July 2015. Horticultural Plant Journal, 1 (1): 11–16.

Horticultural Plant Journal

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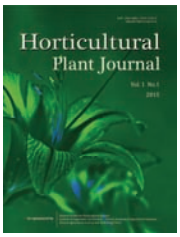

Cloning and Functional Analysis of the MADS-box *CiMADS9* Gene from *Carya illinoinensis*

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Received 1 March 2015; Received in revised form 12 July 2015; Accepted 26 July 2015

Abstract

A MADS-box gene, *CiMADS9*, was cloned from the male flowers of *Carya illinoinensis* by rapid amplification of cDNA ends. The gene was 1 077 bp with a 768 bp open reading frame encoding 255 amino acids. Multiple sequence comparisons revealed that *CiMADS9* is a typical MIKC-type MADS-box gene with a MADS-box domain and a K semi-conserved region. Phylogenetic analysis indicated that *CiMADS9* belongs to the AGL15 group of the MADS-box gene family. Quantitative reverse transcription polymerase chain reaction analysis indicated that the expression levels in reproductive organs (i.e., flowers and young fruits) were considerably higher than in vegetative tissues (i.e., leaves and branches). The highest expression levels were observed in male flowers. An overexpression vector for *CiMADS9* was constructed and the gene was inserted into the *Arabidopsis thaliana* genome. *CiMADS9* expression was confirmed in all transgenic lines. Compared with wild-type plants, transgenic *A. thaliana* plants overexpressing *CiMADS9* exhibited delayed flowering and an increased number of leaves.

Keywords: *Carya illinoinensis*; flower organ development; *CiMADS9*; gene expression; function analysis

1. Introduction

MADS-box transcription factors comprise a supergene family. Previous studies have shown that there are 107, 75, 75, 43, 57, and 167 members in *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Cucumis sativus*, *Brachypodium distachyon*, and *Brassica rapa*, respectively (Rameneni et al., 2014; Wei et al., 2014; Duan et al., 2015). MADS-box transcription factors play key roles in flower development. Genes associated with the ABCDE model are MADS-box genes, except for *AP2* genes (Theißen, 2001; Roux et al., 2006).

The juvenescent phase of pecan trees lasts about 15 years. The male flowers develop earlier than the female flowers. Additionally, pecan is a monoecious species, with male and female reproductive organs present in the same plant. Few molecular biological studies of *Carya illinoinensis* have been performed and the roles of MADS-box genes in pecan development have not been fully characterized.

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http://dx.doi.org/10.16420/j.issn.2095-9885.2015-0023

In this study, a MADS-box gene was cloned using rapid amplification of cDNA ends (RACE) technology. We used a previously cloned conserved fragment (Mo et al., 2013). Additionally, we determined gene expression levels in different organs of three *C. illinoinensis* cultivars based on quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. The cloned gene was also inserted into the *A. thaliana* genome to investigate its function.

2. Materials and methods

2.1. Plant material

Grafted *C. illinoinensis* seedlings (‘Mahan’, ‘Shaoxing’, and ‘Pawnee’ cultivars) were grown in Nanjing, Jiangsu, China. Staminate and pistillate flowers, fruitlets, leaves, and actively growing branches were collected from 6-year-old plants in May 2012. Collected samples were immediately frozen in liquid nitrogen and stored at −80 ℃ until used.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was extracted from the ‘Mahan’ staminate flowers using the Plant Total RNA Extraction Kit (BioTeke, Beijing). First-strand cDNA was synthesized from 1 μg total RNA with an oligo(dT)18 adaptor primer and the PrimeScript RTase kit (TaKaRa, Japan).

2.3. Cloning of CiMADS9

Highly conserved MADS domain amino acid motifs were previously identified in pecan (Mo et al., 2013). The gene-specific primers GSP1 (5′-ATGCAAATAGCAGGCAAGT CACATTCTC-3′) and GSP2 (5′-CGCTGTTCTCTGTGATGCT GAGGTCG-3′) were designed based on the conserved MADS domain sequence. Nested PCR was completed to isolate the 3′ end of the *CiMADS9* gene. The GSP1 primer and the abridged universal amplification primer AUAP (5′-GGCCACGCGTCGA CTAGTAC-3′) were used in one round of PCR. The GSP2 and AUAP primers were used during a second round of PCR, with the first-round PCR product as the template. The PCR program consisted of an initial denaturation step at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 65 ℃ for 30 s, 72 ℃ for 1 min, and a final extension step at 72 ℃ for 10 min. The PCR product was then cloned into the pMD19-T simple vector (TaKaRa, Japan) and sequenced. To ensure that the full-length coding region sequences were present, RT-PCR was performed using the VvMADS9-F (5′-ATGGGGAGAGGGAGGATAG-3′) and VvMADS9-R (5′-CCATCAGCCAAACCTTCT-3′) primers. The PCR products were cloned and sequenced.

