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Characterization and functional analysis of a MADS-box transcription factor gene (*GbMADS9*) from *Ginkgo biloba*

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ABSTRACT

The MADS-box transcription factors play diverse roles in plant development, but little is known about the role of MADS-box genes in *Ginkgo biloba*. Here a MADS-box transcription factor gene, designated as *GbMADS9*, was cloned and functionally characterized from *G. biloba*. The open reading frame of *GbMADS9* comprised 684 bp encoding a polypeptide of 227 amino acids. Phylogenetic analysis showed that GbMADS9 belonged to the GGM13 clade of B_{sister}-class MADS-box proteins. Real-time PCR results revealed that *GbMADS9* was preferentially expressed in strobili and ovules. In addition, its expression was up-regulated with the increase in the size of ginkgo pulp, indicating that *GbMADS9* had a positive function in ginkgo ovule development. The expression of *GbMADS9* in ginkgo was up-regulated in response to salinity, drought, and cold stress, as well as to phytohormones gibberellic acid and abscisic acid. *GbMADS9* transgenic *Arabidopsis* plants presented early flowering phenotypes compared with wild-type plants. The expression of the *FLOWERING LOCUS T* (*FT*), *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) genes involved in flowering was up-regulated, whereas the expression of *AGAMOUS-LIKE24* (*AGL24*) and *SHORT VEGETATIVE PHASE* (*SVP*) was down-regulated in the *GbMADS9* transgenic lines. The *GbMADS9* transgenic plants exhibited better growth than wild-type plants under high osmotic stress. Enhanced tolerance of transgenic *Arabidopsis* plants to osmotic stress was confirmed by changes in the chlorophyll, proline, and malondialdehyde contents. Moreover, increased tolerance to osmotic stress in *GbMADS9* transgenic lines was associated with improved antioxidant enzymatic activities. These findings suggested that *GbMADS9* might be a promising gene to manipulate flowering time and improve abiotic stress tolerance in *G. biloba*.

1. Introduction

Ginkgo biloba has existed since the late Triassic period, more than 200 million years ago. *G. biloba* is the only surviving member of the Ginkgoaceae family and Ginkgoales order, which underscores its unique phylogenetic status (Bilia, 2002). Among living Spermatophyta, *G. biloba* is often called a "living fossil" because it has remarkably retained a number of reproductive features, such as production by the male gametophyte of a pair of multi-flagellated spermatozoids swimming to the female gamete, accumulation of reserves in the ovule before fertilization, and occurrence of fertilization after the ovule has fallen down from the mother tree (Friedman, 1987). *G. biloba*, as a gymnosperm, also possesses unique developmental features such as dichotomous branching veins in the leaf vascular tissue and motile spermatozoids. Furthermore, *G. biloba* has a long juvenile phase. These plants typically do not reach sexual maturity until 20 to 30 years, severely limiting high-quality gingko breeding (Singh et al., 2008; Chen et al., 2008). Thus, *G. biloba* is a key taxon to understand the evolution of reproductive and developmental characters among vascular plants, as well as to comprehend flower evolution among Spermatophytes. In addition, *G. biloba* is a tree of high aesthetic value with a long life span and high resistance to insects, bacterial and viral infections, and air pollution (Chen et al., 2001). The morphology of the *Ginkgo* tree has changed very little for over 200 million years. Some *Ginkgo* trees may live longer than 3000 years, during which they have to survive from various environmental stresses. Plants respond to stress not only through physiological and developmental changes, but also by expressing stress-inducible genes (Shen et al., 2005). Hence, it is important to excavate genes involved in stress tolerance and flowering from *G. biloba*. Such genes are theoretically and practically significant to genetic engineering breeding in *G. biloba*.

The MADS-box gene family encodes transcription factors (TFs) with a conserved DNA-binding domain, called the MADS-box.

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These genes, ubiquitous in living organisms, have extensive functions, such as cardiac muscle development in animals (Olson et al., 1995), pheromone responses and arginine metabolism in yeast (El Bakkoury et al., 2000), and regulation of plant growth and development (Theißen, 2001). The size and complexity of plant MADS-box gene families have already been reported following the studies of a taxonomically broad range of species, such as *Arabidopsis thaliana* (Pařenicová et al., 2003), rice (Arora et al., 2007), petunia (Immink et al., 2003), tomato (Hileman et al., 2006), maize, sorghum (Zhao et al., 2011), soybean (Zheng et al., 2013), *Prunus mume* (Xu et al., 2014), and orchid (Tsai et al., 2014). These studies have revealed that MADS-box genes play many critical regulatory roles, including dehiscence zone formation (Liljegren et al., 2000), embryo development (Thakare et al., 2008), fruit ripening (Vrebalov et al., 2002; Zeng et al., 2011), vegetative organ development, flowering time control, and regulation of floral meristem and organ identity (Theißen, 2001; Smaczniak et al., 2012; Heijmans et al., 2012; Kwantes et al., 2012). Some MADS-box genes have already shown to play key roles to control flowering time. For example, the overexpression of *OsMADS50* caused extremely early flowering at the callus stage in rice (Lee et al., 2004). Over-expression of the gene *BpMADS4* from *Betula pendula* in apple shortened the juvenility and induced flowering during in vitro cultivation (Flachowsky et al., 2007). Recently, Alter et al. (2016) demonstrated that maize ZmMADS1 represented a functional ortholog of the central flowering time integrator *SOC1* of *Arabidopsis*. RNA interference-mediated down-regulation of *ZmMADS1* resulted in a delay of flowering time in maize, while strong overexpression caused an early-flowering phenotype, indicating its role as a flowering activator. Furthermore, several MADS-box genes participate in regulating the biosynthetic pathways of secondary metabolites (Nesi et al., 2002; Lalusin et al., 2006) and tolerance to abiotic stress (Arora et al., 2007; Xu et al., 2014).

