbMADS2* during flower development, we measured the time-course expression pattern of *GbMADS2* in flowers.

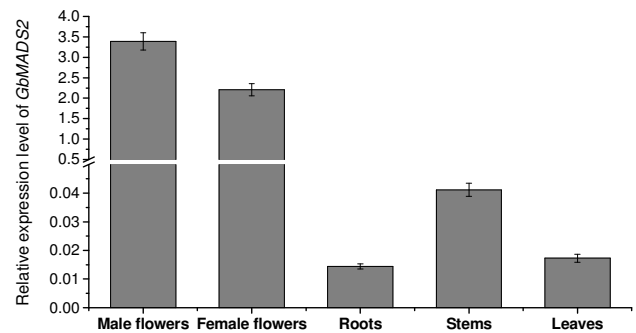


Fig. 5. Expression level of *GbMADS2* in different tissue of *Ginkgo biloba*

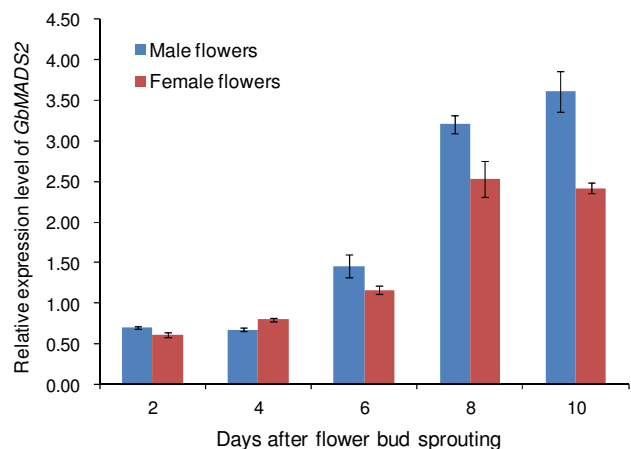


Fig. 6. Time-course expression profile of *GbMADS2* during flower development in *Ginkgo biloba*

The results showed that the transcript level of *GbMADS2* significantly increased along with the development of male and female flowers (Fig. 6), implying that *GbMADS2* might be involved in flower development.

Discussions

MADS-box genes have been divided in two large groups, termed type I and type II genes (Alvarez-Buylla et al., 2000). Type I and type II MADS-box genes were generated by a gene duplication that occurred before the divergence of plants, animals and fungi more than a billion years ago (Becker et al., 2000). In plants, the type I MADS-box genes function are still not clear, type II MADS-box genes which include all plant MADS-box genes for which expression patterns or mutant phenotypes are known (De Bodt et al., 2003; Kaufmann et al., 2005). The type I MADS-box genes only contain MADS domain-box structure (Nam et al., 2004), while Type II genes comprise MADS-box, I, K and C structure with different degree of conservative, and called MIKC-type MADS-box (Ma et al., 1991; Münster et al., 1997; Nam et al., 2004; Kaufmann et al., 2005). The sequence analysis results revealed that *GbMADS2* contained M, I, and K domains and C terminus, which are typical MADS-box structure of plants. Therefore, *GbMADS2* belongs to MIKC type MADS-box gene and might be related to floral organ formation and development (Coen and Meyerowitz 1991; Heijmans et al., 2012).

Most genomes of plants exist in varying quantity of MADS-box genes duplication. Therefore, it is possible for providing the raw materials for the evolution of the organism new form by gene duplication and function diversification. MADS-box genes form a multiple gene families at many genes duplication of the evolution process, especially in angiosperms (Aswath and Kim, 2005). By phylogeny reconstruction, almost all of the angiosperm MADS-box genes can be subdivided into 12 major gene clades (Becker and Theissen, 2003). Phylogenetic tree showed that gymnosperms and angiosperms had seven same subfamilies of MADS domain, that was to say the existing seed plants have at least seven different MIKC type genes: AG, AGL2, AGL6, DEF/GLO, GGM13, STMADS11 and TM3 gene subfamily in their common ancestor (Smaczniak et al., 2012). Most of MIKC type MADS genes are involved in the development of sporophyte reproductive organs, such as the ovule (Busi et al., 2003). The phylogenetic tree analysis revealed that *GbMADS2* gene belonged to AG subfamily, implying that the function of *GbMADS2* is probably similar to AG clade genes.

Real-time PCR results revealed that expression level of *GbMADS2* in reproductive was significantly higher than that in vegetative tissues, similar to the *Arabidopsis* and cotton AG genes (Mizukami et al., 1992; Liu et al., 2010), indicating *GbMADS2* might play a role in the development of reproductive tissues in *G. biloba*. Similar to the time-course expression profile of *OsMADS1* gene in rice (Jeon et al., 2000), we also found the expression level of *GbMADS2* exhibited an increased tendency during flower development, further confirming *GbMADS2* contribute to flower development in *G. biloba*. Taken together, the sequence analysis and the expression pattern data supports the conclusion that *GbMADS2* probably represents a function orthologue of the AG gene family, implicating that the gene may participate in flower development.

Conclusions

In summary, isolation and characterization of *GbMADS2* gene constitute a start point for our studies of flower development in *G. biloba*. We have also recently isolated several MADS-box genes of *G. biloba* and characterized their expression (manuscript in submission). Experiments are underway to further characterize the possible specific role of each gene in *G. biloba* that will enable us to infer a flower development model of this plant and define ways to enhance its productivity exploiting molecular genetic techniques.

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