2.4. Amino acid alignment and phylogenetic analysis

The deduced amino acid sequences were analyzed using the NCBI Blast program (http://www.ncbi.nlm.nih.gov) to search for homologous sequences. Alignment of the deduced amino acid sequences was performed using the ClustalW multiple alignment program from BioEdit. A phylogenetic tree was constructed with the Neighbor-joining method using MEGA 5.1 software with 1 000 bootstrap replications.

2.5. CiMADS9 expression analysis in C. illinoinensis using qRT-PCR

Total RNA was isolated as described. Reverse transcription was performed using 1 μg total RNA and the ReverTra Ace qPCR RT Kit (TaKaRa, Catv#RR047Q) according to the manufacturer’s instructions. The gene-specific primers for *CiAG* were QTMADS9-F (5′-GGTACAACGAGTGTGTAGATTCTC C-3′) and QTMADS9-R (5′-TCCTCTCCTTCACAGACAATA ACCC-3′). The *CiActin* gene was used as a positive internal control with primers ACTIN-F (5′-GCTGAACGGGAAATTGT C-3′) and ACTIN-R (5′-AGAGATGGCTGGAAGAGG-3′)(Zhou, 2010). The qRT-PCR was completed using an Applied Biosystems 7300 Real Time PCR System(Zhang et al., 2011). The final reaction volume was 20 μL, which included 1 μL 10-fold diluted cDNA, 0.3 μL (10 pmol · L^{-1}) each primer, 10 μL SYBR® *Premix Ex Taq*™ (Perfect Real Time) (TaKaRa), and 8.4 μL sterile double distilled water. The PCR program consisted of an initial denaturation step at 95 ℃ for 4 min, followed by 40 cycles of 95 ℃ for 20 s, 57 ℃ for 20 s, and 72 ℃ for 40 s. The specificity of the PCR amplification was confirmed using a heat dissociation curve (55 to 95 ℃) following the final PCR cycle. The relative mRNA abundance of the cloned gene and *CiActin* was analyzed using the 7300 system software and the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Construction of the expression vector

Full length cDNA sequences of the CiMADS9 fragments were amplified using the MADS9-F (5′-CCGGTACCATGGGG AGAGGGAGGATAG-3′) and MADS9-R (5′-CCGAGCTCAG GAATAACGAGATCAG-3′) primers to introduce a *Bam*HⅠ or *Kpn*Ⅰ restriction enzyme site. The digested fragments were cloned into the pMD19-T vector and recombinants were verified by sequence analysis. The fragments were then ligated into the corresponding sites of the pCAMBIA1301 binary vector under the control of the *Cauliflower mosaic virus* 35S promoter (sense orientation) as described by Zhang et al. (2012). The *CiMADS9* binary constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 using a freeze-thaw method and verified by PCR.

2.7. Arabidopsis thaliana transformation

The 35S::CiAG constructs were transformed into *A. thaliana* using an established method (Clough and Bent, 1998). The transformed *A. thaliana* lines were selected in half strength

Murashige and Skoog medium containing hygromycin (20 μg · mL^{-1}). Surviving transgenic plants were grown in the greenhouse at 22 ℃ with a 16 h photoperiod and further verified by RT-PCR analysis. The T_2 generation was used for phenotypic assessments.

3. Results

3.1. CiMADS9 cloning

Primers GSP2 and AUAP were used for 3′ RACE PCR, and a 1 000 bp band was observed (Fig. 1). Sequencing results indicated the fragment was 973 bp and contained a poly-A structure. Assembling this sequence with a conserved sequence using ContigExpress produced a 1 077 bp fragment.

Based on the maximum open reading frame of the spliced sequence, we designed RT-PCR primers and amplified a 750 bp fragment (Fig. 2). Sequencing results indicated the fragment was 768 bp and identical to the spliced sequence. Thus, we obtained a 1 077 bp gene, with a maximum open reading frame of 768 bp that encoded a protein consisting of 255 amino acids.

3.2. CiMADS9 sequence analysis

The BLAST analysis of the amino acid sequence revealed the presence of conserved MADS-box and K-box domains. The sequence was similar to the *AGL15* gene of *Citrus sinensis* L. Osbeck, *Morus notabilis* Schneid, and *Cicer arietinum*, with 73%, 71%, and 70% homology, respectively. Preliminary analyses suggested this cDNA was a MADS-box gene and we named it *CiMADS9* (GenBank Accession KP698118).