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 ortholog of the *AGAMOUS* (*AG*) MADS-box gene of *A. thaliana*. Lovisetto et al. (2013, 2015) also identified a B-sister MADS-box gene (*GBM10*) and an *AGAMOUS* gene (*GBM5*) from *G. biloba*. Transgenic studies showed that *GBM10* and *GBM5* plays an important role in development of the ovule/seed and the fleshy fruit-like structure surrounding the seed, respectively. We recently reported an *AG* clade and a *SEP* clade gene, known as *GbMADS2* and *GbSEP*, respectively, which might be involved in the development of reproductive organs (Wang et al., 2015; Cheng et al., 2016). However, MADS-box genes that control flowering time and response to stress in *G. biloba* have not been reported. In this study, we isolated a novel MADS-box gene, designated as *GbMADS9*, from *G. biloba*. The expression pattern and ectopic expression functional analyses undertaken may contribute to understand the function of *GbMADS9* in control of flowering time and tolerance of abiotic stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Two-year-old seedlings and 15-year-old grafts of *G. biloba* cv. Daguo were grown in a greenhouse at Yangtze University, China. The roots, leaves, stems, male and female strobili, and young and mature ovules of Ginkgo grafts were collected to test the spatial expression profile of *GbMADS9*. Ovule samples at various development stages were collected to measure the time-course expression pattern of *GbMADS9* in the ovules. The female strobilus at one-week after sprouting of 15-year-old ginkgo grafts were sampled for hormonal treatments. Hormonal treatments were performed by spraying the female strobilus of 15-year-old grafts with 20 μM gibberellin (GA_3), 20 μM indole-3-acetic acid (IAA), and 100 μM abscisic acid (ABA) dissolved in 0.01% (v/v) Tween 20. Control ginkgo female strobilus were sprayed with an equal amount of 0.01% Tween 20. Two-year-old seedlings grown in vermiculite soil at the four-leaf stage were selected for stress treatments. Salt stress treatments were conducted using 200 mM sodium chloride (NaCl), and an equal amount of water was used as control. The seedlings were sprayed with polyethylene glycol (PEG, 15% m/v) to simulate the drought stress test, and seedlings were kept at 4 °C for the cold stress test. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analyses.

A. thaliana (ecotype Columbia) seeds were sterilized with 75% (v/v) alcohol for 5 min and then with 0.01% $HgCl_2$ (w/v) for 3 min. Subsequently, the seeds were washed five times in sterile water. Sterile seeds were scattered on MS medium at pH 5.8. The seeds were vernalized for 36 h at 4 °C and transferred to a growth chamber at 23 °C under a 16/8 h (light/dark) photoperiod. Seedlings were transferred into vermiculite soil 14 days later.

2.2. Cloning of the GbMADS9 gene

Total RNA was extracted from female strobilus using the CTAB method (Cai et al., 2007). Primers MADS9-FP and MADS9-RP (sequences in Table S1) were designed and synthesized (Shanghai Sangon, China) based on the transcriptome sequencing data of *G. biloba*. *GbMADS9* cDNA was obtained by the one-step RT-PCR kit (Dalian TaKaRa, China). Primers MADS9FP and MADS9RP were also used to amplify the genomic sequence of *GbMADS9* using ginkgo DNA as a PCR template. The PCR product was cloned into pMD18-T vector and then sequenced.

2.3. 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 vector NTI™ Suite 11 was used for multiple sequence alignment. A phylogenetic tree was constructed using CLUSTAL W 2.0 and MEGA 5.0. Tree reliability was measured by bootstrap analysis with 100 replicates.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR was performed to measure the relative transcript levels of selected genes. First-strand cDNA synthesis was conducted in triplicate for each sample according to the manufacturer's instructions (PrimeScript™ RT Reagent Kit, Dalian TaKaRa, China). qRT-PCR was performed using a Bio-Rad iQ5 thermal cycler with SYBR Premix Ex Taq™ II Kit (Dalian TaKaRa, China) according to the manufacturer's protocol. 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. PCR reaction conditions were 95 °C for 3 min, 40 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s, and 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. Expression was normalized to two reference genes from *Ginkgo* (*Gb18S* and *GbGAPDH*), namely *18S* gene (*Gb18S*, GenBank accession no. D16448) and *GbGAPDH* (GenBank accession no. L26924), respectively, and *Arabidopsis* (*Actin2 and UBQ*) to minimize the variation in the cDNA template levels. Table S1 lists the reference genes and primer sequences designed using Sequence Detection System software. Two different normalization factors were calculated based on (1) the geometric mean of the genes with the lowest Geomean and (2) a single reference gene with the lowest or highest Geomean value (Yang et al., 2015). Relative quantification of gene expression