The multiple sequence alignment results (Fig. 3) showed that the gene has typical MIKC-type structural domains, including a highly conserved MADS-box domain (59 amino acids), an I domain (31 amino acids), a K semi-conserved region (80 amino acids), and a C domain (84 amino acids).

Cluster analysis of representative transcription factors from the 12 main clades of the *A. thaliana* MADS-box gene family and *CiMADS9* (Fig. 4) indicated *CiMADS9* belongs to the AGL15 subgroup.

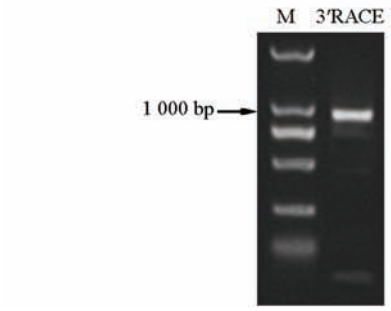

Fig. 1 MADS-box gene amplified by 3′ RACE PCR

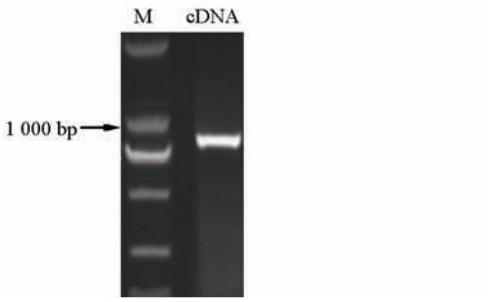

Fig. 2 Full length cDNA of the *CiMADS9* coding region amplified by RT-PCR

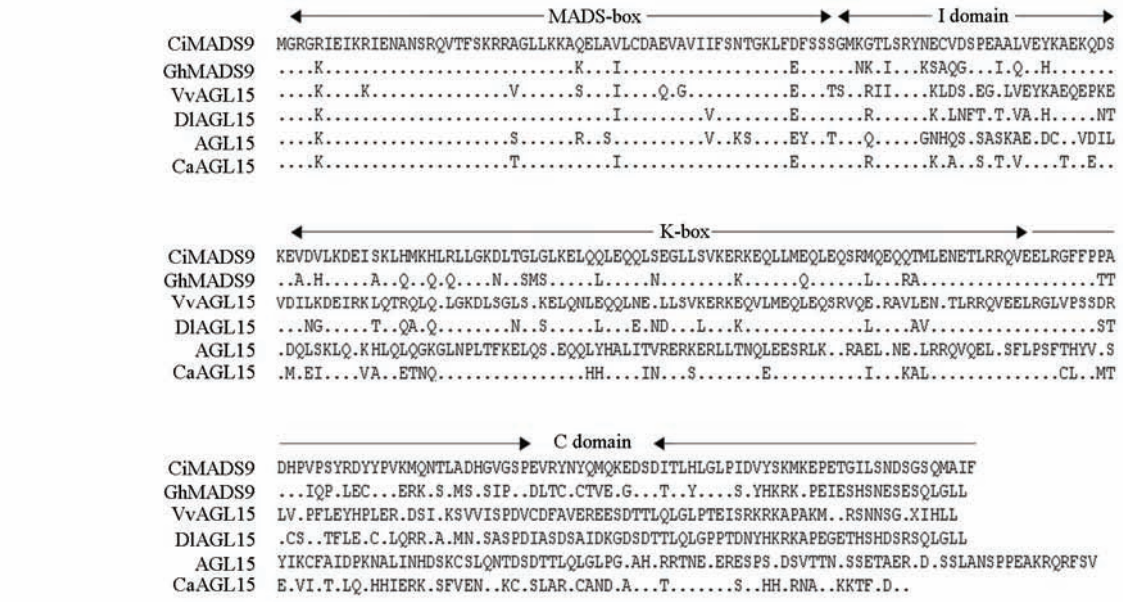

Fig. 3 Amino acid alignment of CiMADS9 and its closest homologs

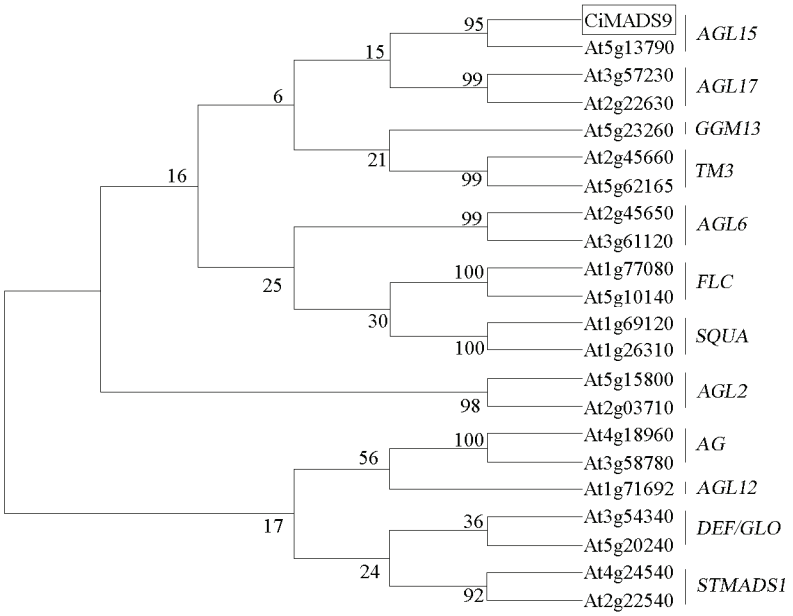

Fig. 4 Phylogenetic tree of the CiMADS9 protein of *C. illinoinensis*

3.3. CiMADS9 expression analysis

The *CiMADS9* expression patterns among different organs in three *C. illinoinensis* cultivars were determined using qRT-PCR analysis (Fig. 5). The highest *CiMADS9* expression levels were observed in male flowers. The gene was expressed to a greater extent in the reproductive organs (i.e., flowers and young fruits) than in the vegetative tissues (i.e., leaves and branches).

3.4. Transgenic Arabidopsis thaliana expressing CiMADS9

A 750 bp fragment corresponding to *CiMADS9* was amplified from genetic material extracted from transgenic plants, while nothing was amplified from wild-type DNA (Fig. 6). This indicated that *CiMADS9* was inserted into the *A. thaliana* genome and was being expressed.

3.5. CiMADS9 functional analysis

Phenotypic assessments were completed with the T_2 generation and wild-type plants. Compared with the wild-type plants, transgenic *A. thaliana* overexpressing *CiMADS9* exhibited delayed flowering (Fig. 7).

Flowering in the transgenic plants occurred 8 days after that in the wild-type plants (Fig. 8, A). Additionally, the transgenic plants had at least six more leaves than the wild-type *A. thaliana* (Fig. 8, B).

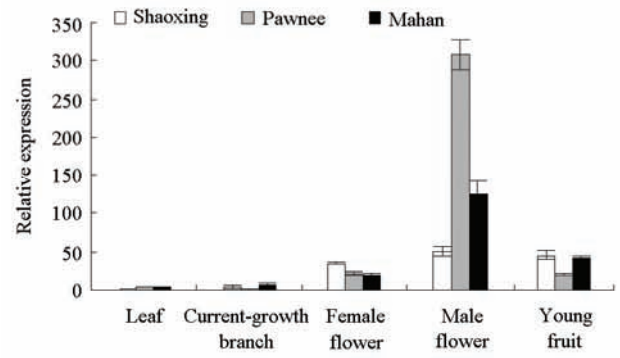

Fig. 5 *CiMADS9* expression patterns revealed by qRT-PCR analysis

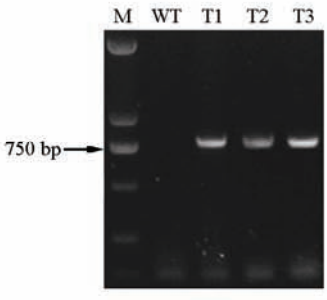

Fig. 6 RT-PCR analysis of *CiMADS9* in *Arabidopsis thaliana*
M: Marker; WT: Wild type; T1–T3: Hygromycin-resistant lines.

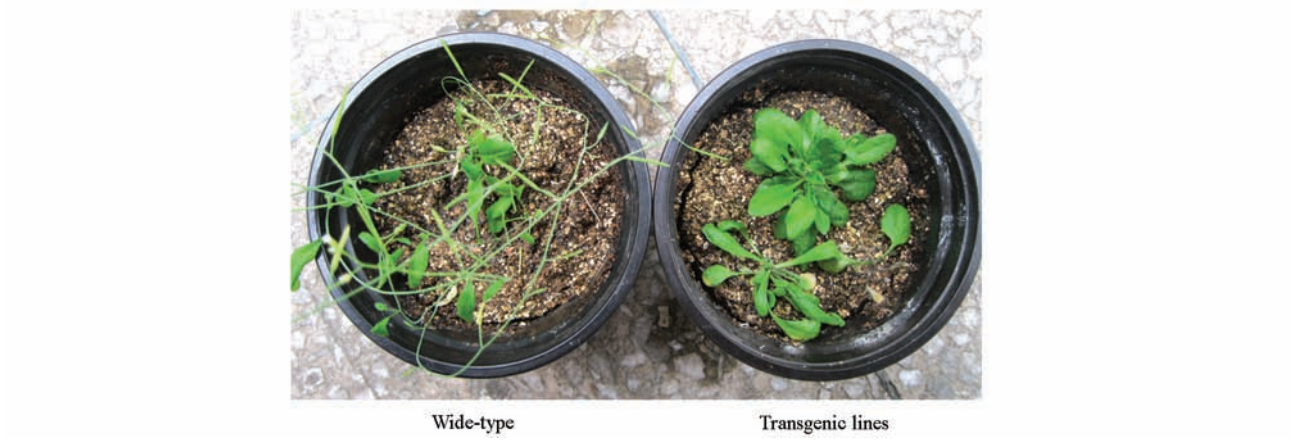

Fig. 7 Phenotypic comparison of transgenic and wild-type *Arabidopsis thaliana*

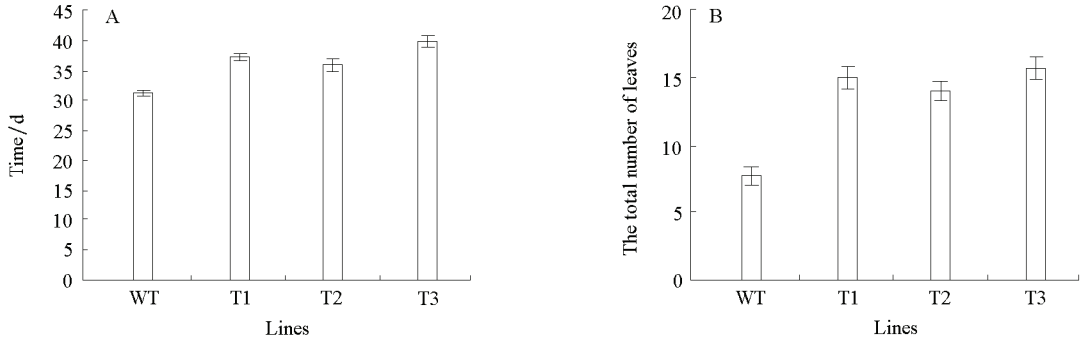

Fig. 8 The number of days between seeding and flowering (A) and the total number of leaves (B) of wild-type and transgenic *Arabidopsis thaliana*

WT: Wild type; T1–T3: Transgenic lines.

4. Discussion

We cloned a MADS-box gene called *CiMADS9* from the male flowers of *C. illinoinensis* using RACE technology. Amino acid sequence alignment and phylogenetic tree analysis indicated *CiMADS9* belongs to the AGL15 group of the MADS-box gene family. *AGL15* genes are expressed in *A. thaliana* vegetative organs at a low level (Fernandez et al., 2000). They are primarily expressed in young reproductive organs (Heck et al., 1995; Perry et al., 1999; Wang et al., 2008). In *Petunia hybrida* Vilm, *AGL15* plays an important role in male and female gamete and zygote developmental processes (Chen et al., 2011). We observed that *CiMADS9* is mainly expressed in reproductive organs (i.e., flowers and young fruits) and not in vegetative tissues (i.e., leaves and branches). This is consistent with the *AGL15* expression pattern in *A. thaliana*, and demonstrates that *CiMADS9* is relevant to flower development. Combined with the findings of previous studies, our results suggest that *CiMADS9* is important in male and female gamete and zygote development. *CiMADS9* was cloned from male *C. illinoinensis* flowers and was highly expressed in the male flowers of three pecan cultivars, indicating that it has a key role in male gamete development.

Other studies have shown that *A. thaliana AtAGL15* inhibits flowering and delays the senescence and shedding of the floral organ and perianth (Fang and Fernandez, 2002; Adamczyk et al., 2007). In this study, *CiMADS9* was inserted into the genome of *A. thaliana*. The RT-PCR results confirmed *CiMADS9* expression. Phenotypic assessments revealed that compared with the wild-type plants, transgenic *A. thaliana* overexpressing *CiMADS9* began flowering 8 days later and had more leaves. Therefore, *CiMADS9* expression results in delayed flowering.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31200502, 31401854) and the Natural Science Foundation of Jiangsu Province (Grant No. BK20140760, BK20150552).

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